Evolution of morphological and climatic adaptations in *Veronica L.* (Plantaginaceae)

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Perennials and annuals apply different strategies to adapt to the adverse environment, based on 'tolerance' and 'avoidance', respectively. To understand lifespan evolution and its impact on plant adaptability, we carried out a comparative study of perennials and annuals in the genus *Veronica* from a phylogenetic perspective. The results showed that ancestors of the genus *Veronica* were likely to be perennial plants. Annual life history of *Veronica* has evolved multiple times and subtrees with more annual species have a higher substitution rate. Annuals can adapt to more xeric habitats than perennials. This indicates that annuals are more drought-resistant than their perennial relatives. Due to adaptation to similar selective pressures, parallel evolution occurs in morphological characters among annual species of *Veronica*.

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7   **Abstract:** Perennials and annuals apply different strategies to adapt to the adverse environment, based on 'tolerance' and

8   'avoidance', respectively. To understand lifespan evolution and its impact on plant adaptability, we carried out a comparative

9   study of perennials and annuals in the genus *Veronica* from a phylogenetic perspective. The results showed that ancestors of the

10 genus *Veronica* were likely to be perennial plants. Annual life history of *Veronica* has evolved multiple times and subtrees with

11 more annual species have a higher substitution rate. Annuals can adapt to more xeric habitats than perennials. This indicates that

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13 evolution occurs in morphological characters among annual species of *Veronica*.

14   INTRODUCTION

15   Flowering plants have repeatedly evolved a shorter life history of less than a year, with a record of less

16 than three weeks from germination to seed set (Cloudsley-Thompson & Chadwick, 1964). The evolution of

17 annual life cycles is combined with a monocarpic habit (i.e. death of the plant after first and only reproduction).

18 Such plants are called annuals irrespective of considerable differences in their life histories (Mortimer, Hance

19 & Holly, 1990) related to different ecology and habitats. The independent evolution of annuality in more than

20 100 different families from more than 30 orders of angiosperms (sensu APG IV, 2016) and often even multiple

21 times independently among closely related species (e.g. Albach, Martinez-Ortega & Chase, 2004; Andreasen

22 & Baldwin, 2001; Hellwig, 2004; Jakob, Meister & Blattner, 2004; Kadereit, 1984) has made characterization

23 of the annual habit difficult. Furthermore, the necessity to complete the life cycle within one season puts

24 enormous constraints on plants that evolutionarily resulted in reduction in size to reach reproductive age faster

25 and more reliably. Such a scenario has led to convergent evolution in several traits in annuals, especially a

26 selfing breeding system but also a variety of other morphological, physiological, karyological and genomic

27 traits (Silvertown & Dodd, 1996). This widespread convergence has given rise to misconceptions about the

28 evolution of annuals, particularly in cases when a rigorous phylogenetic hypothesis is lacking and comparative

29 methods are not employed (Albach, Martinez-Ortega & Chase, 2004).

30   Several environmental factors that are not mutually exclusive can cause circumstances under which

31 annuals have advantages over perennials, and most of these are related to the ability of annuals to survive

32 unfavorable periods as seeds. Proposed factors include seasonal stress such as drought (Macnair, 2007; Whyte,

33 1977), heat (Evans et al., 2005), frost (Tofts, 2004; Whyte, 1977), unpredictable environment (Stearns, 1976),

34 grazing/seed predation (Klinkhamer, Kubo & Iwasa, 1997; Vesk, Leishman & Westoby, 2004), flooding

35 (Kadereit, Mucina & Freitag, 2006), limited maternal resources (Hensel et al., 1994), low competition (Lacey,

36 1988) and escape from pathogens over time (Clay & van der Putten, 1999; Thrall, Antonovics & Hall, 1993).

37 Even anthropogenic selection factors such as regular mowing and cultivation techniques may induce annual

38 life history (Baker, 1974; Hautekèete, Piquot & Van Dijk, 2002). Therefore, it is often unclear whether

39 evolutionary change is associated with annual life history per se or whether it is a reaction to a specific

40 environmental condition. Advances in phylogeny reconstruction and comparative analyses allow investigation

41   of the processes and the pattern of life history variation in more detail. Whereas a number of taxa have been
42   analyzed in detail to infer the number of origins of annual life history and infer climatic circumstances of the
43   shifts (e.g. Datson, Murray & Steiner, 2008; Turini, Bräuchler & Heubl 2010) few employed rigorous
44   comparative methods to analyze these shifts in life history. For example, Drummond et al. (2012)
45   demonstrated increased speciation rates in derived montane perennial clades of *Lupinus* compared to lowland
46   annuals. Ogburn & Edwards (2015) found perennials occupying cooler climatic niches than related annuals.

47   *Veronica* is a good model system to investigate this issue since annual life history has been shown to have
48   evolved with convergent morphological characteristics multiple times in the same geographical region (Albach,
49   Martinez-Ortega & Chase, 2004). *Veronica* comprises about 450 species and is the largest genus in the
50   flowering plant family Plantaginaceae (Albach & Meudt, 2010). Most species—including all annuals—are
51   distributed in the Northern Hemisphere, but there is also an additional prominent radiation in the Australasian
52   region (but without annuals). Life forms include herbaceous annuals or perennials, and also shrubs or small
53   trees. About 10% of *Veronica* species are annuals, a life history which has originated at least six times
54   independently in the genus (Albach, Martinez-Ortega & Chase, 2004). Chromosome numbers, phytochemistry
55   and DNA sequence data support the polyphyly of annuals in the genus (Albach & Chase, 2001; Müller &
56   Albach, 2010). However, despite the fact that many species of *Veronica* are widespread in accessible regions
57   of the world, climate data has, thus far, not been included in any analysis of the genus. Also, morphological
58   characters were mostly mapped on phylogenetic trees (e.g., Albach, Martinez-Ortega & Chase, 2004) but not
59   included in a comparative analysis. Thus, crucial information to understand the evolution of the genus has,
60   thus far, been excluded from analyses. In this study, we implemented a comparative analysis of morphological
61   and climate data using phylogenetic methods to address the following two questions: 1) What convergent
62   morphological trends are displayed in annuals? 2) Are there climatic factors that may favor annual life history?
63   By answering these questions, we aim to expand our understanding of the evolution of life history and its
64   impact on the adaptability of plants. More specifically, we address the hypothesis that annual life history and
65   selfing evolved in parallel in adaptation to drought. Therefore, we tested a correlation of life history with a
66   number of characters, such as corolla diameter, known to be correlated with selfing in *Veronica* (Scalone, Kolf
67   and Albach, 2013) and contrasted these with characters considered unrelated to mating system, such as leaf
68   length. For environmental parameters, we specifically tested a number of bioclimatic parameters associated
69   with precipitation and temperature to test the alternative hypothesis that annual life history is related to hot
70   temperature. By including a range of morphological and climatological data, we want to infer more exactly,
71   which characters are associated with the annual-selfing-syndrome.
72
73   MATERIAL AND METHODS
74   A total of 81 individuals representing 81 species and all 12 subgenera of *Veronica*, were used to establish
75   the phylogenetic tree in this study. Of these, seqences from 67 species were downloaded from GenBank from
76   previous studies (Albach & Meudt, 2010), whereas sequences from 14 species, which were collected in
77   Xinjiang Province of China, were newly generated for this study (see Supplementary Table 1). Six individuals
78   of five other genera of Veroniceae (*Lagotis*, *Picrorhiza*, *Wulfeniopsis*, *Wulfenia*, and *Veronicastrum*) were
79   designated as outgroups. Genomic DNA extraction and purification was carried out using commercial kits
80   according to manufacturer's instructions (D2485-02, OMEGA bio-tek). In accordance with the methods of
81   Albach & Meudt (2010), we carried out PCR, sequencing and phylogenetic tree reconstruction. DNA

82 sequences of four regions were PCR-amplified, including nuclear ribosomal internal transcribed spacer region
83 (ITS) with primers ITSA (Blattner, 1999) and ITS4 (White et al., 1990), plastid DNA (cpDNA) *trnL-trnL-trnF*
84 with primers c and f (Taberlet et al., 1991), *rps16* with primers rpsF and rpsR2 (Oxelman, Lidén & Berglund,
85 1997), *psbA-trnH* with primers psbA (Sang, Crawford & Stuessy, 1997) and trnH (Tate & Simpson, 2003). A
86 PCR program of 95° C for 2 min, 36 cycles of: 95° C for 1 min, 50-55° C for 1 min, and 72° C for 1.5–2
87 min, and finally 72° C for 5 min and 10° C hold, was used for all markers. DNA sequencing was performed
88 by Sangon Biotech Co., Ltd (Shanghai, PR China). Bayesian inference methods were used to analyze the
89 combined data set. Best fitting substitution models for the datasets were inferred using jModelTest 2.1.7
90 (Darriba, 2012). The Bayesian inference tree was built using MrBayes 3.2.5 (Ronquist et al., 2012) with the
91 GTR+Γ model using the Markov chain Monte Carlo (MCMC) for 1,000,000 generations with a burn-in of
92 250,000. The posterior probability (PP) was used to estimate nodal robustness. The stationarity of the runs was
93 assessed using Tracer version 1.6 (Rambaut et al., 2014). We approximated divergence times using the
94 function chronopl in the R package "ape" (Paradis et al., 2015).

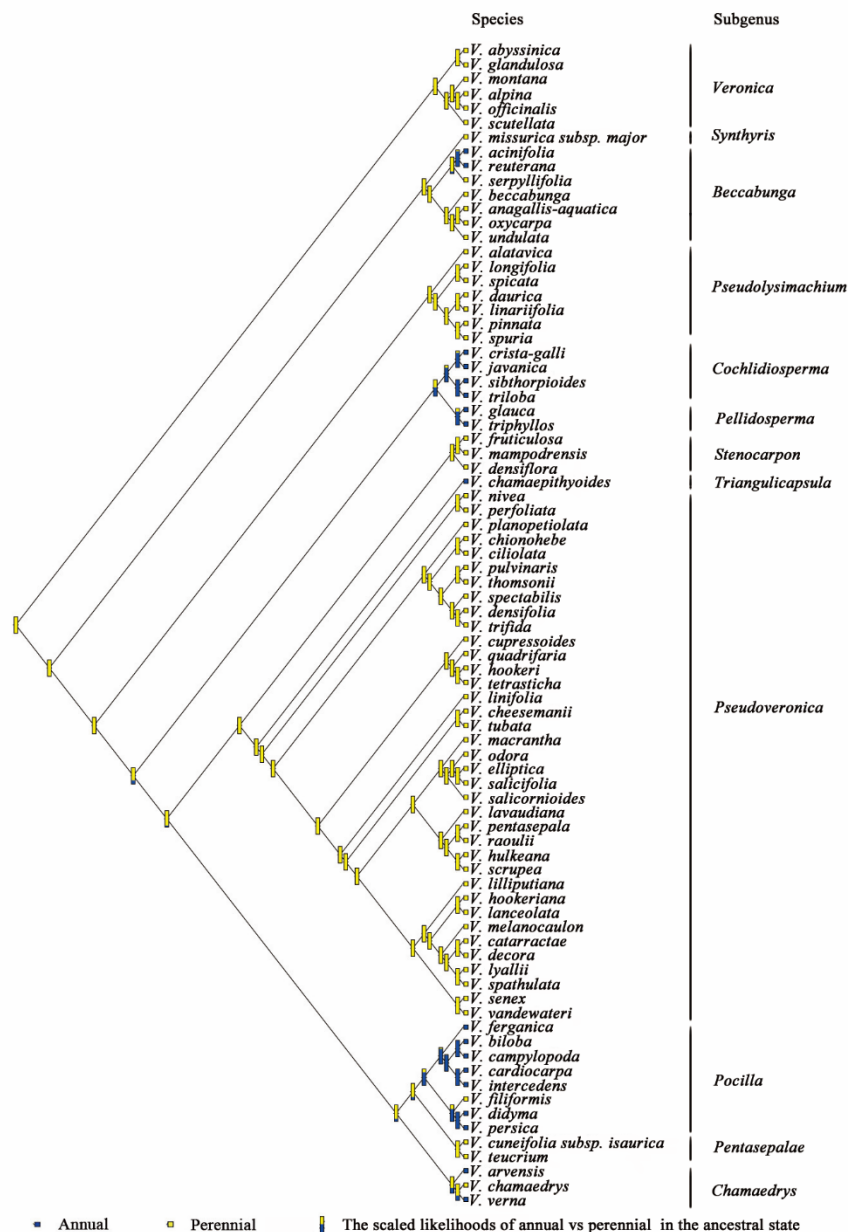
95 We obtained morphological traits from field measurements and referenced from various flora, such as
96 Flora of China (Hong & Fischer, 1998), Flora d´Italia (Pignatti, 1982), Flora of New Zealand (Allan, 1961),
97 New Zealand Plant Conservation Network (http://nzpcn.org.nz/default.aspx). Plant traits were coded for each
98 species according to characters and character states used by Saeidi−Mehrvarz & Zarre (2004). In total 9 binary
99 characters about resource acquisition and reproductive characteristics were taken into consideration (character
100 states and scoring matrix were shown in Supplementary Table 2 and 3).

101 We obtained GPS latitude/longitude data from the GBIF website (http://www.gbif.org/) for up to 500
102 occurrence records for each species using the function occ in the R package "spocc" (Chamberlain, Ram &
103 Hart, 2016). Invalid, low accuracy or duplicate data were removed. GPS data of species collected by us were
104 also added to the analysis. Bioclimatic variables were obtained for each of the geographical coordinates from
105 WorldClim (www.worldclim.org) and processed using ArcGIS version 10.0. Climate data from each locality
106 was acquired using the toolbox function "Extract Values to Points" and average values for each bioclimatic
107 variable was calculated for each species. Drought and heat can affect annual and perennial relative fitness
108 (Macnair, 2007; Whyte, 1977; Evans et al., 2005; Pérez‐Camacho, 2012), and 7 related bioclimatic variables
109 were selected for analysis (GBIF localities and corresponding climate data, average data were shown in
110 Supplementary Table 4 and 5).

111 We used the function ace in the R package "ape" (Paradis et al., 2015) to estimate ancestral character
112 states and the associated uncertainty for life history. Additionally, we also calculated phylogenetic signal using
113 the function phylo.d in the package ''caper'' (Orme et al., 2012). The R package "iteRates" was used to
114 implement the parametric rate comparison test and visualize areas on a tree undergoing differential substitution
115 (Fordyce, Shah & Fitzpatrick, 2014). We have conducted phylogenetic comparative analysis. The function
116 binary PGLMM in the R package "ape" were used to perform comparative tests of morphological traits
117 between annual and perennial plants. We tested climate data differences between annual and perennial plants
118 using the function aov.phylo in the package "geiger" (Harmon et al., 2008).

119 RESULTS

120 Phylogenetic relationships of *Veronica* from Bayesian inference of the four-marker dataset is shown in
121 Supplementary Fig.1. The result of Bayesian phylogenetic analyses was assessed using Tracer with all ESSs
122 >200 (after discarding a burn-in of 25%). The main clades of phylogenetic tree were consistent with previous

123   studies. The evolution and inferred ancestral life history in *Veronica* is shown in Figure 1. Scaled likelihood of
124   perennial life history at the root was 0.99. The D value as calculated in caper is a measure of phylogenetic
125   signal in a binary trait, for which a value smaller than 0 indicates high correlation of the trait with phylogenetic
126   differentiation and greater than 1 corresponds to a random or convergent pattern of evolution. The value of D
127   for life history was -0.55, thus demonstrating relatively strong phylogenetic conservatism. This implies that
128   lifespan is a relatively conservative trait and the change from perennial to annual, despite seven origins in the
129   genus, is not a frequent occurrence.

130

131   Figure 1 Ancestral state reconstruction of life history in *Veronica*. Proportional likelihoods for character states of ancestral life history are shown for nodes.

132   Phylogenetic relationship of *Veronica* was built by Bayesian inference based on four-marker dataset. For Bayesian posterior probabilities, see

133   Supplementary Fig.1.

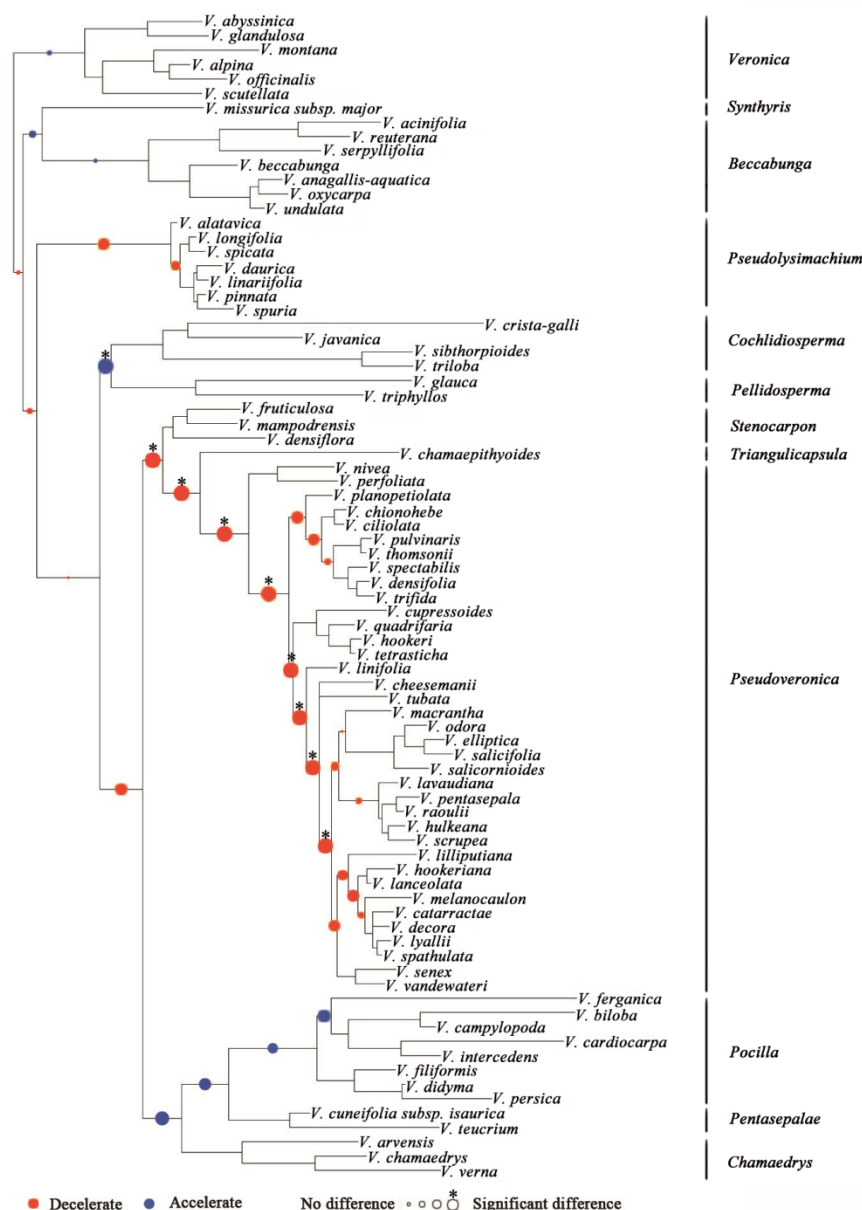
134   Substitution rates (as measured by branch lengths) differ among clades within *Veronica* (Fig. 2). In

135   general, clades with more annual species have faster substitution rates. The only significant increase in

136   substitution rates subtends the clade of annual subgenera *Cochlidiosperma* and *Pellidosperma*, whereas most

137   of the significant decreases in substitution rates are associated with the evolution of the perennial, Australasian

138   subgenus *Pseudoveronica*.

139

140   Figure 2 Shifts in substitution rates in *Veronica* as assessed by the distribution of branch lengths among clades. The blue nodes mean that substitution rates

141   of that clade are faster than that of the remainder tree, whereas red nodes express the opposite meaning. The sizes of the colored nodes indicate the

142   likelihood of rate-shifts.*The asterisk means that a rate-shift is significant. The results are based on limited sampling (<20%).

143   There are obvious differences in some morphological traits between annual and perennial plants (Table 1).
144   Analysis of the morphological traits measured here shows that perennials have larger leaves, longer stamens
145   and larger corollas, whereas annuals tend to have larger bracts and capsules with deeply emarginated apices.

146   Table 1 Comparison of morphological traits between annual and perennial plants.

Mean rank	Annual	Perennial	Z given phy	Pvalue given phy
Leaf length	52.25	37.79	-2.0971	0.03599
Leaf width	42.00	40.71	-5e-04	0.9996
Bract shape	53.25	37.50	-2.8681	0.004129
Bract length	31.50	43.71	2.2606	0.02378
Corolla shape	58.00	36.14	-2.0321	0.04214
Corolla diameter	57.50	36.29	-2.6443	0.008185
Capsule apex	60.50	35.43	-2.7878	0.005307
Stamen length	51.75	37.93	-2.1757	0.029581
Style length	42.50	40.57	-0.0032	0.9975

147

148   Differences in habitats between annual and perennial plants are summarized in Table 2. The results
149   demonstrated that annuals can withstand higher temperature (in warmest month). In terms of precipitation,
150   there are also significant differences in precipitation of driest month. Perennials are found in areas of higher
151   precipitation compared to annuals.

152   Table 2 Comparison of habitats between annual and perennial plants. Temperature unit: (°C * 10); Precipitation unit:(mm).

Variables	Annual	Perennial	Pvalue given phy
Max Temperature of Warmest Month	249.38±8.22	199.59 ±4.22	0.008
Mean Temperature of Warmest Quarter	179.42±6.78	138.05 ±4.14	0.017
Mean Temperature of Driest Quarter	90.90±22.80	76.33 ±9.19	0.741
Annual Precipitation	695.94±89.94	1539.67 ±109.65	0.050
Precipitation of Driest Month	25.85±4.26	80.24 ±6.99	0.047
Precipitation of Driest Quarter	89.14±13.49	287.47 ±24.94	0.042
Precipitation of Warmest Quarter	168.35±43.28	364.30 ±27.03	0.092

153

154   DISCUSSION
155   The evolution of annual life history is a common evolutionary transition in angiosperms,
156   having occurred in more than 100 families. In angiosperms, the perennial habit is believed to be
157   the ancestral condition (Melzer et al., 2008). Nevertheless, secondary evolution of perennial life
158   history from annual herbaceous ancestors has been shown to occur in certain environments, such
159   as islands (Bohle, Hilger & Martin, 1996; Kim et al., 1996) and mountains (Karl et al., 2012).
160   Here, we analyzed a number of hypotheses regarding the evolution of annual life history in more
161   detail based on comprehensive information on morphology and ecological data based on an

162 explicit phylogenetic hypothesis. While many of these hypotheses were inferred in previous
163 studies, modern comparative analytical tools allow to check theses hypotheses in more detail. In
164 this study, the ancestral condition of the genus *Veronica* has been inferred to have been
165 perenniality and the annual life history has evolved multiple times with a single reversal in *V.*
166 *filiformis* of the Caucasus Mountains consistent with previous conclusions (Albach, Martinez-
167 Ortega & Chase, 2004). Overall, we inferred seven origins of annuals. An additional three
168 origins of annuality (in *V. hispidula*, *V. peregrina* and *V. anagalloides* (all subgenus *Beccabunga*;
169 Albach, Martinez-Ortega & Chase, 2004; Müller & Albach, 2010) are not included in the
170 analysis here.

171   The seven to ten independent shifts between life histories are associated with considerable
172 morphological diversity among annual species. However, certain characters are characteristic for
173 annuals (the annuality syndrome) associated with the rapid completion of the life cycle. For
174 example, the generation-time hypothesis, which assumes that mutations are mostly accumulated
175 during recombination, states that organisms that reproduce faster such as annuals also have more
176 DNA substitutions over time (Page & Holmes, 2009). Results of this study demonstrate that
177 clades including annuals have a higher substitution rate and are, thus, consistent with this theory
178 and previous analyses for *Veronica* (Müller & Albach, 2010), although this is significant only for
179 the oldest clade of annuals (*V.* subg. *Cochlidiosperma* (Rchb.) M. M. Mart. Ort. & Albach). On
180 the other side, the perennial clade with the lowest substitution rate (*V.* subg. *Pseudoveronica*, see
181 above) is also the one with the highest diversification rate (Meudt et al. 2015). However, the
182 impact of life history transformation is not restricted to substitution rate.

183   Two of the correlations detected are most likely associated with the smaller stature of
184 annuals. These are the larger leaves of perennials and the larger bracts in annuals (especially in
185 subgenera *Pocilla* and *Cochlidiosperma*) that compensate for the reduced number and size of
186 stem leaves in smaller plants. Also, reduction to a single, terminal inflorescence is likely to be a
187 consequence of small size but may also be related to differences in breeding system. Other
188 inflorescence characters are more clearly associated with differences in breeding system between
189 annuals and perennials.

190   Estimates for selfing among angiosperms as a whole are 25-30% (Barrett & Eckert, 1990)
191 with estimates for annuals alone going up to 50% (Hamrick & Godt, 1996). The association
192 between annual life history and selfing has been known for some time (Henslow, 1879) and has
193 also been thoroughly discussed in the literature (e.g., Barrett, Harder & Worley, 1996; Stebbins,
194 1957). Annual species invest fewer resources into their sexual organs (e.g., number of lateral
195 inflorescences; density of inflorescence, corolla size) than perennials (although not necessarily
196 relative to overall size of the plants). Such changes are likely to be associated with parallel
197 changes in life history and breeding system. A larger corolla and longer stamens have previously
198 been demonstrated to be correlated with an outcrossing breeding system in the genus (Scalone,
199 Kolf & Albach, 2013). Surprisingly, a longer style is here not associated with perenniality as
200 inferred by Scalone, Kolf & Albach (2013). In contrast, we infer that selfing is facilitated by
201 lowering the stigma below the anthers through emargination of the capsule. By that means, the
202 stigma is removed from the anthers without shortening the style. Other characters that may have

203 an influence on breeding system in perennials is the trend towards tubular corollas, which may
204 contain more nectar, and the longer pedicels in perennials that allows better presentation of the
205 flower. Thus, our analysis supports the notion that outcrossing is associated with perennial life
206 history in *Veronica* (Albach & Greilhuber, 2004). Such a correlation in the evolution of annual
207 life history is often argued to be due to reproductive assurance in annuals, depending on
208 reproduction in their single season of flowering (Busch & Delph, 2012). However, to understand
209 the basis for this association, one needs to move beyond such correlation and understand the
210 ecological circumstances of transitions in life history.

211 Several such circumstances have been inferred to be responsible for the evolution of annual
212 life history (see introduction). Here, we inferred higher temperature, higher temperature variation
213 and lower precipitation to be the characteristic environmental conditions for annuals in
214 comparison with perennials. This is consistent with previous suggestions that inferred drought,
215 heat or unpredictable environment are responsible for the evolution of annual life history (Evans
216 et al., 2005; Stearns, 1976; Whyte, 1977). Thus, despite the multiple origins of annuals in the
217 genus, annual clades in *Veronica* may have reacted to the same climatic circumstances favoring a
218 change in life history. Although we did not specifically test for differences among clades of
219 annuals, markedly different climatic circumstances in one clade of annuals should have led to
220 differences between inferences based on phylogenetically informed and non-phylogenetic
221 analyses.

222 Consequently, it is likely that parallel evolution in different groups of *Veronica* led to the
223 evolution of annual life history and a characteristic set of related characters. Parallel evolution is
224 more likely if occurring in the same region at the same time because of the same selection
225 pressure. Based on the molecular dating of *Veronica* in Meudt et al. (2015), however, annual
226 lineages originated over a range of dates starting in the Miocene, similar to other Mediterranean
227 annuals inferred to have originated in response to the evolution of the Mediterranean climate
228 evolution and the Messinian salinity crisis (Fiz, Valcárcel & Vargas, 2002). With the exception
229 of *V. peregrina*, not included here, all groups of annual *Veronica* originated from ancestors in the
230 Mediterranean and southwest Asia. Thus, progressing aridification may have spurred evolution
231 of annual life history at different times in the same region in different groups of *Veronica*.
232 During aridification, competition from related species decreased, and environmental filtering
233 became a major limiting effect on species. Under such circumstances, the avoidance strategy of
234 annuals by drought-tolerant seeds is favored by natural selection (De Bello, LepŠ & Sebastia,
235 2005). However, this hypothesis will be investigated in more detail in the different clades of
236 annual *Veronica* by more detailed investigation of character evolution and ancestral habitat
237 estimation.
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