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Genetic architecture of pollination syndrome transition between hummingbird-specialist and generalist species in the genus *Rhytidophyllum* (Gesneriaceae)

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Adaptation to pollinators is a key factor of diversification in angiosperms. The Caribbean sister genera *Rhytidophyllum* and *Gesneria* present an important diversification of floral characters. Most of their species can be divided in two major pollination syndromes. Large- open flowers with pale colours and great amount of nectar represent the generalist syndrome, while the hummingbird-specialist syndrome corresponds to red tubular flowers with a less important nectar volume. Repeated convergent evolution toward the generalist syndrome in this group suggests that such transitions rely on few genes of moderate to large effect. To test this hypothesis, we built a linkage map and performed a QTL detection for divergent pollination syndrome traits by crossing one specimen of the generalist species *Rhytidophyllum auriculatum* with one specimen of the hummingbird pollinated *R. rupicola*. Using geometric morphometrics and univariate traits measurements, we found that floral shape among the second-generation hybrids is correlated with morphological variation observed between generalist and hummingbird-specialist species at the genus level. The QTL analysis showed that colour and nectar volume variation between syndromes involve each one major QTL while floral shape has a more complex genetic basis and rely on few genes of moderate effect. Finally we did not detect any genetic linkage between the QTLs underlying those traits. This genetic independence of traits could have facilitated evolution toward optimal syndromes.

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1 INTRODUCTION

2 Flower is a key innovation often invoked to explain the radiation and evolutionary success of
3 angiosperms (Stebbins, 1970). Flowers present variable traits such as shape, colour, flowering
4 time from which it is often possible to distinguish groups of traits that evolve jointly for the
5 flower to be effectively pollinated by a given type of pollinator. These groups of traits are called
6 pollination syndromes (Fenster et al., 2004). The selection for these syndromes is often so strong
7 that it is possible to predict which type of pollinator a given plant species relies on via the
8 observed syndrome. For instance, flowers can harbour very different traits depending on whether
9 they are pollinated by wind or animals (Friedman & Barrett, 2009). In animal-pollinated species,
10 major traits involved in pollination syndrome include corolla shape and colour, floral scent, as
11 well as the amount and concentration of nectar produced, and variation in these traits enable
12 species to be distinguish by different groups of pollinating animals. Rosas-Guerrero et al. (2014)
13 reviewed floral traits of 417 species and showed that the concept of pollination syndrome can be
14 very effective at predicting the pollinators of animal pollinated flowers, more so than for non-
15 animal syndromes. Interestingly, syndrome predictability is more effective for tropical plants,
16 probably because of lower pollinator population densities in the tropics that increase selection
17 pressure (Rosas-Guerrero et al., 2014).

18 Pollination syndrome is a set of very dynamic and rapidly evolving characteristics, providing
19 numerous examples of convergent evolution in many groups. In *Penstemon* (Plantaginaceae), for
20 example, ornithophilous pollination evolved multiple times from insect pollinated flowers
21 (Wilson et al., 2007). In *Ruellia*, insect pollination evolved repeatedly from the ancestral
22 hummingbird pollination (Tripp & Manos, 2008). In *Gesneria* and *Rhytidophyllum*
23 (Gesneriaceae), generalist and bat pollinated species evolved several times from a hummingbird
24 syndrome (Martén-Rodríguez et al., 2010). The tribe Sinningieae of the Gesneriaceae also shows
25 an important lability of pollination modes, associated with evolution of traits such as corolla
26 shape and colour (Perret et al., 2007). Because such transitions between syndromes are often
27 linked with species diversification (reviewed in van der Niet and Johnson 2012), understanding
28 how these transitions occur is critical for understanding angiosperms evolution.

29 Observations of such an important lability of flower characteristics, combined with the fact that
30 flower diversification is often linked to species diversification, led us to wonder about the genetic

31 basis of these traits. Studies of the genetic basis of phenotypic evolution are often focused on
32 determining (i) if parallel phenotypic changes rely on parallel genomic evolution and (ii) if these
33 major phenotypic transitions result from major changes at a limited number of genes or from
34 minor changes of multiple genes (reviewed in Hendry 2013). In addition, developmental
35 constraints such as genetic interactions (epistasy) could be important to explain the convergence
36 of different traits to form a particular syndrome. Similarly, there are potentially important roles
37 for genetic correlations between traits and ecological factors – such as pollinator pressures – in
38 the redundant evolution of floral phenotypes among different species. Indeed, the speed at which
39 a population reaches its fitness optimum greatly depends on whether traits composing the
40 pollination syndrome are genetically independent or linked. Three scenarios can be envisaged: (i)
41 if traits are positively correlated, selection on one trait will affect variation at other traits in a
42 positive way and the general fitness optimum should be reached rapidly; (ii) if traits are
43 genetically independent, no developmental constraints should affect the evolution towards the
44 optimum and the speed of adaptation will solely be influenced by the intensity of the selective
45 pressure; and (iii) if traits are negatively correlated, selection at one trait will pull variation at
46 other traits further from the fitness optimum, hence reducing the pace at which this optimum can
47 be reached. Deciphering the degree of genetic correlation among traits is thus a first step toward
48 understanding the relative role of selection versus intrinsic constraints in the evolution of
49 phenotypes (Ashman & Majetic, 2006).

50 To answer these questions, a popular approach is to perform QTL detection on a hybrid
51 population generated from parents with different pollination syndromes. Previous studies have
52 shown that colour transition is generally explained by one major QTL (Quattrocchio et al. 1998;
53 Yuan et al. 2013; Wessinger et al. 2014). In contrast, nectar volume and concentration frequently
54 rely on numerous genomic regions each having a small to moderate effect on phenotype
55 (Goodwillie, Ritland & Ritland, 2006; Galliot et al., 2006; Nakazato, Rieseberg & Wood, 2013).
56 Flower shape variation was also shown to be generally caused by several QTLs with small to
57 moderate effects, with frequent colocalization of those QTLs (reviewed in Hermann and
58 Kuhlemeier 2011). During the past several years, emerging next generation sequencing
59 technologies have enabled the study of the genetic basis of adaptation in non-model species.
60 Also, improvements of methods to study morphology, principally with geometric morphometrics,
61 now enable to study the genetic basis and evolution of these complex characteristics

62 (Klingenberg et al. 2001; Langlade et al. 2005; Klingenberg 2010; Rogers et al. 2012; Franchini
63 et al. 2014; Liu et al. 2014).

64 The closely related genera *Gesneria* and *Rhytidophyllum* consist of approximately 75 species and
65 have rapidly diversified in the Antilles from a common ancestor that existed approximately 8 to
66 11 mya (Roalson et al. 2008). During this rapid species diversification, the group also
67 simultaneously experienced a rapid diversification of floral traits. Floral shape, colour and nectar
68 production have evolved jointly into three evolutionarily labile pollination syndromes (Martén-
69 Rodríguez, Almarales-Castro & Fenster, 2009, Martén-Rodríguez et al. 2010): (i) species
70 pollinated by hummingbirds that have red tubular flowers with diurnal nectar production, (ii)
71 species pollinated by bats harbouring large pale flowers with a bell shape corolla and exhibit
72 nocturnal nectar production, and (iii) generalist species that can either be pollinated by
73 hummingbirds, bats or moths, have generally pale flowers (although often with various spots)
74 with large openings but with a constriction in the corolla, and can have nocturnal and diurnal
75 nectar production. It has been inferred that the hummingbird syndrome is the ancestral pollination
76 mode whereas the bat and generalist syndromes evolved independently several times (with
77 reversals back to the ancestral hummingbird syndrome having been tentatively identified)
78 (Martén-Rodríguez et al., 2010). We intend here to identify the genetic basis of the pollination
79 syndrome transition between the generalist and hummingbird-specialist species in
80 *Rhytidophyllum* using QTL detection in a second-generation hybrid population. *Rhytidophyllum*
81 *auriculatum* is a typical generalist species from Hispaniola and Puerto Rico, and harbours opened
82 yellow flowers producing large amount of nectar. The second species, *R. rupicola*, is a
83 hummingbird specialist with red and tubular flowers that produces only small quantities of
84 nectar. Its endemism to Cuba (Skog 1976; Martén-Rodríguez et al. 2010; pers. obs.) eliminates
85 all potential for natural hybridization with *R. auriculatum*. According to Marten Rodriguez et al.
86 (2010), *R. auriculatum* most likely belongs to a group of generalist that evolved from an ancestral
87 hummingbird syndrome, whereas *R. rupicola* likely represents a reversion to the ancestral
88 hummingbird syndrome; the two species being closely related but not sister species.

89 In this study, we obtained anonymous genetic markers via next generation sequencing (NGS) and
90 built a linkage map from a second generation (F2) hybrid population between *R. rupicola* and *R.*

91 *auriculatum*. We then used geometric morphometrics to study floral shape and test whether QTLs
92 underlying floral trait evolution are few or numerous and whether they are linked or not.

93

94 MATERIAL AND METHODS

95 **Study system:**

96 *Rhytidophyllum auriculatum* (female parent) was crossed with *R. rupincola* (male parent) from
97 specimens from the living collection of the Montreal Botanical Garden (Canada) in 2010 to
98 obtain first-generation (F1) hybrids. An F1 individual was self-fertilized in 2011 to give a
99 second-generation (F2) population of 177 individuals. In parallel, both parents were self-
100 pollinated and gave several viable individuals.

101

102 **Phenotypic measurements:**

103 Phenotypic measures were performed from June of 2013 to April 2014 for morphological and
104 colour traits because of a great heterogeneity of developmental rate in the population. Flower
105 colour was treated as a binary trait: orange or yellow. Given the large variation in intensity and
106 distribution of the orange colour on the corolla (Fig. 1), individuals were considered “orange”
107 when some orange colour was observed on them. Corolla shape was analysed with geometric
108 morphometrics methods designed to capture morphological characteristics of pollination
109 syndromes without a priori hypotheses. In addition, of allowing the determination of shape that is
110 representative of a particular pollination mode, geometric morphometric methods have also been
111 shown to be very efficient at revealing the genetic basis of complex morphological changes
112 (Klingenberg et al. 2001).

113 For each individual, between one and three flowers were photographed. Each photo was analysed
114 twice with the software TpsDIG2 (<http://life.bio.sunysb.edu/morph/soft-dataacq.html>), to
115 evaluate variance due to manipulation errors in our analyses. Photographs from a different study
116 (Joly et al. in prep) were also included to quantify shape variation in the whole
117 *Gesneria/Rhytidophyllum* clade. This was done to characterize the aspects of shape that were the
118 most significant to differentiate generalists from hummingbird specialists (see below). For these

119 photographs, a single flower per individual was included. Six landmarks and 24 semi-landmarks
120 were placed on each photo. Two landmarks were placed at the extremity of the petal lobe (L1,
121 L2), two at the base of the petal lobes (L3, L4) and two at the base of the corolla (L5, L6). Semi-
122 landmarks were evenly dispersed on the contour of the corolla between L3-L4 and L5-L6 (Fig.
123 1). Geometric morphometrics analyses were then performed in R (R Development Core Team
124 2008) with packages *shapes* (Dryden, 2014), *geomorph* (Adams & Otárola-Castillo, 2013) and
125 *ade4* (Dray & Dufour, 2007). A general Procrustes superimposition of all the photos was
126 performed with the function *gpgen* allowing for sliding semi-landmarks in the superimposition,
127 and the mean coordinates of the landmarks and semi-landmarks per individual were extracted to
128 get only one shape per individual. Morphology was then measured using four approaches to
129 address the problem from different facets (see Fig. 2 for more details): (i – *Pollination syndrome*
130 *differences*) A PCA (function *dudi.pca*) of nine generalist and nine hummingbird specialist
131 species from the genera *Gesneria* and *Rhytidophyllum* (see supplementary Table 1 for details)
132 was performed, and the F2 individuals were projected (function *suprow*) on the first PC that
133 represents the shape difference between hummingbird-specialists and generalists. This approach
134 estimates how much each F2 individual resemble to hummingbird specialists or to generalists. (ii
135 – *Parental differences*) A PCA was performed on the two parents, giving only one principal
136 component upon which the F2 individuals were projected. This approach measures how much
137 each F2 individual resemble each parent. (iii – *Morphological variation in the hybrid*
138 *population*) A PCA of the F2 population was performed (including the self-pollinated F1, both
139 parents and three progenies of the self-pollinated parents), from which the scores of the F2
140 individuals were directly obtained. This approach allows investigating the genetic bases of the
141 morphological variation observed in the F2 hybrid population. (iv – *Univariate traits*) Two
142 univariate traits were extracted from the landmarks data before the Procrustes superimposition:
143 corolla tube opening corresponds to the distance between L3 and L4, and corolla curvature as the
144 angle formed by the lines (L1-L2) and (L5-L6) (Fig. 1). Pictures from wild specimens were used
145 to analyse shape only, without any size component because photos did not include a scale.
146 Among the 141 individuals that gave flowers, four F2 individuals with abnormal flowers (disjoint
147 petals or different flower shapes within an individual) and seven individuals presenting flowers
148 with more or less than 5 petal lobes were discarded from the phenotypic measures, leaving 130
149 individuals for shape analysis.

150 Measurements of nectar volume were performed between November and December 2014. Nectar
151 was sampled in early afternoon after flower opening, which generally occurs two days after
152 flower opening. This time was chosen because nectar is released mainly at dawn and dusk in
153 *Gesneria* and *Rhytidophyllum* (Martén-Rodríguez & Fenster, 2008), and because no nectar
154 production was observed during the day for the parental species. To sample nectar, the flowers
155 were removed from the plant, and the volume was measured with a graduated 50 μ L syringe.

157 **Genotyping:**

158 Plant leaves were sampled and dried in silica gel, and DNA was extracted with the Qiagen
159 (Mississauga, Canada) DNeasy Plant Mini Kit. 300 ng of DNA was used to genotype individuals
160 using a Genotyping By Sequencing approach, following the protocol developed by Elshire et al.
161 (2011). Library preparation was performed at Laval University (IBIS platform, Quebec city,
162 Canada) using the restriction enzymes *Pst*I and *Msp*I. We sequenced 177 F2s, duplicating ten
163 individuals to assess genotyping repeatability: four F2s, both parents, the self-pollinated F1 and
164 three other F1s, and two progenies of the self-pollinated parents. Individuals were multiplexed in
165 pools of 96 samples, and sequenced on two lanes on an Illumina Hiseq 2500 at McGill University
166 and Génome Québec Innovation Centre (Montreal, Canada). Stacks pipeline version 1.20 Beta
167 was used to extract genotypes from raw reads (Catchen et al., 2011). Reads were first
168 demultiplexed and trimmed to 82 basepairs with the function process-radtags. Then, unique
169 stacks were generated with the function ustacks, constraining for a minimum read depth (-m) of 2
170 to create a stack, and a maximum inter-read Single Nucleotide Polymorphism (SNP) distance (-
171 M) of 5. The catalog was created with both parents, and SNP calls were first performed with
172 default parameters in sstacks. Then, the error correction module rxstacks was run to perform
173 automated corrections using the bounded SNP model and a cutoff ln likelihood value of -10 to
174 discard unlikely genotypes. The cstacks and sstacks were then repeated with the corrected data,
175 and genotypes data were obtained with the function genotypes. After running genotypes with the
176 -GEN output format and allowing automatic corrections with default parameters, a R script was
177 run to translate those data in an A (parent *R. auriculatum* allele), B (parent *R. rupincola* allele), H
178 (heterozygous) format needed for subsequent analyses. In this script, using the information
179 available from the self-pollinated F1, markers that are aaxab in the parents, and for which the F1
180 is ab were typed in the F2 population, an option not available in the Stacks pipeline.

181
182 Because mutations in some TCP genes are known to be involved in the determination of flower
183 symmetry and in the size and shape of corollas (Hileman and Cubas, 2009), the genes *RADIALIS*
184 and *CYCLOIDEA* were included in the linkage map to test if they could be involved in the
185 variation in flower morphology between the two species. Gene sequences acquired from
186 GenBank (sequence AY363927.1 from *R. auriculatum* for *Gcyc* and sequence AY954971.1 from
187 *Antirrhinum majus* for *RADIALIS*) were compared to the parents' transcriptomes (unpublished
188 data) using BLASTn (Camacho et al., 2009) and primers were designed using software Primer3
189 (Koressaar & Remm, 2007). Gene sequences were deposited in Genbank (accession numbers
190 KP794058, KP794059, KP794060, and KP794061).

191
192 *CYCLOIDEA* was genotyped with the CAPS method (Konieczny & Ausubel 1993). Around 1 ng
193 of DNA was added to a master mix containing 0.375 U of DreamTaq (Termoscientific, Waltham,
194 MA, USA), 1.5 μ L of 10X DreamTaq Buffer, 0.6 μ L of each 10 μ M primer and 0.3 μ L of 10 mM
195 dNTPs in a total reaction volume of 15 μ L. Primers used to amplify *CYCLOIDEA* were *gcycf2*
196 (AAGGAGCTGGTGCAGGCTAAGA) and *gcycr2*
197 (GGGAGATTGCAGTTCAAATCCCTTGA), amplification conditions were 2 min at 94°C,
198 followed by 40 cycles of 94°C 15 sec, 54°C 15 sec, 72°C 30 sec, and then a final extension step
199 of 1 min at 72°C. Circa one μ g of PCR product was then digested with *AfIII* (New England
200 Biolabs, Ipswich, MA, USA) in a 15 μ L volume according to the company's recommendations.
201 The total volume of digestion products was visualized on agarose gel. *RADIALIS* was genotyped
202 with KASPAR (LGC genomics, Teddington, UK), with protocol tuning done by LGC genomics.
203 DNA amplification was done with 75 ng of DNA, 2.5 μ L of KASP master mix, and 0.07 μ L of
204 KASP primer mix in a total volume of 5 μ L. The specific primer for the first parental allele was
205 labelled with a FAM fluorochrome while the second specific primer was labelled with a HEX
206 fluorochrome. Amplification conditions were a first step of 94°C for 15 min, followed by 10
207 cycles of 94°C 20 sec, 61°C decreasing of 0.6°C at each cycle 1 min, and then another 29 cycles
208 of 94°C 20 sec 55°C 1 min. Genotypes were visualized by fluorescence after the amplification
209 procedure on viia7 system (Applied Biosystems, Foster city, CA, USA) with the "genotyping"
210 protocol.

211

212 **Linkage map construction:**

213 GBS markers were filtered to keep only those with less than 25% data missing, and no
214 segregation distortion (χ^2 p -value > 0.05 after Bonferonni correction). A linkage map was built
215 with Carthagene (de Givry et al., 2005). Linkage groups were detected with a maximum two
216 points distance of 30 cM measured with Haldane function and a minimum LOD of 3. Marker
217 ordering in each linkage group was done with the function *lkhd*, which implements the Lin-
218 Kerningham heuristic research algorithm to resolve the travelling salesman problem, optimising
219 the 2 points distances along the linkage group. Once the first map was obtained, manual
220 corrections were made for double-recombinants occurring within 10 cM. Because SNP calls can
221 be erroneous if read depth is small, double recombinants scored as either A or B (homozygous)
222 were replaced into H (heterozygous) if read depth was less than 10 reads. If read depth was more
223 than 10, homozygous double recombinants were replaced by missing data as proposed by
224 Kakioka et al. (2013), because those genotypes have a great probability of being erroneously
225 typed. H (heterozygous) double recombinants were not replaced if both alleles were effectively
226 detected in the sequencing data, but were replaced into A or B if only one allele was detected in
227 the data (this case occurred because of mistakenly corrected calls from automatic correction in
228 stacks). Remaining markers were then filtered again for missing data and segregation distortion,
229 and a new map was built. This was repeated until no double-recombinants within 10cM were
230 found in the linkage map. After these cleaning steps, genotypes of both candidate genes were
231 included in the dataset, and a final linkage map was built.

232

233 **QTL detection:**

234 Before performing QTL detection, correlation between colour, nectar volume and shape traits
235 was tested in the F2 population using pearson coefficient for quantitative traits correlation and F-
236 tests for colour. Among the 177 individuals, 141 gave flowers and were kept for colour tests.
237 One hundred and thirty individuals were kept for shape QTL detection after inappropriate data
238 was removed (see Phenotypic measurements section). Nectar volume was transformed into a
239 binary trait for QTL detection given its large intra-individual variation and non-normal
240 distribution in the F2 population (difference between the maximum and minimum volume for
241 each individual ranged from 4 to 64 μ L with a mean difference value of 25 μ L). Individuals with
242 mean volume inferior to 15 μ L were classified as “0” and those with a mean volume superior to

253 25 μ L as “1”, leaving 67 individuals to detect QTLs for nectar volume. QTL detection was
254 performed with R/qtl version 1.33-7 (Broman et al., 2003). Genotypes probabilities were
255 calculated every 1cM with the function *calc.genoprob*. QTLs were looked for with *scaneone* with
256 the normal model and the Haley-Knott method for the quantitative traits whereas the binary
257 model and the EM method were used for nectar volume and colour. LOD scores were compared
258 to the LOD threshold value obtained with 10,000 permutations. Then, if a QTL was detected, it
259 was added as an additive covariate and the procedure rerun to detect minor QTLs. For non-binary
260 variables, percentage of variance explained by the QTLs and size effects were checked with *fitqtl*,
261 adding in the model one QTL at a time. Given the limited number of individuals scored for nectar
262 volume, a supplementary Spearman correlation test between nectar volume (codes 0/1) and
263 genotypic data for each marker (codes 1/2/3) was performed to confirm the QTL results.

264 **Pleiotropy and epistasy detection:**

265 Pleiotropic QTLs were searched by considering the principal axes of the PCA performed on the
266 hybrid population as proposed by Mangin et al. (1998). The computation of the pleiotropic test
267 statistics was limited to the first three principal axes, which explained most of the variance, as
268 suggested by Weller et al. (1996). Briefly, the test was obtained by computing the LOD scores
269 for each principal component and summing the result of all the three principal components. To
270 access the threshold value of the pleiotropic test statistics, 10,000 permutations were performed
271 (Doerge & Churchill, 1996) with the three principal components being permuted all together in
272 order to get a null distribution, while preserving the initial intra-individual relation between
273 phenotypic traits. QTL detection was based on the 95th quantile. Confidence regions were
274 estimated with a 2-LOD support, as suggested by Van Ooijen (1992). Epistasy among QTLs as
275 well as among QTLs and other markers were tested using MCQTL (Jourjon et al., 2005).

276 Scripts and data used for morphometric analyses, map building and QTL detection are available
277 as supplementary data.

278 **RESULTS**

279 **Correlation between traits and morphological variation:**

273 For the PCA performed on the 18 species with divergent pollination syndromes (approach i), the
274 first principal component (PC1: 68.55%) discriminated hummingbird specialist species from
275 generalists (Fig. 3a). Both parents were positioned within their respective pollination syndrome
276 group while the self-pollinated F1 and the F2 population were intermediate between both
277 syndromes for the first principal component. As only the first principal component separated the
278 two syndromes, only this component was used for QTL detection. The first three principal
279 components of the PCA performed on the hybrid population (approach iii) explained the majority
280 of morphological variability found in the hybrid population (PC1: 35%, PC2: 22.7%, PC3:
281 14.2%, total=71.9%; Fig. 3b). For this PCA, parents were at the extremities of the distribution,
282 while the self-pollinated F1 and the F2s were intermediate between parents. Interestingly, the F2
283 individuals were closer to *R. rupicola* than *R. auriculatum* (Fig. 3b).

284 The correlation between the morphological principal components, two univariate traits
285 (constriction size, the corolla curvature) and two binary traits (corolla colour, nectar volume)
286 were measured. Traits corresponding to different pollination syndrome components (shape,
287 colour, nectar) were not correlated among individuals of the F2 population (Fig. 5). However, the
288 first principal component of each PCA (performed on the genus, both parents or the hybrid
289 population) were correlated with each other with high correlation coefficient (first PC on the
290 genus – first PC on the hybrid population: $r=0.98$; first PC on the genus – PC on the parents:
291 $r=0.901$; first PC on the hybrid population – PC on the parents: $r=0.811$, Fig. 5). Principal
292 components of PCA were also sometimes correlated with univariate shape measures (second PC
293 on the hybrid population-corolla curvature: $r=-0.92$; constriction size-first PC on the genus: $r=-$
294 0.633 , Fig. 5), and this correlation is also visible on Fig. 4 as flowers at the extreme of PC1
295 harbour different opening size and flowers at the low extreme of hybrids PC2 are more incurved
296 than flowers at the high extreme.

297 **Molecular data and linkage map:**

298 Starting from ca. 422 millions raw reads, the stacks pipeline initially gave 2,257 markers. After
299 removing markers with more than 25% missing data and with segregation distortion, 845 markers
300 remained to construct a genetic map. Then, with a third step of iterative map building, following
301 correction for double recombinants and filtering for missing data, we finally obtained 557 clean
302 GBS makers plus the two candidate genes. With a maximum distance of 30cM between

303 consecutive markers and a minimum LOD score of 3, 16 linkage groups were identified. Groups
304 remained stable even if the LOD threshold was changed from 1 to 10, which is suggestive of
305 relatively good stability of our linkage groups. The linkage map represents a total length of
306 1650.6 cM with an average distance between adjacent markers of 3.39 cM and relatively
307 heterogeneous linkage group size (Table 1 and Fig. 6). Recombination fractions and 2-points
308 LOD scores can be visualised on supplementary Fig. 2.

309 **QTL analysis:**

310 *QTLs for simple traits*

311 The ratio of yellow to orange flowered individuals in the F₂s was of 42:99, which is not
312 significantly different from a 1:3 ration expected for a dominant Mendelian marker (χ^2 test: $\chi^2 =$
313 1.7234; d.f=1; p -value = 0.1893). A single QTL, on linkage group LG16, was found to explain
314 colour variation in the F₂ population (Fig. 6).

315 One QTL explaining nectar volume differences was detected on LG12, with a very large
316 confidence region (123.4 cM). These results were confirmed by correlation between the traits and
317 markers as only two markers, both on LG12, were significantly correlated to nectar after a
318 Bonferonni correction (position 46.1, p -value = 2.78E-06; position 56.3, p -value = 4.19E-05). As
319 for colour, the amount of variance explained by this QTL couldn't be measured because the data
320 were transformed (binary model).

321 Shape was analysed with geometric morphometrics and with univariate measures. For the shape
322 variation between pollination syndromes (approach i) three distinct QTLs on LG1, LG11, and
323 LG14 were detected and explained respectively 12.8%, 13.6% and 8.8% of variance (Fig. 6;
324 Table 2). For the shape variation between parents (approach ii) also three QTLs were detected on
325 LG13, LG11 and LG14 explaining 6.7%, 10.2% and 12.8% of the variance (Fig. 6; Table 2).

326 When measuring morphological variation in the F₂ hybrids (approach iii), one QTL was
327 identified as controlling the first component on LG1 and explained 15.1% of the variance,
328 another QTL on LG2 explained 14% of the variance for the second component, and a third QTL
329 on LG9 explained the 14.9% of variance for the third component (Fig. 6; Table 2). Corolla tube
330 opening variation was explained by 2 QTLs on LG1 and LG16, explaining 12.5% and 12.4% of

331 the variance, respectively. Corolla curvature was underlain by one QTL on LG2 explaining
332 12.8% of the variance.

333 Interestingly, the same QTLs were detected irrespective of the way morphology was quantified
334 (Fig. 6), that is, co-localizing QTLs were detected for co-varying traits. For instance, the QTL on
335 LG1 was detected with the different methods used to measure shape. Specifically, it was detected
336 using the principal component that distinguished generalists and specialists as well as using
337 corolla tube constriction. Considering all shape analyses together, a total of seven different QTLs
338 were detected, which explained a small to moderate part of morphological variance (Table 2).

339 We found that one candidate gene for floral shape, *CYCLOIDEA* (at position 76.2 cM on LG16),
340 co-localized with a QTL confidence region for corolla constriction, although the position of the
341 gene does not correspond to the maximum LOD value (which corresponds to position 85 cM,
342 Fig. 6 and Table 2). *RADIALIS* did not co-localize with any QTL.

343 *Pleiotropic and epistatic QTLs*

344 When analyzing QTLs acting pleiotropically on the first three shape components obtained from
345 the PCA on the hybrid population, one QTL was detected on LG1, co-localizing with QTLs for
346 simple traits. Epistasis analysis was conducted with MCQTL and no epistatic interaction was
347 detected among QTLs and neither among QTLs and other markers.

348

349 DISCUSSION

350 **Detection of moderate QTLs involved in pollination syndrome transition:**

351 Our linkage map construction was able to recover 16 linkage groups. This is two more than the
352 haploid chromosome number ($n=14$) for *Rhytidophyllum* (Skog 1976). However, while karyotype
353 information exists for *R. auriculatum* (Skog 1976), none exist specifically for *Rhytidophyllum*
354 *rupincola*. Yet, an $n=14$ for *R. rupincola* appears likely because all *Rhytidophyllum* species
355 studied so far are $n=14$. In addition, differences in chromosome number between the parents
356 seem unlikely given the viability of second generation hybrids. Finding more linkage groups than
357 chromosomes might result from low genome coverage, however, we do not favour this

358 hypothesis as the average distance between consecutive markers is of 3.39 cM. The parents of the
359 cross are from distinct species and chromosomal rearrangements could have occurred between
360 them. These could create difficulties in assigning some chromosomal segments to the rest of the
361 chromosome; the smallest linkage groups could thus correspond to rearranged chromosomal
362 regions between both species.

363

364 *Colour differences QTL*

365 We detected one QTL explaining colour transition between *R. auriculatum* and *R. rupincola*. The
366 results presented here are consistent with previous studies on pollination syndrome transitions
367 that investigated the genetic basis of colour variation. Wessinger et al. (2014) found one QTL for
368 colour, corresponding to a gene involved in anthocyanin biosynthesis pathway. Similarly, the
369 well-known case of colour transition between *Mimulus lewisii* and *M. cardinalis* showed that a
370 single mutation at the *YUP* locus can both affect flower colour and pollinators behaviour
371 (Bradshaw & Schemske 2003). However, an important variation of colour patterns among orange
372 flowers was observed in the hybrid population, both in terms of intensity and localisation of
373 pigments (Fig. 1). This suggests that other genes could be involved in the intensity and
374 distribution pattern of pigments, probably through differential gene expression over the corolla.
375 Other studies on the genetic basis of colour transitions suggests that colour transitions generally
376 involves down-regulation of genes of pigment biosynthesis pathway, often via the action of
377 transcription factors (Galliot, Stuurman & Kuhlemeier, 2006). Future work will then involve the
378 study of the association between colour pattern and the expression of major genes in the
379 anthocyanin biosynthesis pathway.

380

381 *Nectar volume QTL*

382 Categorizing individuals as “low producing” or “high producing”, and using a binary QTL
383 detection model, permitted the detection of one QTL. We also tried to study sugar concentration
384 in nectar (using a Hand Held Brix Refractometer 0-32°, Fisher) but faced the same variability
385 problems as for volume and did not succeed in detecting any QTL (data not shown). The
386 confidence region of the QTL for nectar volume was very large. Other QTLs could likely be
387 detected with a larger sample size and stricter growing conditions to decrease intra individual
388 variation. Indeed, similar studies generally detected several QTLs explaining nectar volume

389 variation. Bradshaw et al. (1998) detected two QTLs for nectar volume explaining together
390 63.4% of total variance. Similarly, Stuurman et al. (2004) also detected two QTLs associated
391 with nectar volume in *Petunia* pollination syndromes. In contrast, Wessinger et al. (2014)
392 detected only one QTL for nectar volume variation.

393

394 *Multiple QTLs for corolla shape*

395 Floral shape was measured in order to first understand the genetic basis of the component of
396 corolla shape associated with pollination syndrome transition and second, to understand the
397 genetic basis of the components of corolla shape that are representative of differences between
398 both parents, but not necessarily important for pollination syndrome identity. For the shape
399 component defined by pollination mode differences, three independent QTLs were detected.
400 While only few QTLs were expected for this shape component, to our knowledge, no other
401 studies have successfully identified QTLs for pollination syndrome with geometric
402 morphometrics and PCA methods. However we can compare our results with studies analysing
403 shape differences in divergent environments in other organisms. For instance, Franchini et al.
404 (2014) used the same method to study the relationship between body shape evolution and trophic
405 ecology between two fish species. Their results are similar to ours in that they also detected
406 relatively few QTL (4), each one explaining less than 8% of variance.

407

408 Regarding shape differences that are not necessarily associated with pollination syndromes
409 (obtained with the PCAs on the parents and on the hybrid population), seven distinct QTLs were
410 detected. Removing those that co-localized with QTLs found with shape differences associated
411 with the pollination syndromes, four shape QTLs remained. Conceivably, shape may have
412 initially evolved dramatically during the process of pollination syndrome transition, followed by
413 gradual, small changes along the evolutionary tree (the two species studied are not sister species).
414 Such a hypothesis could be tested with repeated QTL studies involving closely related species
415 and phylogenetic comparison methods (Moyle and Payseur, 2009).

416

417 These results suggest that the genetic basis of shape evolution is more complex than those of
418 colour and nectar volume. Other studies of floral morphology detected multiple QTLs with small
419 to moderate effects explaining morphological changes linked to pollination syndrome evolution,

420 supporting this idea of increased complexity. For example, Hodges et al. (2002) detected multiple
421 QTLs for spur length and flower orientation differences between two *Aquilegia* species.
422 Wessinger et al. (2014) detected multiple QTLs between two *Penstemon* species associated with
423 morphological differences (explaining between 7.3 and 24.3% of the shape variance). Galliot et
424 al. (2006) detected six QTLs explaining several component of flower size in *Petunia* representing
425 each 2.7 to 41.6% of the variance, as well as four QTLs for nectar volume (explaining 4.2 to
426 39.1% of the variance). The same was detected for five morphological traits in *Leptosiphon* (each
427 one represented by two to seven QTLs explaining two to 28% of the variance) (Goodwillie,
428 Ritland & Ritland, 2006).

429
430 A candidate gene approach is always interesting as it can provide clues as to which genes might
431 be involved in the morphological variation observed in a system. *RADIALIS* and *CYCLOIDEA*
432 are two genes thought to be involved in the determination of floral zygomorphy (Preston,
433 Martinez & Hileman, 2011). More specifically, their localized expression during flower
434 development determines petal size and shape (Feng et al. 2006, Luo et al. 1999, Wang et al.
435 2008). As such, they represented strong candidate genes in the present study for controlling floral
436 shape, especially for the curvature of flowers. Neither were found to be clearly linked to the
437 morphological differences between *R. auriculatum* and *R. rupincola*. Indeed, although
438 *CYCLOIDEA* is situated within the confidence region of one QTL explaining corolla tube
439 opening, it had a LOD score that did not pass the rejection threshold (Fig. 6 and supplementary
440 Fig. 2) and is therefore unlikely to be highly involved in trait variation. This suggests that these
441 candidate genes are not directly responsible for corolla shape variation in our system, at least not
442 for the major shape differences between the pollination syndromes or in the hybrid population.
443 However, this does not mean that they are not involved at all as critical changes could involve the
444 regulation of their expression. If these candidate genes are trans-regulated, then the QTL would
445 not be expected to localize with them. Clearly, further studies will be needed to better understand
446 the genetic basis of flower shape variation in this system.

447

448 **Pollination syndrome evolution in the genus is summarized by morphological transition**
449 **between *R. auriculatum* and *R. rupincola*:**

450

451 Our results showed that generalist and hummingbird specialist species can be differentiated with
452 only one shape component in *Rhytidophyllum* and *Gesneria*, which concurs with a broader study
453 of the group (Joly et al. in prep). This shape component correlates with corolla tube opening in
454 our hybrid population and discriminates the columnar shape of hummingbird pollinated species
455 from the cup shape of generalists (Marten-Rodriguez et al., 2009). The strong correlation
456 between the shape components obtained with the PCA at the genera level, among the parents and
457 on the F2 hybrid population suggest that similar drivers may lie behind flower shape variation.
458 This implies that morphological transition between *R. auriculatum* and *R. rupicola* is
459 representative of the major morphological disparity between pollination syndromes at the genus
460 level.

461
462 To compare the genetic basis of simple traits and global shape, both univariate traits and
463 multivariate traits were measured. Our results showed that univariate morphological traits were
464 strongly correlated with geometric morphometric shape components, suggesting that the
465 information contained in simple traits is generally contained in geometric morphometrics data.
466 Moreover, geometric morphometric approaches allowed the detection of more QTLs,
467 demonstrating that they contain more information than simple traits. However, one QTL obtained
468 with an univariate trait (QTL on LG16 for corolla tube opening) was not detected with geometric
469 morphometric approaches. This could suggest that due to their complexity, geometric
470 morphometric traits may not catch exactly the same variation as univariate traits, but in the
471 present study these results could also be caused by lack of statistical power due to the small
472 segregating population size. Our results are similar to those of Franchini et al. (2014) who also
473 detected similar QTLs for geometric morphometric and simple traits measured with inter-
474 landmark distances. Their study, however, also showed that additional QTLs were identified for
475 univariate characteristics obtained independently from landmark data. Altogether, although these
476 observations strongly favour the use of geometric morphometric in QTL studies, they reveal that
477 it could also be beneficial to include univariate traits when analysing genetic architecture of
478 shape evolution, particularly when using small population sizes.

479

480 **The role of selection in pollination syndrome transition**

481 For most traits measured, only one major QTL was detected. However, for corolla tube opening,
482 inter-parents PC and inter-syndromes PC, two, three, and three QTLs were detected, respectively.
483 All the effects of these QTLs had the same direction. While such a result cannot be validated
484 statistically because of the small number of QTLs, it suggests that those traits evolved under
485 directional selection rather than by drift.

486 The existence of relationships between traits could impact the rate of adaptation. While some
487 authors argue that genetic correlation between traits could slow down adaptation, other showed
488 that it can facilitate it (reviewed in Hendry 2013). Several studies found more or less important
489 correlations between some traits involved in pollination syndrome transitions. Wessinger et al.
490 (2014) found no correlation between shape components, flower colour or nectar volume,
491 although they found weak but significant correlations between nectar concentration and some
492 morphological traits. Galliot et al. (2006), in their segregating *Petunia* population, detected a
493 correlation between nectar volume and floral tube width. In a study of monkeyflowers, Bradshaw
494 et al. (1998) detected epistatic interactions between the locus *YUP* involved in flower colour via
495 carotenoid concentration and two other putative QTLs. Hermann et al. (2013), who studied QTLs
496 of pollination syndromes in *Petunia*, found that QTLs involved in flower scent, colour and
497 morphology were tightly clustered in one genomic region.

498 In our study, flower shape was found to be totally independent from colour and nectar
499 characteristics. Detected QTLs for nectar, colour and shape are localized on different linkage
500 groups or on different regions of the same group, suggesting these traits are genetically
501 independent. However, it is not possible to completely rule out genetic correlations between traits
502 for two reasons. Firstly, some correlations among floral characters might exist in our study
503 system, but we didn't measure components that are linked with each other (such as nectar
504 concentration, pigments intensity and patterning on the corolla, style and stamen length, etc.).
505 Secondly, correlations could involve minor QTLs that were not detected because of our small F2
506 population. It is also possible that strong correlations do not exist in our system. In such a case,
507 we could consider that neither genetic constraints nor canalization played an important role in the
508 pollination syndrome transition between *R. rupincola* and *R. auriculatum*. This would tend to
509 show that selection pressure exerted by pollinators – that is, extrinsic factors – played a greater
510 role in pollination syndrome evolution than intrinsic factors. Indeed, selection could have been

511 exerted independently on each trait, and no developmental mechanism seems to have forced
512 concerted evolution of pollination syndrome traits.

513 However, we still wonder if the same sequence of trait evolution could have taken place with
514 replicated evolutions in the whole group? This question could be answered with replicated QTL
515 studies on independent transitions and with the help of phylogenetic comparative methods.
516 Accordingly, we agree with Moyle & Payseur (2009) that propose a combination of comparative
517 methods with QTL analysis to better understand evolutionary patterns of reproductive isolation or
518 evolution at a larger scale.

519

520 **Conclusion:**

521 The present study enabled the detection of major QTLs underlying the three major traits
522 composing divergent pollination syndromes between two *Rhytidophyllum* species. Even if several
523 minors QTLs potentially remain undetected, few major and independent regions for pollination
524 syndrome transition were identified. The hypothesis raised by our study is that directional
525 selection pressure exerted by different pollinators, rather than developmental constraints, was
526 strong enough to make the different traits converge on a pollination syndrome.

527

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Identification of major quantitative trait loci underlying floral pollination syndrome divergence in *Penstemon*.

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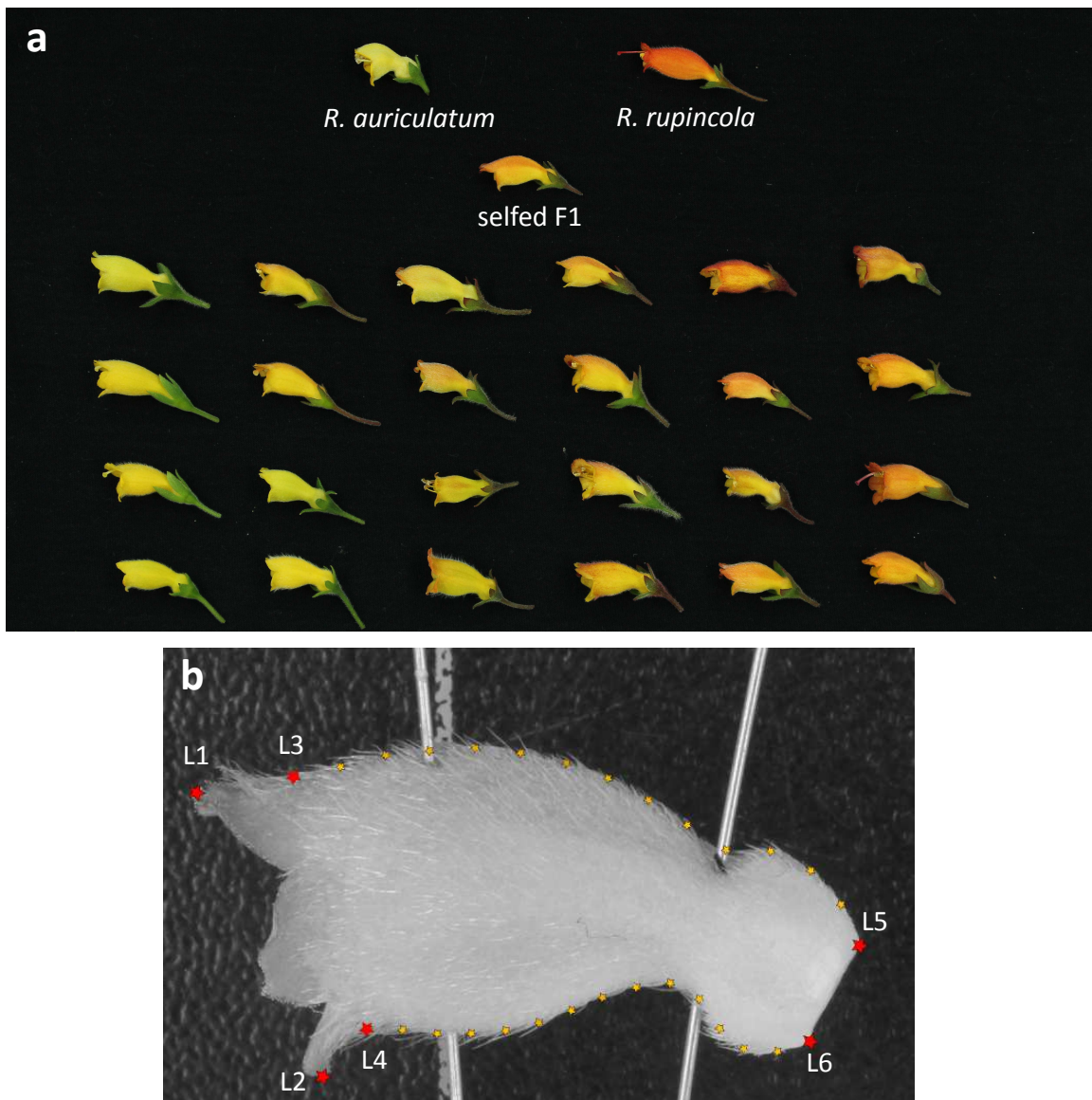


Figure 1. Measure of shape variation in the hybrid population and parents.

(a) Flowers from both parents (top row), the self-pollinated F1 and samples from the F2 population; (b) position of landmarks on corolla pictures- red stars represent landmarks and small orange stars are semi-landmarks.

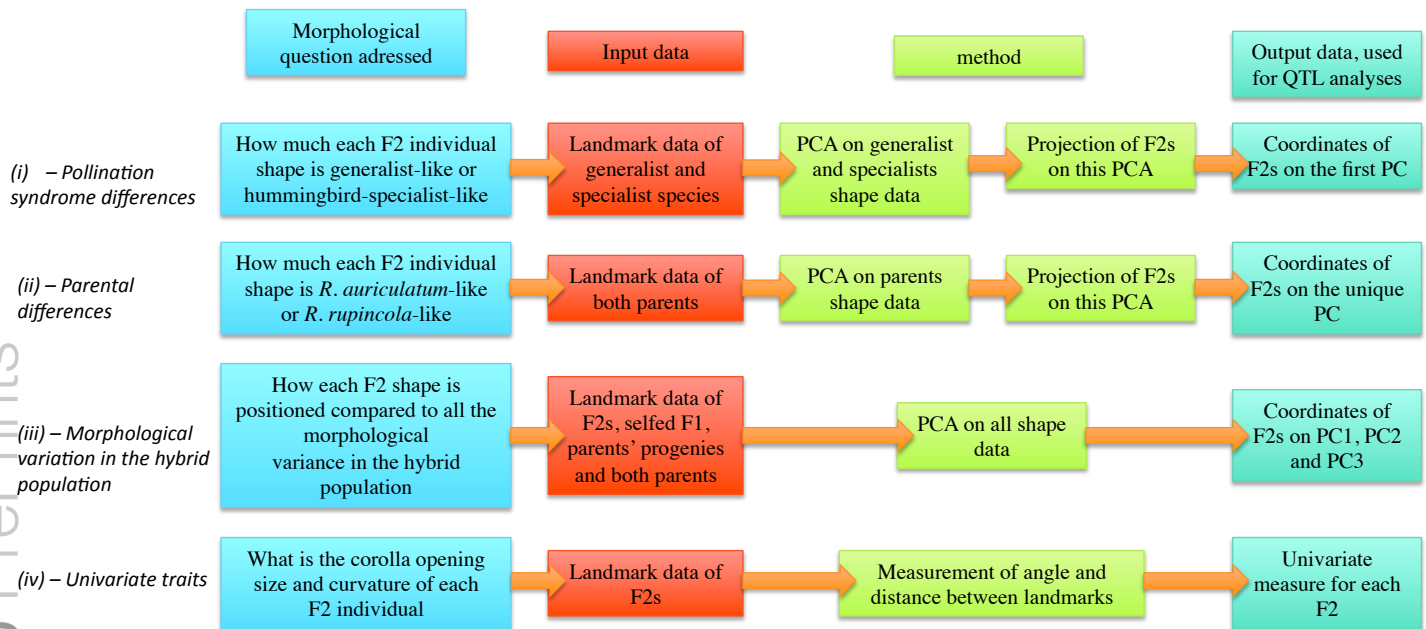


Figure 2. Diagram presentation of the four morphological measurement approaches.

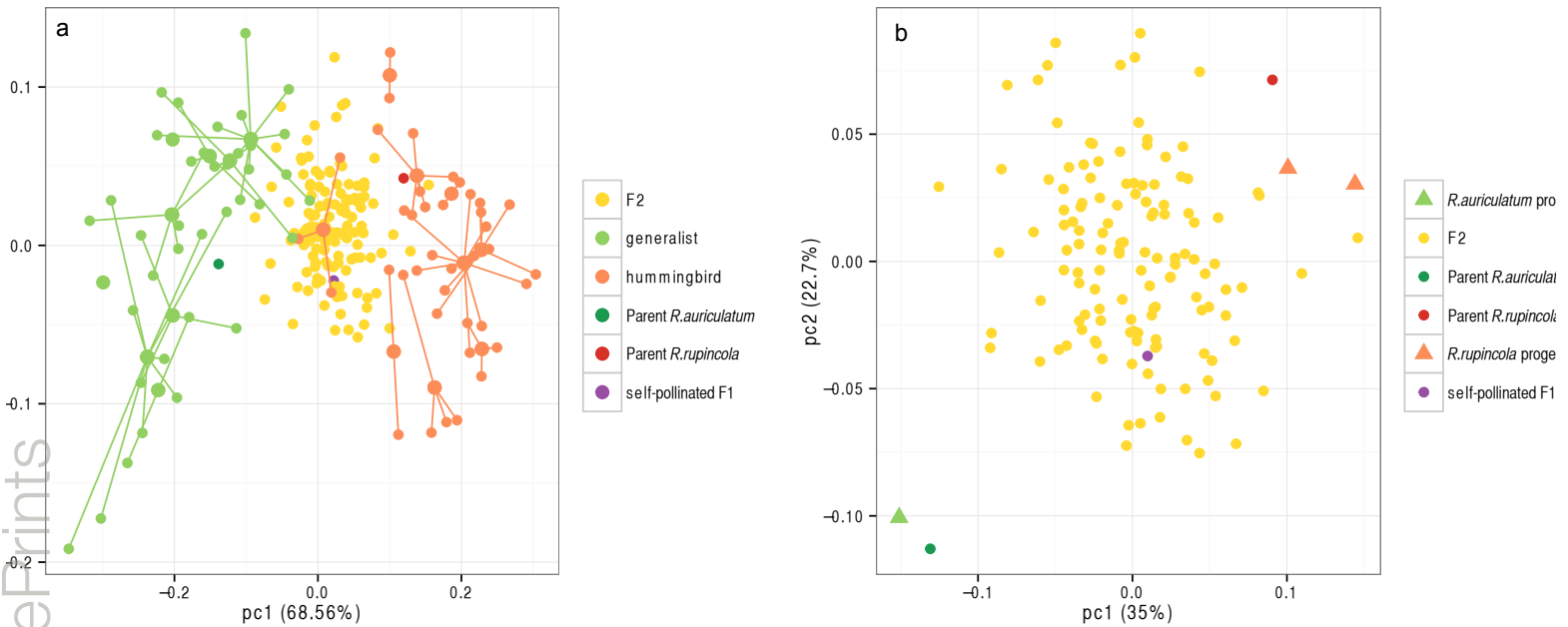


Figure 3. Principal component analyses of shape.

(a) PCA performed on wild specimens from species with different pollination syndromes (methods i- *Pollination syndrome differences*); Large and small dots represent species mean shapes and individual shapes, respectively, and individuals that belong to a given species are linked to it with a line. (b) PCA performed on the hybrid population (methods iii- *Morphological variation in the hybrid population*) where triangles represent self-pollinated parents' progeny. Numbers between brackets are percentage of shape variance represented by each axis.

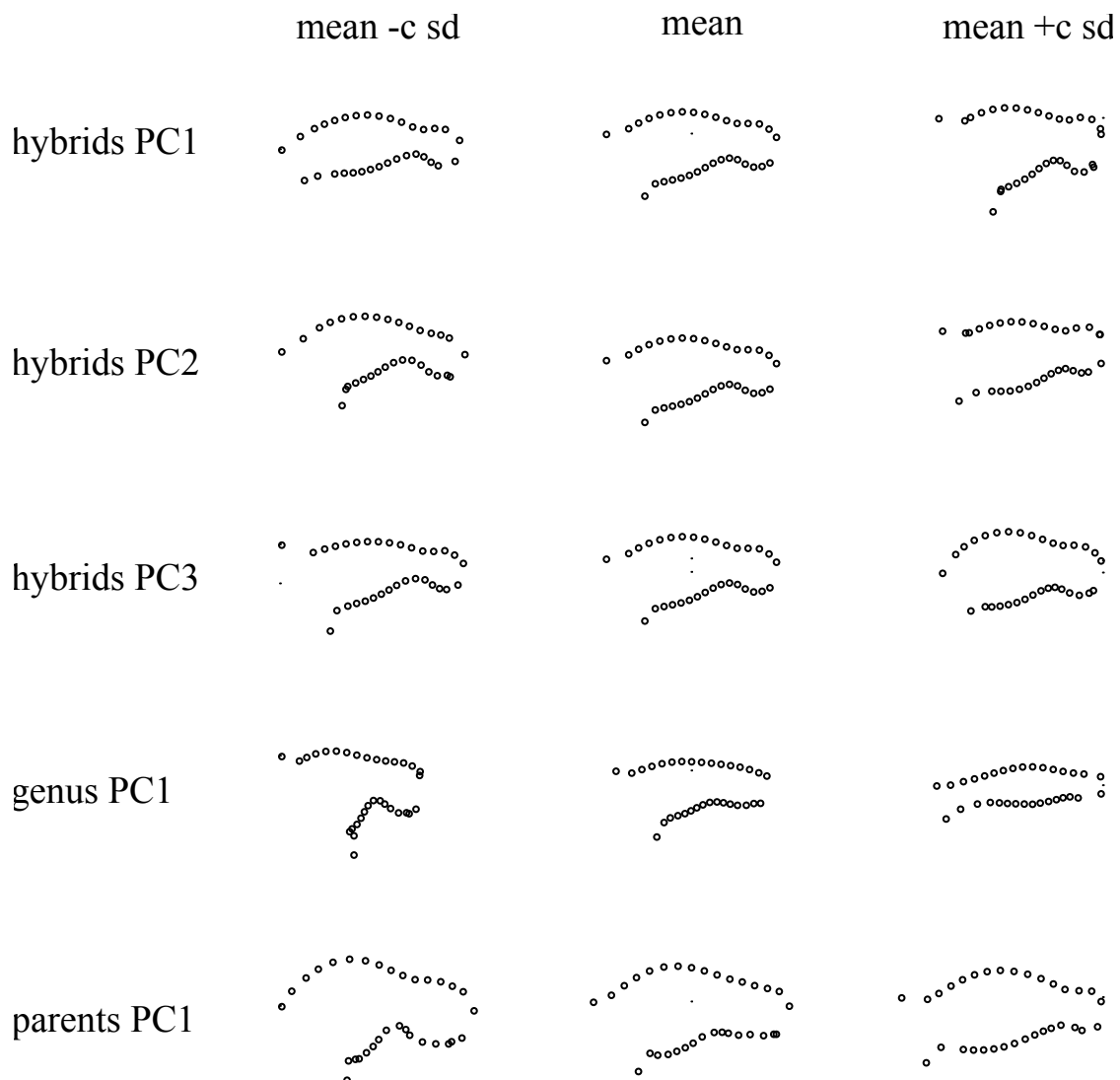


Figure 4. Shape variation associated with each principal component.

Each point represent a landmark (or semi-landmark) position on the profile of the corolla, as shown in Fig. 1b. Sd: standard deviation, $c=1$ for hybrid population PCA, 0.5 for between syndrome PCA and 0.2 for between parents PCA.

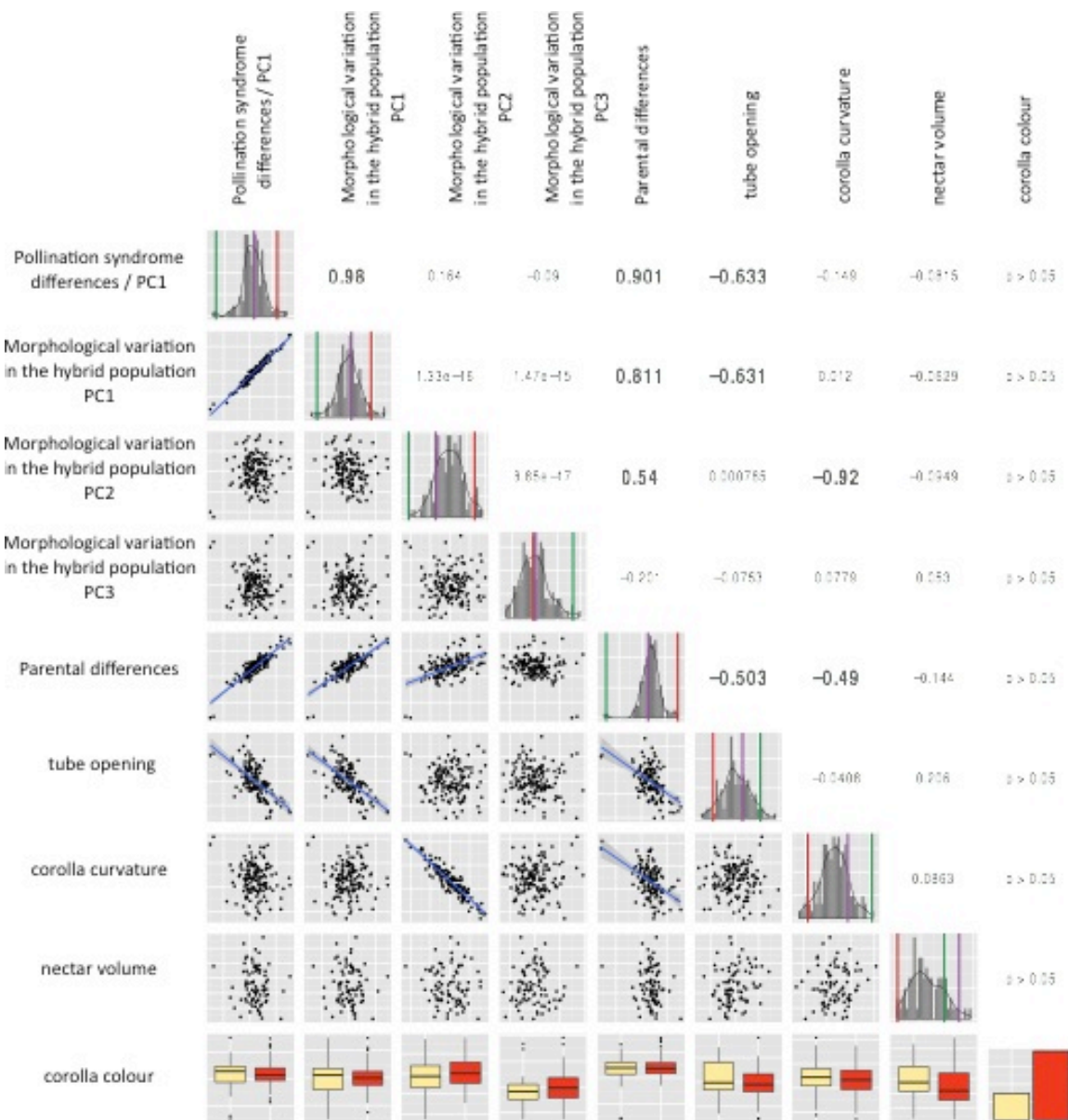


Figure 5. Distribution and correlation among traits in the hybrid population.

Diagonal: traits distribution, the vertical lines correspond to the value of parent *R. rupincola* (red), parent *R. auriculatum* (green) and the self-pollinated F1 (purple). Lower triangle: correlation among traits, if covariation is significant after Sidak correction, the regression line was plotted. Upper triangle: regression coefficient, in bold if correlation is significant.

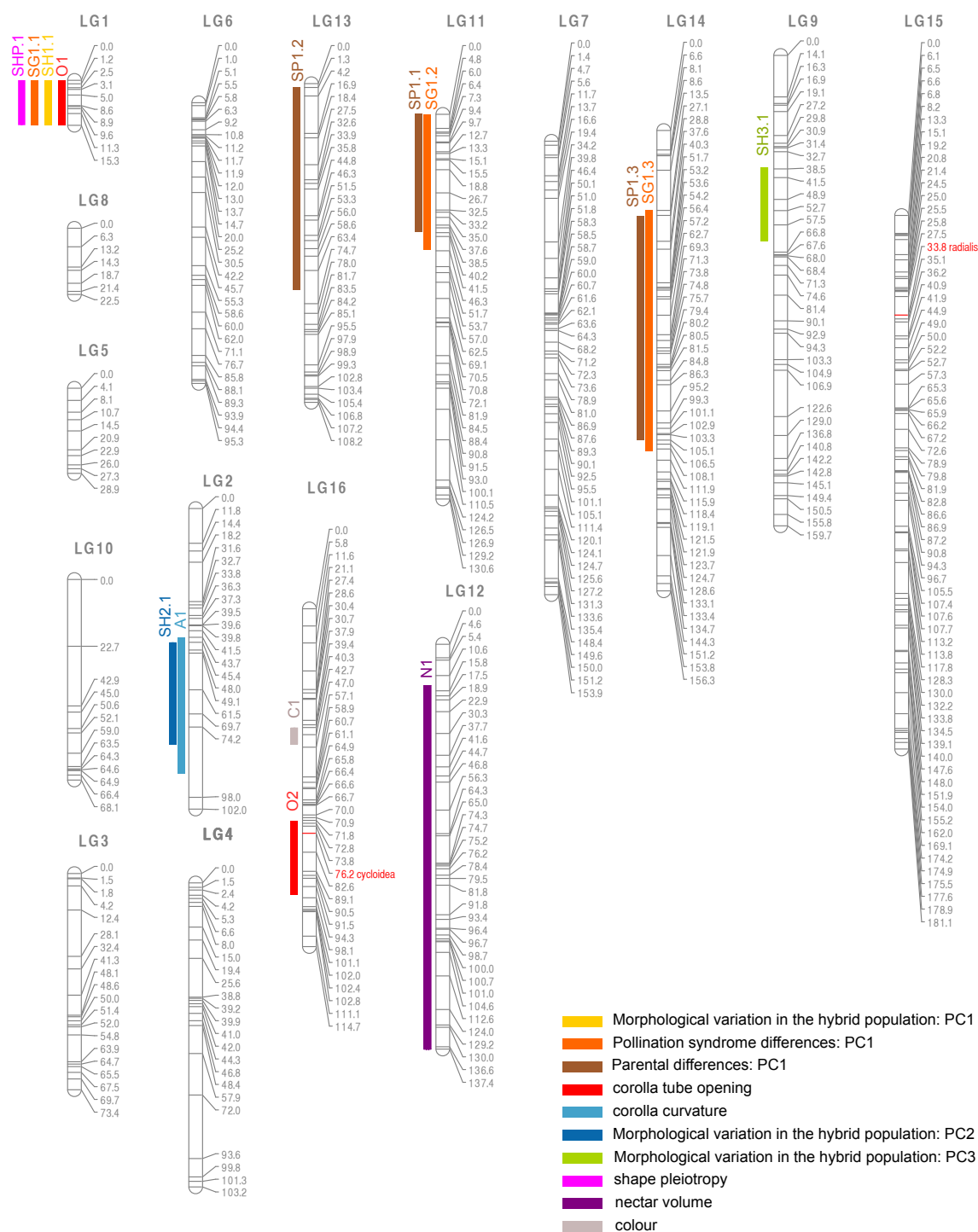


Figure 6. Linkage map and position of QTLs.

QTLs positions are marked with 2-LOD confidence region, numbers right to the linkage groups represent markers position in cM. Unique names for QTLs correspond to the names given in Table 2.

Table 1. Information about linkage groups

Linkage group	Number of markers	Size (cM)	Average distance between markers (cM)
LG1	12	15.3	1.7
LG2	23	102	4.86
LG3	22	73.4	3.86
LG4	26	103.2	4.49
LG5	11	28.9	3.21
LG6	36	95.3	3.07
LG7	59	153.9	3.02
LG8	7	22.5	3.75
LG9	46	159.7	4.2
LG10	13	68.1	5.68
LG11	48	130.6	3.19
LG12	40	137.4	3.71
LG13	34	108.2	3.49
LG14	57	156.3	3.13
LG15	81	181.1	2.62
LG16	44	114.7	2.94
total	559	1650.6	3.39

Table 2. Position and effects of QTLs.

Positions are given in cM from the beginning of the linkage group; confidence regions are calculated with 2 LOD score decrease. QTL names are the same as in Fig. 6. The relative homozygous effect is the additive effect divided by the mean phenotypic difference between both parents.

trait	QTL name	linkage group	position	confidence region	variance % explained	additive effect	relative homozygous effect	t value
colour	C1	LG16	43	40.3-46	-	-	-	-
nectar volume	N1	LG12	41.6	14-137.4	-	-	-	-
PCA on hybrid population	pc1	SH1.1	LG1	11.3	0-15.3	15.13	0.023	4.485
	pc2	SH2.1	LG2	57	45.4-80	14.06	0.020	4.120
	pc3	SH3.1	LG9	19	38.63	14.90	-0.017	-4.70
	pleiotropy	SHP.1	LG1	0	0-15	-	-	-
PCA on parents	pc1	SP1.1	LG11	28	0-40	10.26	0.017	4.209
		SP1.2	LG13	18	1.3-70	6.68	0.013	3.493
		SP1.3	LG14	91	29-105	12.88	0.015	4.345
PCA on genus	pc1	SG1.1	LG1	11.3	0-15.3	12.76	0.020	4.698
		SG1.2	LG11	29	0-46	13.60	0.023	4.938
		SG1.3	LG14	86.3	27.1-109	8.85	0.015	3.625
corolla curvature	A1	LG2	54	43.7-90	12.84	-5.987	0.10	-3.68
corolla tube opening	O1	LG1	15.3	0-15.3	12.48	-0.061	0.14	-4.59
	O2	LG16	85	72-97	12.36	-0.066	0.15	-4.11