1 **Evolutionary history of the buildup and breakdown of**

2 **the heterostylous syndrome in Plumbaginaceae**

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Summary

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29　● The evolutionary pathways leading to the heterostylous syndrome are not well

30　understood and models concerning the origins of distyly differ in the order in which

31　reciprocal herkogamy and self-incompatibility evolve. We investigated the evolution

32　and breakdown of distyly in Plumbaginaceae, a family with considerable diversity of

33　floral traits and reproductive systems.

34　● Using Bayesian Markov chain Monte Carlo analyses and stochastic character

35　mapping, we examined the evolutionary assembly and breakdown of the

36　heterostylous syndrome based on a well-resolved phylogeny of 121 species of

37　Plumbaginaceae and six outgroup taxa using five nuclear and plastid gene regions.

38　We used the distribution of reproductive traits and reconstructed ancestral characters

39　across phylogenies to evaluate competing models for the evolution of distyly.

40　● The most likely common ancestor of Plumbaginaceae was self-incompatible and

41　monomorphic for sex-organ arrangement and pollen-stigma characters. Character

42　state reconstructions indicated that reciprocal herkogamy evolved at least three times

43　and that shifts to selfing and apomixis occurred on multiple occasions.

44　● Our results provide comparative support for the early ideas of H.G. Baker on

45　evolutionary pathways in Plumbaginaceae, and the more recent selfing avoidance

46　model by D. & B. Charlesworth in which distyly evolves from self-incompatible

47　ancestors.

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49　**Key words:** ancestral state reconstructions, heteromorphic self-incompatibility, floral

50　evolution, heterostyly, pollen-stigma dimorphism, Plumbaginaceae, reciprocal

51　herkogamy.

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Introduction

Heterostyly is a remarkable example of a convergent floral polymorphism that has evolved numerous times in at least 28 angiosperm families (Ganders, 1979; Lloyd & Webb, 1992a; Barrett & Shore, 2008). Heterostylous populations have a reciprocal arrangement of stigma and anther heights, a condition referred to as reciprocal herkogamy (Webb & Lloyd, 1986), and are usually comprised of two (distyly) or three (tristyly) style morphs. The polymorphism in stigma and anther height is usually accompanied by a heteromorphic incompatibility system under sporophytic control, and by ancillary polymorphisms of stigmatic papillae and pollen. The evolution and breakdown of heterostyly has received sustained interest since Darwin's (1877) classic book on floral polymorphisms (reviewed in Barrett, 1992; Weller, 2009; Cohen, 2010). However, it was not until the latter part of the last century that evolutionary biologists began to investigate the evolutionary buildup of this floral syndrome using theoretical models (Charlesworth & Charlesworth, 1979; Lloyd & Webb, 1992b). The models differ in the sequence in which the morphological characters and physiological incompatibility system are established, and also in the emphasis placed on various selective forces.

Charlesworth & Charlesworth's (1979) 'selfing avoidance' model postulates that the ancestral condition for distyly involves a monomorphic population with plants possessing long styles and long-level anthers. Self-incompatibility with two mating types (often referred to as diallelic or heteromorphic incompatibility) establishes first to avoid selfing and inbreeding depression, with reciprocal herkogamy subsequently evolving to improve cross-pollination between the two cross-compatible mating types. The second step in this model is often described as "Darwin's cross-promotion hypothesis" (Darwin, 1877). By contrast, the 'pollen transfer' model of Lloyd & Webb (1992b) assumes that the promotion of cross-pollination is the principal selective force for the evolution of distyly, as Darwin (1877) proposed. In their model, the ancestral condition is approach herkogamy (stigmas positioned above anthers in flowers) and reciprocal herkogamy establishes to promote more proficient cross-pollen transfer among plants in the population. Subsequently, heteromorphic incompatibility may or may not evolve, depending on the severity of inbreeding depression. In both models, stigma-height dimorphism is an intermediate and transitory stage.

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90   Comparative data from phylogenetic reconstructions and character mapping of a

91 few heterostylous lineages have provided some support for various aspects of Lloyd and

92 Webb's model (e.g., *Narcissus*, Graham & Barrett, 2004; *Lithodora* and related

93 *Glandora*, Ferrero *et al.*, 2009a, 2012; *Exochaenium*, Kissling & Barrett, 2013). In

94 addition, experimental work on pollination (Stone & Thomson, 1994; Cesaro &

95 Thompson, 2004; Keller *et al.*, 2014) and mating patterns using genetic markers (Kohn

96 & Barrett, 1992; Zhou *et al.*, 2015) have also provided support for Darwin's cross-

97 promotion hypothesis but do not allow rejection of either model because both involve

98 this selective force at different stages in the buildup of the polymorphism. Because

99 heterostyly has clearly evolved on numerous occasions, it is possible that both models

100 for the evolution of distyly will be supported once more data become available from

101 additional families.

102   Plumbaginaceae is of worldwide distribution and is comprised of 27 genera and

103 c. 650 species (Kubitzki, 1993). The family exhibits great diversity in morphological

104 characters associated with the heterostylous syndrome, thus providing an opportunity to

105 investigate the evolutionary buildup and breakdown of floral polymorphisms. This was

106 recognized by the renowned plant evolutionist Herbert G. Baker (1948a,b, 1966), who

107 pointed out that species from subfamily Staticoideae displayed significant

108 morphological differences in pollen and stigma characters when compared with those in

109 subfamily Plumbaginoideae. Taxa in Plumbaginoideae are largely distylous, self-

110 incompatible and display relatively uniform patterns of pollen exine ornamentation and

111 stigmatic papillae shape between style morphs (Baker, 1948a, 1966; Dulberger, 1975a;

112 Erdtman, 1986; Ghobary, 1986; Ferrero *et al.*, 2009b). By contrast, species in subfamily

113 Staticoideae exhibit considerable morphological variation and particularly striking

114 pollen-stigma dimorphisms (i.e., ancillary pollen and stigma characters).

115   Generally referred to as *Armeria*-type pollen, as opposed to *Plumbago*-type

116 pollen from the Plumbaginoideae (Erdtman, 1986), dimorphic pollen in Staticoideae

117 commonly has a coarse or finely reticulate sexine, termed pollen type A or B,

118 respectively. Plants producing pollen type A have cob-like stigmatic papillae, whereas

119 plants with pollen type B have papillate stigmas (Baker, 1948a,b, 1966; Dulberger,

120 1975a,b, 1992; and see Figs. 1, 2 in Costa *et al.*, 2017). In common with other

121 heterostylous plants (reviewed in Dulberger, 1992), pollen-stigma dimorphism in the

122 Plumbaginaceae is very closely associated with heteromorphic self-incompatibility. In

123 Staticoideae, where the dimorphisms are especially well developed, they presumably

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124 function to promote disassortative pollination because of the absence of reciprocal

125 herkogamy (see Dulberger, 1975a; Mattsson, 1983; Dulberger, 1992), and indeed there

126 is experimental evidence supporting this hypothesis (Mattsson, 1983; Costa *et al.*,

127 2017).

128   The first detailed hypothesis for the buildup and breakdown of the heterostylous

129 syndrome in Plumbaginaceae was proposed by Baker (1948a, 1966), based on patterns

130 of geographical distribution of species and studies of variation in morphological

131 characters, particularly of pollen and stigmas. However, Baker's hypothesis was not

132 developed from explicit phylogenetic methods; he simply ordered species and

133 characters in a particular sequence, based on his own ideas on the most plausible

134 evolutionary pathways (e.g., Baker, 1966). He postulated that the heterostylous

135 syndrome evolved from a common ancestor with heteromorphic incompatibility and

136 monomorphic pollen and stigmas, but by different pathways in the two subfamilies

137 (Baker, 1948a, 1966).

138   In Plumbaginoideae, Baker proposed that reciprocal herkogamy was the only

139 morphological addition to the ancestral incompatibility system, whereas in Staticoideae,

140 the floral polymorphisms evolved in three stages. Specifically, he proposed that pollen

141 dimorphism established first in species with monomorphic capitate stigmas, as in

142 *Goniolimon*, *Acantholimon* and some *Limonium*. Subsequently, filiform dimorphic

143 stigmas and dimorphic pollen evolved in species of *Armeria*, *Limoniastrum* and

144 *Limonium*. Finally, reciprocal herkogamy evolved in the pollen-stigma dimorphic

145 *Limonium vulgare*. Baker assessed the compatibility status of a large number of species

146 of *Limonium* by performing controlled hand-pollinations (Baker, 1953a,b) and inferred

147 that self-incompatibility was widespread in the family, apart from a few self-compatible

148 or apomictic species of *Limonium* with "secondary monomorphism" of pollen and

149 stigmas. He considered this secondary monomorphism as derived from pollen-stigma

150 dimorphism as a result of either the evolution of homostyly [small-flowered selfing

151 forms derived from heterostylous ancestors (see Darwin, 1877; Ganders, 1979)], or

152 through the replacement of sexual reproduction by apomixis (Baker, 1966).

153 Significantly, Baker's hypothesis (Baker, 1948a, 1966) shares features in common with

154 the model of D. & B. Charlesworth (1979) for the evolution of distyly as both involve

155 the evolution of self-incompatibility prior to the establishment of reciprocal herkogamy.

156   Here, we investigate Baker's ideas on the evolutionary buildup and breakdown

157 of the heterostylous syndrome in Plumbaginaceae using models of character evolution

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158   and stochastic character mapping. We used molecular data to construct a phylogenetic

159   tree for the family and an extensive survey of herbarium specimens and the literature to

160   investigate the evolution of heteromorphic morphological traits. Our study addressed

161   the following specific questions: (1) Was the most likely common ancestor of

162   Plumbaginaceae self-incompatible with monomorphic pollen and stigmas, as Baker

163   (1948a, 1966) originally proposed? (2) How many times has reciprocal herkogamy

164   evolved in the family and was this prior to or after the establishment of self-

165   incompatibility and pollen and stigma dimorphisms? (3) Is there phylogenetic evidence

166   of breakdown of the distylous syndrome to alternative reproductive systems involving

167   homostyly and apomixis? (4) Is reciprocal herkogamy more likely to be selected in

168   lineages with pollen-stigma dimorphism? (5) Does apomixis evolve following the loss

169   of pollen-stigma dimorphism in subfamily Staticoideae? Our results provide support for

170   several of Baker's proposals and also for the order in which self-incompatibility and

171   reciprocal herkogamy are established in the Charlesworth and Charlesworth's (1979)

172   model of the evolution of distyly.

173

Materials and Methods

Characterization of floral polymorphisms and associated characters

176   To characterize the distribution of reproductive traits among taxa of Plumbaginaceae,

177   we combined extensive literature searches and sampling of herbarium specimens

178   (number of specimens per species: mean ± SD 3.10 ± 1.23, median 3, range 1-6) for a

179   total of 121 species. Details are provided in Supporting Information Methods 1 and the

180   herbarium specimens sampled are listed in Supporting Information Table S1.

181   Information from the literature was available for 61 species in the dataset and for 33 this

182   was combined with herbarium sampling. The characterization of floral polymorphisms

183   for 59 species was based on herbarium sampling only. The resulting character matrix

184   contained six traits (see Supporting Information Table S2): sex-organ arrangement

185   (reciprocal herkogamy vs style-stamen height monomorphism), incompatibility status

186   (self-incompatibility vs self-compatibility), exine sculpturing (*Armeria*-type pollen vs

187   monomorphic pollen), stigmatic papillae condition (stigma dimorphism vs stigma

188   monomorphism), stigma morphology (filiform vs capitate vs others) and reproductive

189   mode (sexual vs apomixis).

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190   We followed several criteria in constructing the character matrix: (1) Because

191   most species included in the analyses were not tested experimentally for their

192   incompatibility status, we used two approaches to infer whether they were self-

193   incompatible or self-compatible. For species in Plumbaginoideae, data on pollen type is

194   uninformative with regards to incompatibility status and thus we used information in the

195   literature, whereas for Staticoideae information from the literature (e.g., Baker, 1966)

196   was combined with microscopic observations of pollen exine sculpturing and stigmatic

197   papillae shape from herbarium specimens. Species were scored as self-incompatible if

198   they possessed A/cob and B/papillate pollen-stigma combinations, and as self-

199   compatible if they had A/papillate or B/cob pollen-stigma combinations. This approach

200   follows criteria established by Baker (1948a, 1966) based on controlled pollinations and

201   also Erben (1979). (2) As *Plumbago*-type pollen shows only very slight differences

202   between style morphs in exine sculpturing (e.g., Ghobary, 1986; Ferrero *et al.*, 2009b),

203   this condition was considered monomorphic in all analyses. (3) Species of *Limonium*

204   were scored as sexual or apomictic based on observations of pollen exine sculpturing

205   and stigmatic papillae shape combined with information on chromosome counts from

206   the literature. Dimorphic species with A/cob and B/papillate pollen-stigma

207   combinations and even chromosome counts of $2n = 12, 14, 16, 18, 32, 34, 36, 54$ were

208   considered sexual, whereas species monomorphic for pollen and stigmas (A/cob or

209   B/papillate) with odd chromosome counts were scored as apomictic. This approach also

210   follows Erben (1979) and data available from Baker (1966). We confirmed and assigned

211   all species names following *The Plant List* (2013) and *Species 2000 & ITIS Catalogue*

212   *of Life* (Roskov *et al.*, 2016) available online.

213

214   Phylogenetic analyses

215   We used a supermatrix approach (de Queiroz & Gatesy, 2007; Roquet *et al.*, 2013) to

216   construct a phylogenetic hypothesis for evolutionary relationships of species in the

217   ingroup. Sequences of the five most common markers used in previous phylogenetic

218   studies of Plumbaginaceae were downloaded from GenBank using Geneious v9.0.5

219   (Kearse *et al.*, 2012). We used one marker of the nuclear genome (*ITS*) and four of the

220   plastid genome (*rbcL*, *matK*, intergenic spacers *tnrL-trnF* and *trnT-trnY*) (Supporting

221   Information Table S3). Our outgroup included six species from three families

222   considered to be the closest relatives of Plumbaginaceae: *Polygonum amphibium*, *P.*

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223 *capitatum*, *Rumex obtusifolius* and *Triplaris americana* (Polygonaceae), *Tamarix*

224 *aphylla* (Tamaricaceae) and *Frankenia pulverulenta* (Frankeniaceae) (Soltis *et al.*,

225 2000).

226   We aligned sequences using MAFFT v7 available online

227 (https://mafft.cbrc.jp/alignment/server/; Katoh & Standley, 2013). All alignments were

228 improved by removing poorly aligned or ambiguous regions by setting "automated 1"

229 function on trimAl v1.3 software (Capella-Gutiérrez *et al.*, 2009) available online at the

230 Phylemon 2.0 server (http://phylemon.bioinfo.cipf.es/index.html; Sánchez *et al.*, 2011).

231 To construct the supermatrix, trimmed alignments were concatenated by using

232 FASconCAT v1.0 (Kück & Meusemann, 2010) resulting in a supermatrix of 4480

233 characters and 94 taxa. We analyzed the supermatrix using a maximum likelihood (ML)

234 approach following the GTR model as implemented in RAxML v8.2.8 (Stamatakis,

235 2014) available at the CIPRES server (http://phylo.org, Miller *et al.*, 2010) by running

236 1000 bootstrap replicates. We kept the best ML phylogenetic tree and nodes with

237 bootstrap values lower than 50% were collapsed using TreeGraph 2 (Stöver & Müller,

238 2010).

239   To obtain an ultrametric phylogenetic tree, we used molecular dating by

240 likelihood methods as implemented in the *chronos* function available from the R

241 package "ape" (Paradis *et al.*, 2004; Paradis, 2013) on the ML tree previously obtained.

242 We obtained minimum and maximum ages for Plumbaginaceae from Bell *et al.* (2010)

243 and for calibration purposes used minimum = 27 mya and maximum = 57 mya. The

244 selection of the model of substitution rate was based on the information criterion PHIIC

245 and the model with the lowest PHIIC was selected (Paradis, 2013). PHIIC values for the

246 tested models are available in Supporting Information Table S4. There were no

247 differences when changing the smoothing parameter for the model "strict" and therefore

248 the simplest one was chosen (model "strict", lambda value "0").

249   For 35 species included in the character matrix for which there were no

250 sequences available in GenBank, we searched in the literature for information regarding

251 monophyly of the genus. If that was the case, species were grafted onto their respective

252 genera in the ultrametric tree by using *add.species.to.genus* function from R package

253 "phytools" (Revell, 2012). When no information regarding monophyly was available,

254 we searched the literature for the putatively closest relative and added the species to a

255 specific node by setting *bind.tip* function from R package "phytools" (Revell, 2012).

256 For *Limonium minutiflorum*, the closest relatives [i.e., species from the same section

257   according to Boissier (1848)] were in two different positions in the tree (Supporting

258   Information Fig. S1). To reduce inaccuracies in character reconstructions associated

259   with the grafting of this species, two trees each with *L. minutiflorum* in a different

260   position were created. The grafting of species introduced some polytomies in the final

261   trees that were randomly resolved by creating two sets of 100 trees using Mesquite

262   v3.04 (Maddison & Maddison, 2015). The ingroup included 121 species, seven of

263   Plumbaginoideae and 114 species of Staticoideae, from 14 genera representing ~19% of

264   the total species in Plumbaginaceae.

265

266   Inferences on ancestral states and character mapping

267   To assess the ancestral states of the six characters investigated, we conducted Bayesian

268   Markov chain Monte Carlo (MCMC) analyses in two sets of 100 trees (see *Phylogenetic*

269   *analyses*), thus accounting for phylogenetic uncertainty. We used the MultiState

270   continuous-time Markov model of character evolution for discrete data as implemented

271   in BayesTraits v2 (Pagel *et al.*, 2004). To reduce the uncertainty and randomness

272   associated with setting the prior distribution, we used hyperpriors as recommended by

273   Pagel *et al*. (2004). The hyperprior is a uniform distribution that is used to seed values

274   of the gamma or exponential priors (Pagel *et al*., 2004).

275   To test for the most likely ancestral character states, we implemented MCMC

276   analyses by running Markov chains for one million iterations that were sampled each

277   1000 steps, with a burn-in of 10 000, and by setting the stepping stone sampling to use

278   100 stones and run each for 10 000 iterations. The node containing all species of

279   Plumbaginaceae was fixed to each possible state of a given character under two models

280   of transition rates between character states: (1) equal rates (ER), and (2) all rates

281   different (ARD). For incompatibility status and reproductive mode, two additional

282   analyses were run by restricting back transitions (i.e., SC to SI and apomixis to sexual)

283   to zero. In total, we tested four models for each trait (five for incompatibility status and

284   reproductive mode, six for stigma morphology), and the best fitting model was selected

285   based on the Bayesian Information Criterion (BIC).

286   We mapped transitions between states for the six characters by means of

287   stochastic character mapping (SCM; Huelsenbeck *et al.*, 2003) in the two sets of 100

288   trees using *make.simmap* function from R package "phytools" (Revell, 2012). This

289   function simulates stochastic character histories on a phylogenetic tree or sets of trees.

9

290   We used the most likely value of the transition matrix to fit the prior distribution, except

291   for incompatibility status and reproductive mode. The loss of complex characters such

292   as these is generally irreversible (Dollo, 1893; Bull & Charnov, 1985; Goldberg & Igić,

293   2008), and thus low transition rates were defined in the Q matrix (SI to SC, sexual to

294   apomictic: $1E^{-3}$; SC to SI, apomictic to sexual: $1E^{-5}$). The best fitting model of character

295   evolution (ER vs ARD) as revealed by MCMC analyses was used for each trait. We

296   performed Markov chains for 100 simulations for each tree, that is 10 000 simulations

297   for each set of trees for each character. Because the *make.simmap* function allows

298   estimation of the posterior probability (PP) of all possible states for a character that is

299   considered uncertain, we used a binary matrix, i.e., a matrix of probabilities ranging

300   from 0 to 1 for each trait. Species with missing data for a given character were assumed

301   to have equal prior probability of taking one or the other character state.

302   Character mapping allows the estimation of the PP of states of the most recent

303   common ancestor of each node, and the calculation of the average number of changes

304   between states, as well as the time spent in each state. We obtained the relative rate of

305   transition between states by dividing the frequency of each transition (i.e., the ratio

306   between one specific change and the total number of changes) by the frequency of time

307   spent in each state (Torices & Anderberg, 2009).

308

309   Correlated evolution between characters

310   To test for: (1) correlated evolution between reciprocal herkogamy and pollen-stigma

311   dimorphism, and (2) whether apomixis originates following the loss of pollen-stigma

312   dimorphism, we conducted MCMC analyses using *Discrete independent* (traits are

313   assumed to evolve independently) and *Discrete dependent* (the rate of change of one

314   trait depends on the state of the other) options as implemented in BayesTraits v2 (Pagel

315   *et al.*, 2004). We scored species as pollen-stigma dimorphic based on the possession of

316   pollen and stigmatic papillae dimorphism simultaneously. The stepping stone sampling

317   was set to use 100 stones and run each for 10 000 iterations. Under these models,

318   transitions between states are only allowed for one character at each time. We compared

319   the likelihoods of the two models by means of Bayes Factor (BF) and values Log BF >

320   2 indicated evidence for correlated evolution.

321

322   **Results**

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323 ## Phylogenetic distribution of character states

324 Reciprocal herkogamy occurs in the two subfamilies of Plumbaginaceae, but its overall

325 frequency differs between them (Fig. 1, Supporting Information Table S2). All species

326 of Plumbaginoideae in the dataset ($n = 7$) possess reciprocal herkogamy and are

327 therefore distylous. In the Staticoideae, distyly occurs in *Goniolimon*, some

328 *Acantholimon* species and *Limonium vulgare* for a total of 12 species of the 114

329 investigated in the subfamily. All species of Plumbaginoideae in the dataset were self-

330 incompatible ($n = 7$), whereas self-incompatibility, as inferred based on pollen-stigma

331 character state combinations, occurred in 101 species of Staticoideae. Self-compatibility

332 was inferred in four species, all *Limonium,* in subfamily Staticoideae, and nine species

333 had missing data for this trait (Supporting Information Table S2).

334 Regarding ancillary polymorphisms of pollen and stigma (Fig. 1, Supporting

335 Information Table S2), *Armeria*-type pollen was only found in Staticoideae and

336 monomorphic exine of the *Plumbago*-type appeared to be restricted to Plumbaginoideae

337 ($n = 7$). However, eight species of *Limonium* produced only one pollen type, mainly

338 type A (*L. echioides* produced pollen type B) and these species were inferred to possess

339 monomorphic pollen. Similarly, stigma dimorphism only occurred in species of

340 subfamily Staticoideae and 11 species of *Limonium* had monomorphic stigmas, which

341 were either cob or papillate. Capitate stigmas were characteristic of all Plumbaginoideae

342 species included in this study, and also species of *Acantholimon* ($n = 38$),

343 *Cephalorrhizum* ($n = 1$), *Dictyolimon* ($n = 1$) and *Goniolimon* ($n = 3$), all in the

344 Staticoideae (Fig. 1, Supporting Information Table S2).

345 Almost all species investigated were inferred to be sexual based on data from the

346 literature and the combination of pollen-stigma dimorphism with chromosome counts.

347 A few *Limonium* species ($n = 8$) were inferred to be apomictic as they exhibited

348 monomorphic pollen and stigmas and odd-numbered chromosomes (Fig. 1, Supporting

349 Information Table S2). Species of *Aegialitis* shared few of the character states with

350 other species of Staticoideae. *Aegialitis annulata* and *A. rotundifolia* lacked reciprocal

351 herkogamy, possessed monomorphic pollen and capitate stigmas and were inferred to

352 be sexual (Fig. 1, Supporting Information Table S2).

353

354 ## Character evolution

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355 The results obtained from MCMC analyses and SCM, using the two sets of 100 trees

356 differing in the position of *Limonium minutiflorum*, were very similar with respect to

357 the most likely ancestral condition of Plumbaginaceae and the number of transitions

358 between character states. Therefore, only results for one set of trees are described

359 below. Results for the second set are provided in Supporting Information Tables S5-S7.

360 Figure 2 illustrates the most likely pathways of reproductive character evolution

361 in Plumbaginaceae revealed by our analyses. The most recent common ancestor of

362 Plumbaginaceae was most likely monomorphic for sex-organ arrangement, self-

363 incompatible, pollen and stigma monomorphic with capitate stigmas and was sexual

364 (Table 1). Our analysis indicates that reciprocal herkogamy evolved at least three times

365 in the family (Table 2), and was the most likely condition of the most recent common

366 ancestor of species in subfamily Plumbaginoideae and the clade containing genera

367 *Acantholimon* and *Goniolimon* (Fig. 1). In addition, the most likely change in sex-organ

368 arrangement was the breakdown of reciprocal herkogamy to homostyly (relative rate of

369 transition: 1.80). The loss of incompatibility was estimated to have occurred at least five

370 times (4.99 ± 0.004) in the family.

371 The average number of transitions (± SE) from the ancestral monomorphic state

372 to dimorphic pollen and stigmas was 2.39 ± 0.01 and 7.55 ± 0.03, respectively. The

373 opposite pattern, involving reversion from dimorphism to monomorphism for pollen

374 and stigmas was estimated to be 9.28 ± 0.02 and 11.08 ± 0.03, respectively. The loss of

375 pollen dimorphism was estimated to be three times more likely to occur than the

376 opposite transition, whereas the loss of stigmatic papillae dimorphisms was only

377 slightly larger than their gain in the Staticoideae (Table 2). The transition from capitate

378 to filiform stigmas most likely occurred only once (1.37 ± 0.01), whereas the reversion

379 was found to be even less likely (Table 2). The change in reproductive mode from

380 sexual to asexual was restricted to *Limonium* species (Fig. 1) and we estimate that this

381 transition most likely occurred five times (5.01 ± 0.02).

382

383 Correlated evolution between characters

384 We found evidence that reciprocal herkogamy and pollen-stigma dimorphism evolved

385 independently of one another in Plumbaginaceae (BF = -21.111). In contrast, we

386 detected a strong correlation between the joint evolution of reproductive mode and

387 pollen-stigma dimorphism (BF = 12.771; Fig. 3). In subfamily Staticoideae, the shift

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388   from sexual to apomictic reproduction (q_{24} = 0.0179) appears to precede the loss of

389   pollen-stigma dimorphism (q_{21} = 0.0112) and most likely involved an intermediate stage

390   of apomixis with pollen-stigma dimorphism.

391

392   **Discussion**

393   Our comparative analysis of the evolutionary history of the origin and breakdown of the

394   heterostylous syndrome in Plumbaginaceae revealed several main findings: (1) The

395   most recent common ancestor of the family was likely self-incompatible with a uniform

396   arrangement of sex organs and monomorphic pollen and stigmas. (2) Reciprocal

397   herkogamy evolved on at least three different occasions from an ancestor with

398   monomorphic style and stamen lengths. (3) Pollen-stigma dimorphism is likely to have

399   evolved before the establishment of reciprocal herkogamy in Staticoideae. (4)

400   Transitions from pollen-stigma dimorphism to monomorphism, associated with the

401   evolution of self-compatibility and apomixis, occurred on multiple occasions in

402   *Limonium.* Our study is the first comprehensive re-evaluation of Baker's hypotheses for

403   the evolutionary buildup and breakdown of the heterostylous syndrome in

404   Plumbaginaceae. It provides some support for Baker's proposals and more broadly to

405   aspects of the selfing avoidance model for the evolution of distyly (Charlesworth &

406   Charlesworth, 1979; Fig. 2). Below, we discuss our findings and provide several

407   refinements to the evolutionary pathways originally proposed by Baker (1948a, 1966).

408

409   Evolution of reciprocal herkogamy

410   The results of stochastic character mapping indicated that reciprocal herkogamy evolved

411   on at least three occasions from a common ancestor with a monomorphic arrangement

412   of sex organs; once in Plumbaginoideae and twice in Staticoideae. Earlier phylogenetic

413   reconstructions of heterostylous groups have provided evidence for multiple origins of

414   reciprocal herkogamy in various taxa including including Boraginaceae (Ferrero *et al.*,

415   2009a; Cohen, 2013), *Linum* (Armbruster *et al.*, 2006; McDill *et al.*, 2009), *Narcissus*

416   (Pérez *et al.*, 2003; Graham & Barrett, 2004) and *Nymphoides* (Tippery & Les, 2011).

417   Concerning Plumbaginoideae, we included species from three of the four currently

418   accepted genera – *Plumbago, Dyerophytum* and *Ceratostigma* – and all investigated

419   species in these genera were distylous. Evidence from SCM suggested that the ancestral

420   condition in this subfamily is reciprocal herkogamy. The fourth genus in

13

421 Plumbaginoideae is *Plumbagella,* which is Asian in distribution and is comprised of a

422 single species, *P. micrantha* (Kubitzki, 1993). This species is annual, the corolla is very

423 reduced in size (ca. 4-5 mm; eFloras, 2008) and its monomorphic arrangement of sex

424 organs is most probably a derived condition involving the evolutionary breakdown of

425 distyly to homostyly (see Baker, 1948a, 1966), rather than an ancestral monomorphic

426 condition. In common with other homostylous taxa in heterostylous groups (Darwin,

427 1877; reviewed in Ganders, 1979; Barrett, 1989), *P. micrantha* is probably autogamous

428 and the facility for autonomous self-pollination may have enabled establishment

429 following long-distance dispersal to Asia.

430   The most likely common ancestor of subfamily Plumbaginoideae had a

431 reciprocal arrangement of sex-organs, whereas the common ancestor of all Staticoideae

432 most likely possessed a monomorphic arrangement of sex organs, although this does not

433 imply that they were necessarily selfing, as seems likely for derived homostylous taxa

434 in the family. Reciprocal herkogamy appears to have evolved at least twice from stylar

435 monomorphism in Staticoideae. *Limonium vulgare* was originally described as distylous

436 and this was thought to be the only case of reciprocal herkogamy in Staticoideae (Baker,

437 1948a, 1966). Our results support the hypothesis that reciprocal herkogamy is derived

438 from style-stamen monomorphism (and see Baker, 1948a, 1966). However, later studies

439 of *Acantholimon* (Bokhari, 1972), *Goniolimon tataricum* (Schill *et al.*, 1985) and *G.*

440 *italicum* (Morretti *et al.*, 2015), as well as our own sampling of herbarium specimens,

441 demonstrate that the occurrence of distyly is more widespread in Staticoideae than

442 originally thought, occurring in at least 12 species. *Acantholimon* and *Goniolimon* form

443 a clade (Fig. 1; and also see Lledó *et al.*, 1998, 2001, 2005) for which reciprocal

444 herkogamy is the most probable basal condition, as revealed by SCM. The lack of

445 reciprocal herkogamy in some species of *Acantholimon* is therefore likely to be a

446 derived condition, and these taxa are probably homostylous resulting from the

447 breakdown of distyly, as inferred for *Plumbagella*.

448

449 ## Pollen-stigma dimorphism and incompatibility

450 The most likely common ancestor of Plumbaginaceae had monomorphic pollen, capitate

451 stigmas and was self-incompatible. To infer the compatibility status of species in

452 Plumbaginaceae, we combined experimental evidence available from controlled hand

453 pollinations (e.g., Baker, 1953b; Dulberger, 1975a; Ferrero *et al.*, 2009b; reviewed in

454 Dulberger, 1992), and assumptions based on pollen exine ornamentation and stigmatic

455 papillae shape in Staticoideae. Species of Plumbaginoideae resemble the basal condition

456 in the family in having no obvious signs of pollen-stigma dimorphism and capitate

457 stigmas (Fig. 1, Supporting Information Table S2; Baker, 1948a,b, 1966). Although

458 there are very slight differences in exine sculpturing, as revealed by scanning electron

459 microscopy of pollen from the long- and short-styled morphs of *Dyerophyton africanum*

460 and *D. indicum* (Ghobary, 1986), these have not be considered sufficiently well

461 developed to classify *Plumbago*-type pollen as dimorphic (Erdtman, 1986).

462 Experimental tests for the presence of self-incompatibility have been conducted in some

463 species of Plumbaginoideae [e.g., *Plumbago capensis* and *P. europaea* (Dulberger,

464 1975a); *P. auriculata* (Ferrero *et al*., 2009b); *Ceratostigma willmottianum* (Dulberger,

465 1975a)], and with the exception of *Plumbagella micrantha* (Dahlgren, 1918), they were

466 all found to be self-incompatible setting no or few seeds upon self-pollination. As

467 mentioned above, self-compatibility in *P. micrantha* is considered a derived condition

468 allowing reproduction by seed in this annual, likely homostylous, species (see Baker,

469 1948a).

470   In contrast, pollen-stigma dimorphism is highly developed in Staticoideae,

471 except for *Aegialitis*. The only two species in this genus, *A. annulata* and *A.*

472 *rotundifolia*, are distinct from the remaining Staticoideae in having *Plumbago*-type

473 pollen (Baker, 1966; Ghobary, 1984) and monomorphic capitate stigmas (Baker, 1966;

474 Lledó *et al.*, 2001). Indeed, this genus is probably one of the earliest branching lineages

475 in Staticoideae (Baker, 1948a,b, 1966). More recently, the phylogenetic position of

476 *Aegialitis* has been investigated by parsimony analyses and the conclusion reached that

477 the genus constitutes a distinct lineage within Staticoideae (Lledó *et al*., 2001), which

478 has diverged from the most likely common ancestor before pollen-stigma dimorphism

479 evolved in the subfamily (and see Baker, 1948a,b, 1966).

480   Stochastic character mapping revealed a single origin of pollen-stigma

481 dimorphism in Plumbaginaceae that was "superimposed" on a previously established

482 self-incompatibility system. This may have occurred soon after the divergence of the

483 clade containing *Aegialitis* in Staticoideae. Investigations in *Limonium* (Baker, 1953a;

484 Dulberger, 1975a) and *Armeria maritima* (Iversen, 1940; Dulberger, 1975a; Mattsson,

485 1983; Costa *et al*., 2017) have indicated that species with pollen-stigma dimorphism

486 possess heteromorphic incompatibility, a widespread association in most heterostylous

487 families (Dulberber, 1992). In addition to marked differences in exine sculpturing,

15

488 pollen types A and B also differ in their lipid composition (Mattsson, 1983), which

489 probably plays a key role in the initial interaction between pollen and stigmas, during

490 the adhesion phase (Mattsson, 1983; Heslop-Harrison & Heslop-Harrison, 1985).

491 Heteromorphic incompatibility and pollen and stigma polymorphisms are tightly

492 associated in this subfamily and both physiological and morphological characters

493 appear to participate in the rejection of self-pollen (Dulberger, 1975a,b; Costa *et al.*,

494 2017), a situation that does not occur in species with homomorphic incompatibility

495 (reviewed in Franklin-Tong, 2008), where *S*-gene protein specificities govern self-

496 rejection.

497

498 ## The breakdown of heterostyly to uniparental reproduction

499 The shift from obligate outcrossing to predominant selfing is generally considered the

500 most common mating-system transition in angiosperms (Stebbins, 1974), with selfing

501 commonly selected when it confers reproductive assurance (Lloyd, 1992), especially

502 after long-distance dispersal and during repeated colonization episodes (Baker, 1955,

503 1959, 1967; Pannell *et al.*, 2015). Our analysis of the distribution of characters on

504 phylogenetic trees suggests that the transition from pollen-stigma dimorphism and self-

505 incompatibility to monomorphism and self-compatibility most likely occurred at least

506 four times independently in *Limonium* (Fig. 1). In addition, pollen-stigma

507 monomorphism and self-compatibility are also reported for *Armeria maritima* subsp.

508 *sibirica* (Baker, 1948c, 1966) and some *Limonium* species from North America and

509 Australia (Baker, 1953a,b), but these were not included in our character mapping.

510 Monomorphic self-compatible species most frequently produce type A pollen and have

511 papillate stigmas, whereas the combination B/cob has only been found in the annual

512 Mediterranean species *L. echioides.* It is probable that in *Limonium* self-compatibility

513 has arisen in association with dispersal events from Europe (Baker, 1953a,b, 1959),

514 especially from the western Mediterranean, which is the main center of diversification

515 of the genus (Erben, 1993; Kubitzki, 1993), to America and Australia, where species

516 with pollen-stigma monomorphism and self-compatibility are most frequent (Baker,

517 1953a,b). However, these hypotheses concerning migration need to be substantiated by

518 phylogeographical analyses, which should provide valuable insights on the

519 biogeography of reproductive transitions in this group.

16

520   In addition to sexual mating strategies involving outcrossing or selfing, asexual

521   reproduction via apomixis has been described in several *Limonium* species (e.g.,

522   D'Amato, 1940, 1949; Baker, 1953a, 1966; Erben, 1979; Ingrouille & Stace, 1985;

523   Cowan *et al.*, 1998; Róis *et al.*, 2016). Apomictic species are most commonly polyploid

524   (Asker & Jerling, 1992; Carman, 1997), which is also the case for apomictic *Limonium*

525   (e.g., D'Amato, 1949; Erben, 1979; Cowan *et al.*, 1998; Caperta *et al.*, 2016; Róis *et al.*,

526   2016). Apomixis is often associated with hybridization between species and can

527   overcome the sexual sterility that is a feature of many interspecific hybrids (Asker &

528   Jerling, 1992). Reproductive barriers between species of *Limonium* are often weak and

529   hybridization is frequent (Erben, 1993). The shift from sexual to asexual reproduction

530   via apomixis in Plumbaginaceae most likely preceded the loss of pollen-stigma

531   dimorphism. High transition rates between apomictics with pollen-stigma dimorphisms

532   and those with monomorphic pollen and stigmas suggest that this transition most likely

533   involved an unstable intermediate condition of apomictics with dimorphic pollen and

534   stigmas. Indeed, there is no report in the literature of this condition in Plumbaginaceae

535   suggesting rapid loss of pollen-stigma dimorphism following shifts from sexual to

536   apomictic reproduction. *Limonium* apomictics have monomorphic pollen and stigmas

537   with combinations A/cob or B/papillate, which would normally involve a self-

538   incompatible pollen-stigma combination. In addition, pollen production per flower is

539   very low and pollen grains are mainly aborted (Baker, 1966; Erben, 1979; Cowan *et al.*,

540   1998). As previously reported in other angiosperm taxa (e.g., Souza *et al.*, 2012; Aliyu

541   *et al.*, 2013), the character mapping in our study revealed multiple independent origins

542   of apomixis in lineages composed of mainly sexual species. The mixture of sexual and

543   asexual reproductive strategies in *Limonium* may have contributed to the diversification

544   of this genus in the western Mediterranean (Lledó *et al.*, 2005) and also to its taxonomic

545   complexity.

546

547   The evolutionary buildup of the heterostylous syndrome

548   Our findings are generally in accord with most of Baker's hypotheses (Baker, 1948a,

549   1966) on the evolutionary buildup and breakdown of the heterostylous syndrome. Our

550   analyses revealed two distinct pathways associated with the assembly of the

551   heterostylous syndrome; a relatively simple one for Plumbaginoideae and a more

552   complex pathway for Staticoideae (Figs. 1, 2). In Plumbaginoideae, reciprocal

17

553 herkogamy evolved in a self-incompatible ancestor without the development of pollen-

554 stigma dimorphism (Figs. 1, 2). By contrast, in Staticoideae the first step in the

555 evolution of heterostyly was the acquisition of pollen-stigma dimorphism in a self-

556 incompatible ancestor followed by the evolution of reciprocal herkogamy. In contrast to

557 Staticoideae, ancillary characters are not exceptionally well-developed in some

558 heterostylous species (Dulberger, 1992), and this is the most likely reason why previous

559 phylogenetic reconstructions of heterostylous lineages have not examined the

560 evolutionary history of pollen-stigma dimorphisms. Our results suggest that pollen and

561 stigma dimorphisms have evolved together in concert rather than sequentially. In

562 contrast, Baker (1948a, 1966) proposed that pollen dimorphism evolved first in a self-

563 incompatible species with monomorphic stigmas (e.g. *Goniolimon*, *Acantholimon* and

564 some *Limonium*) and stigma polymorphism evolved subsequently. However, this order

565 seems unlikely as later investigations of *Goniolimon* (Schill *et al.*, 1985; Morretti *et al.*,

566 2015) and *Acantholimon* (Bokhari, 1972), and our own observations of herbarium

567 specimens, have demonstrated that taxa in these genera all exhibit pollen-stigma

568 dimorphism and we found no evidence of any species with only pollen dimorphism. In

569 fact, pollen-stigma dimorphism is widespread in Staticoideae, and with the exception of

570 *Aegialitis*, monomorphic pollen and stigmas are most likely derived and associated with

571 the transition from outcrossing to selfing or apomixis. Our results further suggest that

572 reciprocal herkogamy most likely resulted from two independent origins after the

573 establishment of pollen-stigma dimorphism in the clade formed by *Acantholimon* and

574 *Goniolimon*, and separately in *Limonium*.

575   The findings of this study have general relevance for competing models of the

576 evolution of distyly. One of the main findings of our reconstructions is that reciprocal

577 herkogamy evolved after the establishment of heteromorphic self-incompatibility in

578 Plumbaginaceae. This is the order of establishment proposed in the inbreeding

579 avoidance model of Charlesworth & Charlesworth (1979) but is the opposite polarity to

580 that predicted in the pollen transfer model of Lloyd & Webb (1992b). Our results

581 therefore represent the first comprehensive comparative evidence on the evolution of

582 distyly supporting the sequence proposed in the Charlesworth & Charlesworth (1979)

583 model.

584   Despite the difference in polarities of the two models for the evolution of

585 distyly, both involve a transient polymorphic stage of stigma-height dimorphism on the

586 pathway from stylar monomorphism to distyly. Stigma-height dimorphism occurs in

18

587 species of *Narcissus* (Graham & Barrett, 2004), *Lithodora* and *Glandora* (Ferrero *et al*.,

588 2009a, 2012), genera in which heterostylous species also occur; however, we found no

589 evidence that any species of Plumbaginaceae possesses this form of stylar

590 polymorphism. Stigma-height dimorphism is rather infrequent among distylous families

591 and it is quite possible that this putative intermediate stage is ephemeral and is rapidly

592 replaced once distyly evolves in a lineage (reviewed in Barrett *et al.*, 2000). Given the

593 numerous independent origins of the heterostylous floral syndrome across angiosperm

594 families, it is possible that features of both models of the evolution of heterostyly are

595 correct for specific lineages. Future comparative work on the evolutionary history and

596 molecular genetic architecture of characters in the heterostylous syndrome will

597 undoubtedly shed light on which of the two pathways by which heterostyly evolves is

598 more commonly followed and what reproductive and genetic features of lineages cause

599 the particular pathway that is taken to occur.

600

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611

612 Author contributions

613 J.C., R.T. and S.C.H.B. designed the research questions, J.C. conducted data collection,

614 J.C. and R.T. performed data analyses, J.C. wrote the paper with input and revisions

615 from all co-authors.

616

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857 **Figure captions**

858

859 **Figure 1** Phylogenetic distribution of the six characters investigated in Plumbaginaceae.

860 Subfamily Plumbaginoideae is represented by the clade containing *Plumbago*, whereas

861 subfamily Staticoideae is represented by the clades containg the genera *Acantholimon*,

862 *Goniolimon*, *Armeria*, *Psylliostachys* and *Limonium*. **(a)** Reciprocal herkogamy: black –

863 reciprocal herkogamy, grey – style-stamen height monomorphism. **(b)** Incompatibility

864 system: blue – self-incompatible, yellow – self-compatible. **(c)** Exine sculpturing: green

865 – monomorphic, red - *Armeria*-type pollen. **(d)** Stigmatic papillae condition: dark blue –

866 dimorphic, light blue – monomorphic. **(e)** Stigma morphology: brown – capitate, light

867 pink – filiform, dark pink – others (i.e., fimbriate, pear-shaped, lanceolate). **(f)**

868 Reproductive mode: yellow – sexual, orange – apomictic. In all cases, white represents

869 missing information for a given species. Pie diagrams show the posterior probability of

870 reciprocal herkogamy (black) vs style-stamen height monomorphism (grey), self-

871 incompatibility (blue) vs self-compatibility (yellow), monomorphic (green) vs *Armeria*-

872 type pollen (red), and dimorphic (dark blue) vs monomorphic (light) stigmas for a given

873 node.

874

875 **Figure 2** Evolutionary buildup and breakdown of heterostyly in Plumbaginaceae: **(c)-**

876 **(d)** subfamily Plumbaginoideae, **(e)-(g)** subfamily Staticoideae. Steps (a)-(c) correspond

877 to the selfing avoidance model of Charlesworth & Charlesworth (1979); stigma-height

878 dimorphism is omitted as there is no evidence of this intermediate stage in the family.

879 Hypothesized selective forces are indicated in italics next to solid arrows, which

880 indicate transitions between states. Dashed arrows in (c) and (g) indicate compatible

881 pollinations. **(a)** Self-compatible (SC) ancestor with stigmas and anthers at the same

882 height. **(b)** Evolution of heteromorphic incompatibility with two mating groups with

883 monomorphic sex organs, pollen and stigmas. **(c)** Evolution of distyly, heteromorphic

884 incompatibility and monomorphic pollen and stigmas in *Plumbago*, *Dyerophytum* and

28

885 *Cerastostigma* species. **(d)** Evolution of homostyles, SC with monomorphic pollen and

886 stigmas and smaller-flowered *Plumbagella micrantha*. **(e)** Evolution of pollen-stigma

887 dimorphism in *Acantholimon*, *Goniolimon*, *Armeria*, *Psylliostachys*, *Ceratolimon*,

888 *Limoniastrum* and *Limonium* species. Top line, left to right: pollen type A, pollen type

889 B. Bottom line, left to right: cob stigmas, papillate stigmas. Pollen and stigma images

890 from Figs. 1-2 in Costa *et al*. (2017). Transition to homostyly in Staticoideae is based

891 on Baker (1948c). **(f)** Apomictic small-flowered species of *Limonium* possessing A/cob

892 and B/papillate pollen-stigma combinations. **(g)** Distylous, SI and pollen-stigma

893 dimorphism in *Limonium vulgare, Acantholimon* and *Goniolimon* species. Downwards

894 arrow from (g) to (e) indicates the breakdown of distyly in *Acantholimon* species.

895

896 **Figure 3** Transition rates between the four character-state combinations of reproductive

897 mode and pollen-stigma dimorphism for species of Plumbaginaceae in the phylogeny (*n*

898 = 121 species). For seven species, there was no information regarding character state-

899 combinations considered. q_{ij} – transition from state *i* to state *j*; reproductive system: Sex

900 – sexual, A – apomictic; pollen-stigma dimorphism: M – monomorphic pollen and

901 stigmas, PSD – pollen-stigma dimorphism; *n* – number of taxa in each character state

902 combination.

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905 **Supporting Information**

906 **Fig. S1** Ultrametric maximum likelihood phylogenetic tree of Plumbaginaceae.

907 **Methods S1** Characterization of the floral polymorphism and associated characters.

908 **Table S1** Information on each herbarium specimen sampled for data on reciprocal

909 herkogamy and ancillary characters.

910 **Table S2** Character states used in the ancestral character state reconstruction of

911 Plumbaginaceae.

912 **Table S3** GenBank accession number for all taxa included in the supermatrix for

913 maximum likelihood analysis of Plumbaginaceae.

914 **Table S4** Models of substitution rate variation among branches and values of the

915 smoothing parameter, lambda, tested for creating an ultrameric tree of Plumbaginaceae.

916   **Table S5** Support for models of character trait evolution for the most recent common

917   ancestor of the Plumbaginaceae following Bayesian analyses (BIC) and stochastic

918   character mapping (PP) for the second set of trees.

919   **Table S6** Rate of change between states for the six characters investigated for the

920   second set of 100 trees of Plumbaginaceae.

921   **Table S7** Support for models of correlated evolution between traits in Plumbaginaceae

922   and the values of transition rates (q_{ij}) obtained from the models.

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923 **Table 1** Support for models of character state evolution for the most recent common

924 ancestor of the Plumbaginaceae following Bayesian Markov chain Monte Carlo

925 analyses (BIC) and stochastic character mapping (PP).

Model	BIC	PP
(a) Sex-organ arrangement, ER		
Monomorphism	67.622	0.966
Reciprocal herkogamy	72.706	0.034
(b) Incompatibility status, ARD		
Self-incompatible	50.052	1.000
Self-compatible	55.480	0.000
(c) Exine sculpturing, ER		
Monomorphic	106.564	0.773
Dimorphic (i.e., *Armeria*-type)	108.670	0.227
(d) Stigmatic papillae shape, ER		
Monomorphic	99.594	0.570
Dimorphic	100.444	0.430
(e) Reproductive mode, ARD		
Sexual	45.518	1.000
Apomictic	51.102	0.000
(f) Stigma morphology, ER		
Filiform	67.382	0.091
Capitate	63.754	0.881
Others	69.968	0.027

926 ER – Equal rates model, ARD – All rates different model, BIC – Bayesian Information Criterion, PP –

927 Posterior Probability.

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932   **Table 2** Number, frequency and rate of change between states for the six characters

933   investigated for the first set of 100 trees of Plumbaginaceae.

Character state	No. of transitions	Frequency of transition	Relative rate of transition
(a) Sex-organ arrangement			
Monomorphic to RH	3.70 ± 0.01	0.57	0.75
RH to homostylous	2.83 ± 0.01	0.43	1.80
(b) Incompatibility status			
SI to SC	4.99 ± 0.003	0.99	1.02
SC to SI	0.05 ± 0.01	0.01	0.35
(c) Exine sculpturing			
Armeria-type to monomorphic	9.28 ± 0.02	0.80	1.42
Monomorphic to *Armeria*-type	2.39 ± 0.01	0.20	0.47
(d) Stigmatic papillae shape			
Dimorphic to monomorphic	11.08 ± 0.03	0.59	1.06
Monomorphic to dimorphic	7.55 ± 0.03	0.41	0.92
(e) Reproductive mode			
Apomictic to sexual	0.29 ± 0.03	0.05	0.95
Sexual to apomictic	5.01 ± 0.02	0.95	6.88
(f) Stigma morphology			
Filiform to capitate	0.86 ± 0.03	0.14	0.33
Filiform to others	0.58 ± 0.03	0.10	0.22
Capitate to filiform	1.37 ± 0.03	0.23	0.54
Capitate to others	1.31 ± 0.04	0.22	0.51
Others to filiform	0.75 ± 0.02	0.13	1.01
Others to capitate	1.08 ± 0.03	0.18	1.45

934   Values are mean ± SE. RH – reciprocal herkogamy, SI – self-incompatible, SC – self-compatible. For

935   details of the calculation of frequency of transitions and relative rates of transition see *Material and*

936   *Methods*.

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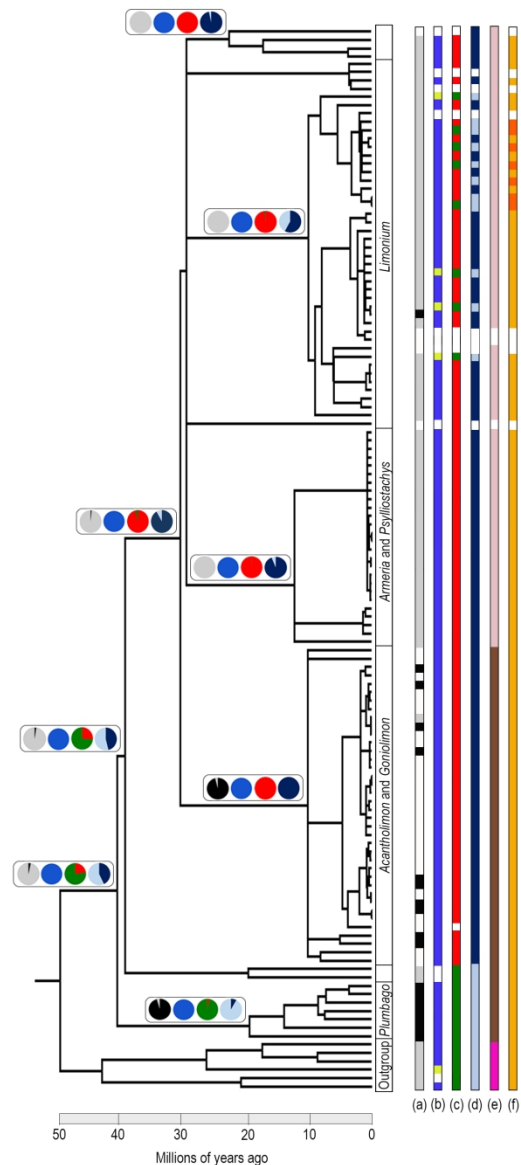
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Figure 1 Phylogenetic distribution of the six characters investigated in Plumbaginaceae. Subfamily Plumbaginoideae is represented by the clade containing *Plumbago*, whereas subfamily Staticoideae is represented by the clades containg the genera *Acantholimon*, *Goniolimon*, *Armeria*, *Psylliostachys* and *Limonium*. **(a)** Reciprocal herkogamy: black – reciprocal herkogamy, grey – style-stamen height monomorphism. **(b)** Incompatibility system: blue – self-incompatible, yellow – self-compatible. **(c)** Exine sculpturing: green – monomorphic, red - *Armeria*-type pollen. **(d)** Stigmatic papillae condition: dark blue – dimorphic, light blue – monomorphic. **(e)** Stigma morphology: brown – capitate, light pink – filiform, dark pink – others (i.e., fimbriate, pear-shaped, lanceolate). **(f)** Reproductive mode: yellow – sexual, orange – apomictic. In all cases, white represents missing information for a given species. Pie diagrams show the posterior probability of reciprocal herkogamy (black) vs style-stamen height monomorphism (grey), self-incompatibility (blue) vs self-compatibility (yellow), monomorphic (green) vs *Armeria*-type pollen (red), and dimorphic (dark blue) vs monomorphic (light) stigmas for a given node.
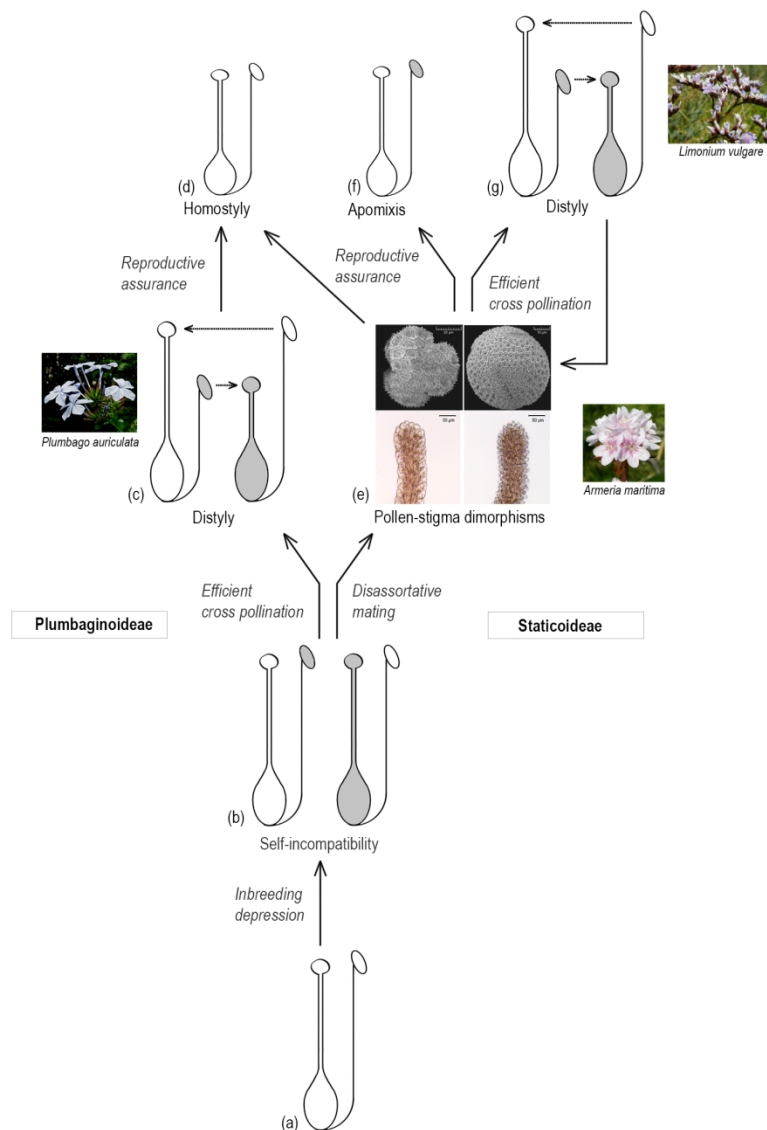
Figure 2 Evolutionary buildup and breakdown of heterostyly in Plumbaginaceae: **(c)-(d)** subfamily Plumbaginoideae, **(e)-(g)** subfamily Staticoideae. Steps (a)-(c) correspond to the selfing avoidance model of Charlesworth & Charlesworth (1979); stigma-height dimorphism is omitted as there is no evidence of this intermediate stage in the family. Hypothesized selective forces are indicated in italics next to solid arrows, which indicate transitions between states. Dashed arrows in (c) and (g) indicate compatible pollinations. **(a)** Self-compatible (SC) ancestor with stigmas and anthers at the same height. **(b)** Evolution of heteromorphic incompatibility with two mating groups with monomorphic sex organs, pollen and stigmas. **(c)** Evolution of distyly, heteromorphic incompatibility and monomorphic pollen and stigmas in *Plumbago*, *Dyerophytum* and *Cerastostigma* species. **(d)** Evolution of homostyles, SC with monomorphic pollen and stigmas and smaller-flowered *Plumbagella micrantha*. **(e)** Evolution of pollen-stigma dimorphism in *Acantholimon*, *Goniolimon*, *Armeria*, *Psylliostachys*, *Ceratolimon*, *Limoniastrum* and *Limonium* species. Top line, left to right: pollen type A, pollen type B. Bottom line, left to right: cob stigmas, papillate stigmas. Pollen and stigma images from Figs. 1-2 in Costa *et al.* (2017). Transition to homostyly in Staticoideae is based on Baker (1948c). **(f)** Apomictic small-flowered species of *Limonium* possessing A/cob and B/papillate pollen-stigma combinations.

(g) Distylous, SI and pollen-stigma dimorphism in *Limonium vulgare*, *Acantholimon* and *Goniolimon* species. Downwards arrow from (g) to (e) indicates the breakdown of distyly in *Acantholimon* species.
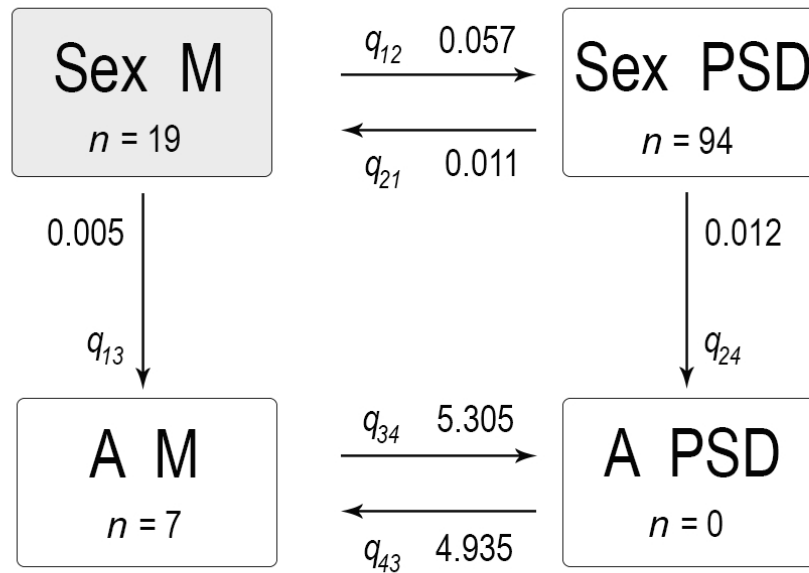
Figure 3 Transition rates between the four character-state combinations of reproductive mode and pollen-stigma dimorphism for species of Plumbaginaceae in the phylogeny ($n = 121$ species). For seven species, there was no information regarding character state-combinations considered. qij – transition from state i to state j; reproductive system: Sex – sexual, A – apomictic; pollen-stigma dimorphism: M – monomorphic pollen and stigmas, PSD – pollen-stigma dimorphism; n – number of taxa in each character state combination.