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Strong conservatism of floral morphology during the rapid diversification of the genus *Helianthemum*

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Abstract

Premise: Divergence of floral morphology and breeding systems are often expected to be linked to angiosperm diversification and environmental niche divergence. However, available evidence for such relationships is not generalizable due to different taxonomic, geographical and time scales. The Palearctic genus *Helianthemum* shows the highest diversity of the family Cistaceae in terms of breeding systems, floral traits, and environmental conditions as a result of three recent evolutionary radiations since the Late Miocene. Here, we investigated the tempo and mode of evolution of floral morphology in the genus and its link with species diversification and environmental niche divergence.

Methods: We quantified 18 floral traits from 83 taxa and applied phylogenetic comparative methods using a robust phylogenetic framework based on genotyping-by-sequencing data.

Results: We found three different floral morphologies, putatively related to three different breeding systems: type I, characterized by small flowers without herkogamy and low pollen to ovule ratio; type II, represented by large flowers with approach herkogamy and intermediate pollen to ovule ratio; and type III, featured by small flowers with reverse herkogamy and the highest pollen to ovule ratio. Each morphology has been highly conserved across each radiation and has evolved independently of species diversification and ecological niche divergence.

Conclusions: The combined results of trait, niche, and species diversification ultimately recovered a pattern of potentially non-adaptive radiations in *Helianthemum* and highlight the idea that evolutionary radiations can be decoupled from floral morphology evolution even in lineages that diversified in heterogeneous environments as the Mediterranean Basin.

KEYWORDS

breeding system, Cistaceae, evolutionary radiation, floral morphology, floral trait, *Helianthemum*, Mediterranean Basin, trait conservatism, trait evolution

The study of the evolution of floral morphology along phylogenies provides clues about the role of breeding systems in angiosperm diversification (Vallejo-Marín et al., 2014) because floral design is tightly linked to flower function (McDonald et al., 2011; Wright et al., 2012; Vamosi

et al., 2018). In fact, suites of floral traits are frequently used as proxies of breeding systems (Goodwillie et al., 2010; Sicard and Lenhard, 2011). Firstly, larger flowers are more easily detected by pollinators (Bell, 1985; Ohashi and Yahara, 2001; Lázaro et al., 2013) and usually contain a

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greater amount of nectar (Cruden and Lyon, 1985; Herrera, 1992; Jones, 2001; Willmer, 2011), favoring higher pollen transfer between flowers and individuals (Bell, 1985; Conner and Rush, 1996; Aigner, 2005; Nattero et al., 2011; Barrio and Teixido, 2015). Secondly, the spatial separation of stigmas and anthers (herkogamy) drives the level of outcrossing by preventing physical contact between male and female floral structures, remarkably influencing the outcome of pollen transport (e.g., Breese, 1959; Barrett and Shore, 1987; Simón-Porcar et al., 2022). And thirdly, the amount of pollen and ovules produced by a single flower (i.e., the pollen to ovule ratio) is inversely related to the likelihood that a pollen grain reaches a stigma, thus determining pollination efficiency (Cruden, 1977, 2000; Pellmyr et al., 2020).

Shifts of floral morphology are expected to be linked to diversification rates in angiosperms (Vamosi and Vamosi, 2010; O'Meara et al., 2016). This expectation arises from the higher levels of inbreeding, a lack of potential for novel allelic combinations and the increased risk of extinction among populations reported in selfing species (Schoen and Brown, 1991; Hamrick and Godt, 1996; Wright et al., 2008). Consistent with these effects, lineages with floral traits enhancing outcrossing often have greater rates of net diversification than do lineages lacking such a system (e.g., Solanaceae: Goldberg et al., 2010; Primulaceae: de Vos et al., 2014; *Bulbophyllum*, Orchidaceae: Gamisch et al., 2015). However, selfing may ensure reproduction if pollinators are scarce favoring local adaptation and the emergence of local endemics (Lavergne et al., 2004). Indeed, selfing lineages with greater diversification rates than their outcrossing relatives have also been documented (e.g., *Linanthus*, Polemoniaceae: Goodwillie, 1999; Delphinieae, Ranunculaceae: Bosch et al., 2001; *Erodium*, Geraniaceae: Alarcón et al., 2011), and some have even proposed that lineages that have undergone evolutionary radiations (i.e., rapid and recent diversification rates) should derive more often from selfing ancestors than from outcrossing ones (Naciri and Linder, 2020), based on their ability to colonize new territories (Baker, 1955).

The disparity of diversification patterns when comparing outcrossing vs. selfing lineages suggests that the effect of breeding systems is likely clade specific or, perhaps even more interestingly, dependent on the ecological context (Ansaldi et al., 2018). Indeed, many plant lineages show mixed mating systems that can switch to selfing or outcrossing under different environmental conditions (e.g., Carta et al., 2016). In particular, the level of selfing may increase in response to abiotic stress as well as the natural heterogeneity of the environment (reviewed by Levin, 2010). For instance, some studies have revealed that higher rates of selfing are associated with survival in extremely arid areas (e.g., Cactaceae: Mandujano et al., 2010; Asteraceae: Tadey et al., 2009; Zygophyllaceae: Naghiloo et al., 2019), where the reduced soil nutrient availability and the reduction of pollinator service impose severe limitations on plant reproductive

ability (Batanouny, 2001; Drenovsky and Richards, 2004). Alternatively, outcrossing may be a mechanism prevalent in more humid environments (Wang et al., 2020 and references therein). Altogether, whether floral traits systematically shift with changes in ecological niches has received little attention, in particular using a phylogenetic framework.

Transitions affecting the breeding system of the species are also frequent, particularly in the Mediterranean flora, because the spatial template of mosaic landscapes and environments characterizing the Mediterranean Basin influences the evolutionary lability of floral traits in plant lineages (Gómez et al., 2014, 2015; Thompson, 2020). Moreover, many plant lineages have undergone rapid diversification in the Mediterranean Basin and are considered prime evolutionary radiations (e.g., *Dianthus*, Caryophyllaceae: Valente et al., 2010; *Erodium*, Geraniaceae: Alarcón et al., 2011; *Helianthemum*, Cistaceae: Martín-Hernanz et al., 2019a; *Linaria*, Plantaginaceae: Fernández-Mazuecos and Vargas, 2011; Blanco-Pastor et al., 2012; *Narcissus*, Amaryllidaceae: Santos-Gally et al., 2012). Unfortunately, the potential role of floral morphology and putative breeding systems in the rapid diversification of these species-rich Mediterranean lineages have been rarely tested under a macroevolutionary perspective. Thus, they represent interesting biological models to analyze floral evolution and its relation to speciation and environmental niche divergence.

Within the family Cistaceae, the Palearctic genus *Helianthemum* shows the greatest variation in floral traits such as petal color and size, number of stamens and ovules, style shape and size, herkogamy level, and flower opening (cleistogamous, chasmogamous). Although all species of *Helianthemum* display showy, open, disk-shaped, scentless flowers (Figure 1) primarily pollinated by generalist pollinators belonging to Hymenoptera and Coleoptera (Herrera, 1992; Tébar et al., 1997), the scarce empirical studies on breeding systems have shown high variability among species, from strict autogamy to facultative and strict xenogamy (Tébar et al., 1997; Arrington and Kubitzki, 2003; Rodríguez-Pérez, 2005; Aragón and Escudero, 2008; Agulló et al., 2015; Martín-Hernanz et al., 2019b). How this high floral variability has played a role in the recent and rapid radiation of the genus remains unclear. *Helianthemum* has indeed recently diversified as the outcome of three evolutionary radiations that happened since the Late Miocene and formed the three largest taxonomical sections (i.e., sects. *Eriocarpum*, *Helianthemum*, and *Pseudocistus*; Martín-Hernanz et al., 2019a). A striking pattern is that, despite this explosive diversification, environmental niches along lineages have remained quite conserved along an aridity gradient. In particular, Mediterranean ancestors colonized three more extreme environmental niches (i.e., subdesert, humid-montane, and subtropical-insular) during the paleo-environmental changes that occurred in the Mediterranean Basin since

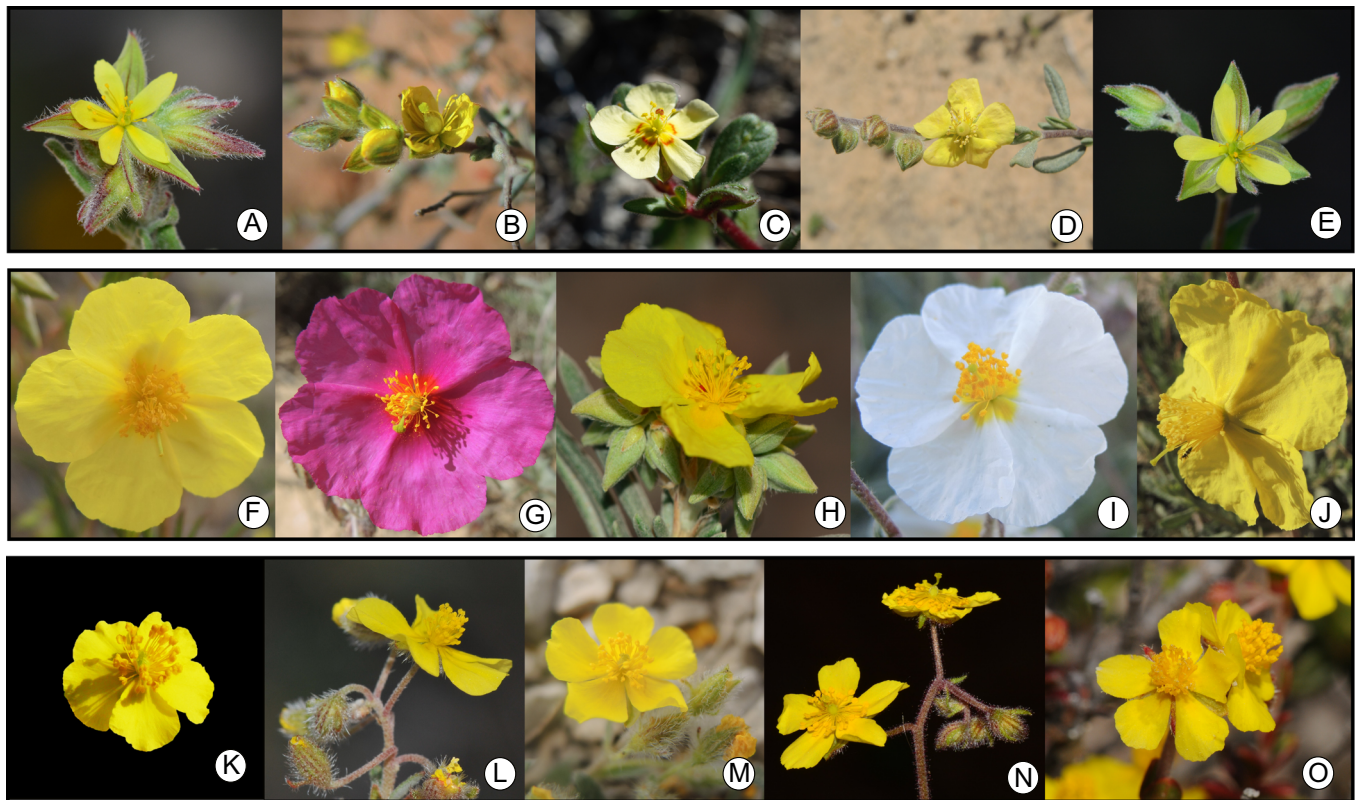


FIGURE 1 Variability in floral morphology of *Helianthemum*. (A–O) Representative floral morphologies defined by the PCA–Hill Smith. (A–E) Representative flowers of type I floral morphology, characterized by small flowers without herkogamy and low pollen to ovule ratio. (A) *H. angustatum*; (B) *H. lippii*; (C) *H. sanguineum*; (D) *H. sessiliflorum*; (E) *H. salicifolium*. (F–J) Representative flowers of type II floral morphology, characterized by large flowers with approach herkogamy and intermediate pollen to ovule ratio. (F) *H. inaguae*; (G) *H. vesicarium*; (H) *H. syriacum*; (I) *H. apenninum*; (J) *H. ventosum*. (K–O) Representative flowers of type III floral morphology, featured by small flowers with reverse herkogamy and the highest pollen to ovule ratio. (K) *H. polyanthum*; (L) *H. cinereum* subsp. *rotundifolium*; (M) *H. oelandicum* subsp. *pourretii*; (N) *H. marifolium* subsp. *molle*; (O) *H. oelandicum* subsp. *conquense*.

the Pliocene. From this period onward, niche conservatism prevailed during species diversification in the three major subclades (Martín-Hernanz et al., 2021a).

Overall, *Helianthemum* stands out as an excellent study system to understand the tempo and mode of floral morphology evolution in the Mediterranean Basin and to evaluate its potential role in the diversification of species-rich plant lineages. In this study, we characterized the floral diversity of *Helianthemum*, evaluated its putative relationship with the breeding system reported in empirical studies for different species, and reconstructed floral morphology evolution of the genus using a robust phylogenetic framework based on genotyping-by-sequencing data (Martín-Hernanz et al., 2019a). We applied phylogenetic comparative methods and addressed the following questions: (1) Has floral morphology been subjected to strong divergent evolution during the process of speciation? The strong environmental niche conservatism recovered in previous studies of the genus (Martín-Hernanz et al., 2021a) suggests that the diversification of floral morphology may have been similarly constrained. (2) Is the evolution of floral morphology associated with shifts in the environmental niche? We expect a phenotype–environment–correlated evolution in which

floral traits related to autogamy are positively associated with increased aridity conditions, as previously reported in other arid-adapted lineages (e.g., Tadey et al., 2009; Mandujano et al., 2010; Naghiloo et al., 2019). (3) And have shifts in floral morphology triggered the rapid diversification of *Helianthemum*? Floral morphology can promote evolutionary radiations under two different underlying processes: the acquisition of a floral key innovation triggering speciation or floral divergence directly involved in the process of speciation. On the one hand, we would expect a key innovation may have promoted the rapid diversification of the genus if a given type of floral morphology has prevailed during the evolution of radiations (Hodges and Arnold, 1995). On the other hand, we would expect that floral divergence may be directly involved in driving speciation if rapid floral trait divergence is coupled with species diversification (Skeels et al., 2021). The combined results of floral trait evolution, environmental niche divergence, and species diversification will ultimately provide important clues as to whether the processes explaining rapid diversification in the Mediterranean Basin are adaptive or non-adaptive with regard floral traits (Gittenberger, 1991; Schenk, 2021 and references therein).

MATERIALS AND METHODS

Data collection

We collected 1122 field samples, including flower buds (673) and fully open flowers (449) from 467 individual plants among 226 populations belonging to 83 species and subspecies (65 species, 10 sections, three subgenera; see Martín-Hernanz et al., 2021b) (Appendix S1). This sampling represents more than 65% of the total number of species of the genus and about 60% of the taxa (Martín-Hernanz et al., 2021b). We measured 18 quantitative and two qualitative variables depicting floral traits. To do so, we dissected the sampled material (flower buds and fully open flowers) using a stereomicroscope and mounted the different floral pieces on microscope slides to take scaled digital micrographs and quantify the continuous variables using ImageJ 1.44p (Abramoff et al., 2004). We counted the number of stamens and ovules to calculate the anther to ovule (A-O) ratio. We measured stigma width in both immature (buds) and mature (fully open) flowers and computed the differences in stigma width between the two types of flowers for each individual. For each fully open flower, we also measured the petal length and width, the length of the shortest and longest stamen, the ovary height and width, the style and stigma height and the stigma width. We calculated the distance between the longest stamen and the pistil length to represent the stigma–anther separation (i.e., herkogamy). Thus, stigma–anther separation had positive values when stamens did not reach the stigma height (approach herkogamy) and negative values when the stigma was below the anthers (reverse herkogamy; Appendix S2). We measured pollen grain diameters and calculated minimum and maximum diameters.

We also estimated the number of pollen grains per flower. To that end, we squashed five randomly selected anthers per sample with entomological needles, then inserted the anthers into an Eppendorf tube containing Isoton II (Beckman Coulter, Fullerton, California, USA). We obtained the anthers from the 673 buds instead from open flowers to ensure that anthers had not dehisced and pollen had not been dispersed. For effective pollen release, anthers were pricked with a sterile needle, then sonicated for 40 min at 42 kHz using a Cole-Parmer 8890 Ultrasonic Bath (Cole-Parmer, Vernon Hills, IL, USA; Astuti et al., 2017). Then, we counted the pollen grains in 500- μ L subsamples using a particle counter (Coulter Multisizer 3, Beckman Coulter, Fullerton, CA, USA). Pollen production per flower was estimated as the number of pollen grains per anther (mean value of two replicates and five subsamples from each floral bud) multiplied by the number of anthers in that flower. We computed the pollen to ovule (P-O) ratio (Cruden, 1977) as the total number of pollen grains per flower divided by the number of ovules. Finally, petal color (yellow, white, or pink) and style type (straight, sigmoid, geniculate, or curly; Appendix S2) were recorded for each sample.

Floral morphology

To depict the relationships between floral traits in a multivariate space of the 83 study taxa, we performed a multivariate analysis including petal color, style type, and the mean values of all quantitative floral traits for each taxon. Because we used both discrete and continuous variables, we used the Hill and Smith (1976) principal component method and the R package *ade4* v.1.7 for PCAs (hereafter referred to as the PCA–Hill Smith; Chessel et al., 2004). To classify floral variations into morphological and potential functional groups, we determined the optimal number of clusters using two approaches: (1) the elbow method, which maps the within-cluster sum of squares onto the number of possible clusters, thus the optimum number of clusters corresponds with the value displaying the sharpest decrease in the within-cluster dissimilarity (called elbow); and (2) hierarchical clustering, which graphically represents the structure and relationships in the data using a dendrogram that allows determining the appropriate number of clusters based on the branch lengths. Once we established the optimal number of clusters (which we refer to as floral morphologies), we assigned each taxon to one type of floral morphology and determined whether the values of the first two axes from the PCA–Hill Smith differed significantly among the floral morphologies. To that end, we used phylogenetic generalized least square (PGLS) analyses with the R package *nlme* v.3.1 (Pinheiro et al., 2016) with the *corPagel* function in the R package *ape* v.5.6 (Paradis and Schliep, 2019) to control for the shared ancestry of species in the residual error (i.e., the phylogeny). Post hoc pairwise comparisons tests between floral morphologies were done with the R package *multcomp* v.1.4 (Hothorn et al., 2008). Additionally, we followed the same methodology to test for significant differences in every single quantitative floral trait among floral morphologies. All data analyses was conducted using R studio (R Core Team, 2022).

Comparative analyses of floral evolution

To reconstruct the evolution of floral traits in *Helianthemum*, we used the robust phylogenetic framework based on genotyping-by-sequencing (GBS) data and generated by Martín-Hernanz et al. (2019a) and proceed as follows. Firstly, we selected the dated consensus GBS tree and a randomly chosen subset of 100 Bayesian dated GBS trees to account for phylogenetic uncertainty. Then, any tips that represented taxa for which floral trait data were not available were pruned in the consensus GBS tree and in the subset of 100 Bayesian dated GBS trees (hereafter respectively “pruned GBS tree” and “RD pruned GBS trees”) using the R package *ape*. We included both species and subspecies following the nomenclature and taxonomic circumscriptions of taxa of López-González (1993) and Martín-Hernanz et al. (2021b).

Phylogenetic signal

To assess the level of phylogenetic signal for floral traits (i.e., the tendency of related species to display similar floral traits), we estimated Pagel's λ (Pagel, 1999) of each floral trait separately. Phylogenetic signal was also estimated for the two axes extracted in the PCA-Hill Smith (related to floral size and male–female function, see Results) on both the pruned GBS-tree and averaged through the RD pruned GBS-trees using the R package *phytools* v. 0.6 (Revell, 2012). Additionally, we estimated the phylogenetic signal of the three types of floral morphology (see Results) with the R package *geiger* v. 2.0 (Harmon et al., 2008).

Evolution of floral morphology

To reconstruct the evolutionary history of floral morphology, we fitted three evolutionary models using the R package *geiger*: the equal state transition rates (ER), symmetrical (SYM), and all rates unequal (ARD) models. We then identified the best-fitting model based on the corrected Akaike information criterion (AICc) and likelihood ratio test (LRT). We performed stochastic character mappings (Huelsenbeck et al., 2003) using *phytools* to estimate the posterior probability of states of the most recent common ancestor of each node, the average number of changes between states, and the time spent in each state. We launched 1000 simulations over the RD pruned GBS-trees using an estimated prior distribution on the root node and the best-fitting model inferred in the previous analysis.

Species diversification rates

To compare diversification rates among floral morphologies in *Helianthemum*, we conducted multistate trait-based analyses inferred by Multi-State Speciation and Extinction (MuSSE) method to estimate simultaneously speciation, extinction, and transition rates between states on a phylogeny (FitzJohn et al., 2009). These were performed using the R package *diversitree* v. 0.9 (FitzJohn, 2012) and the pruned GBS-tree. We first fitted 27 models from a simple model with no difference in speciation, extinction, and transition rates to the most complex model with different speciation, extinction, and transition rates for each character state, selecting the best-fitting model using the AICc. The transition diagram was fitted in the models according to that estimated and plotted using *geiger* (see Material and methods: *Evolution of floral morphology*). We performed subsequent Bayesian analyses for the most complex model to estimate the parameters and plot them respectively through a Markov chain Monte Carlo (MCMC) run for 10,000 generations on the 100 RD pruned GBS-trees (100 steps per tree) with exponential prior distributions and discarding the first 10% of the generations as burn-in.

Posterior probability (PP) distributions of all parameters were summarized and plotted using *diversitree*.

To reconstruct the diversification dynamics of floral traits and compare it with the overall timing of species and environmental niche diversification in the genus *Helianthemum* (Martín-Hernanz et al., 2019a, 2021a), we used BMM v. 2.5.0 (Rabosky, 2013, 2014a). We applied the BMM trait model over the first two axes extracted from the PCA-Hill Smith analysis (i.e., floral size and male–female function, see Results) using the pruned GBS-tree. We additionally applied the BMM trait model over the first two axes of environmental data (i.e., aridity and continentality) retrieved from Martín-Hernanz et al. (2021a). We derived prior settings from the R library *BMMtools* v. 2.1.0 (Rabosky et al., 2014b) and ran 10 million generations of MCMC sampling every 5000 generations. The first 10% generations were discarded as burn-in. We estimated the maximum shift credibility (MSC) configuration for each axis, and we generated rate-through-time plots for the whole genus and for the three major clades to assess whether the global diversification patterns are general or driven by different subclades.

Phenotype–environment correlations

To test the existence of significant correlations between floral morphology and environmental conditions in the genus *Helianthemum*, we used Pagel's method (1994) and phylogenetic generalized least squares (PGLS) regressions. Pagel's method was used to detect correlated evolution between different floral morphologies (see Results) and environmental niches (i.e., Mediterranean, humid-montane, subdesert, and subtropical-insular; Martín-Hernanz et al., 2021a). This analysis was carried out using *phytools* (Revell, 2012). Since Pagel's method does not reflect the variability within each trait state, we also applied PGLS regressions to incorporate such variability. We conducted PGLS regressions in the R package *nlme* using the first two axes extracted from the PCA Hill-Smith with environmental data (i.e., aridity and continentality) retrieved from Martín-Hernanz et al. (2021a), and the first two axes extracted in this paper using floral traits (i.e., floral size and male–female function; see Results). Regressions were compared to a null model through LRT. Phylogenetic relationships used for both analyses were derived from the pruned GBS-tree.

RESULTS

Floral morphology

Appendix S3 shows a phylogenetic heat map with the standardized mean values calculated for the reconstruction of 18 quantitative floral traits from the 83 studied taxa of *Helianthemum* useful in defining floral morphologies by the PCA–Hill Smith (see below). The first two axes of the

PCA–Hill Smith explained 49.25% of total trait variance (Appendix S4). The first principal component (33.43% of total variance) mainly reflected variation in floral size (i.e., petal length and width, stamens length, ovary height and width, style height, and pollen diameter) and herkogamy level (i.e., stigma–anther separation) (Figure 2A; Appendix S4), with positive values representing taxa with larger flowers and approach herkogamy and negative values representing small flowers and reverse or null herkogamy. Consequently, we denoted axis 1 as floral

size. The second principal component (15.82% of total variance) was related to the relative allocation to male and female functions (i.e., number of ovules, number of stamens, anther to ovule ratio, and pollen to ovule ratio) and the type of style. Positive values indicated a high number of ovules and straight style, and negative values indicated a high number of stamens, anther to ovule ratio, and pollen to ovule ratio, and curly, sigmoid, or geniculate styles (Figure 2A; Appendix S4). Thus, we denoted axis 2 as male–female function.

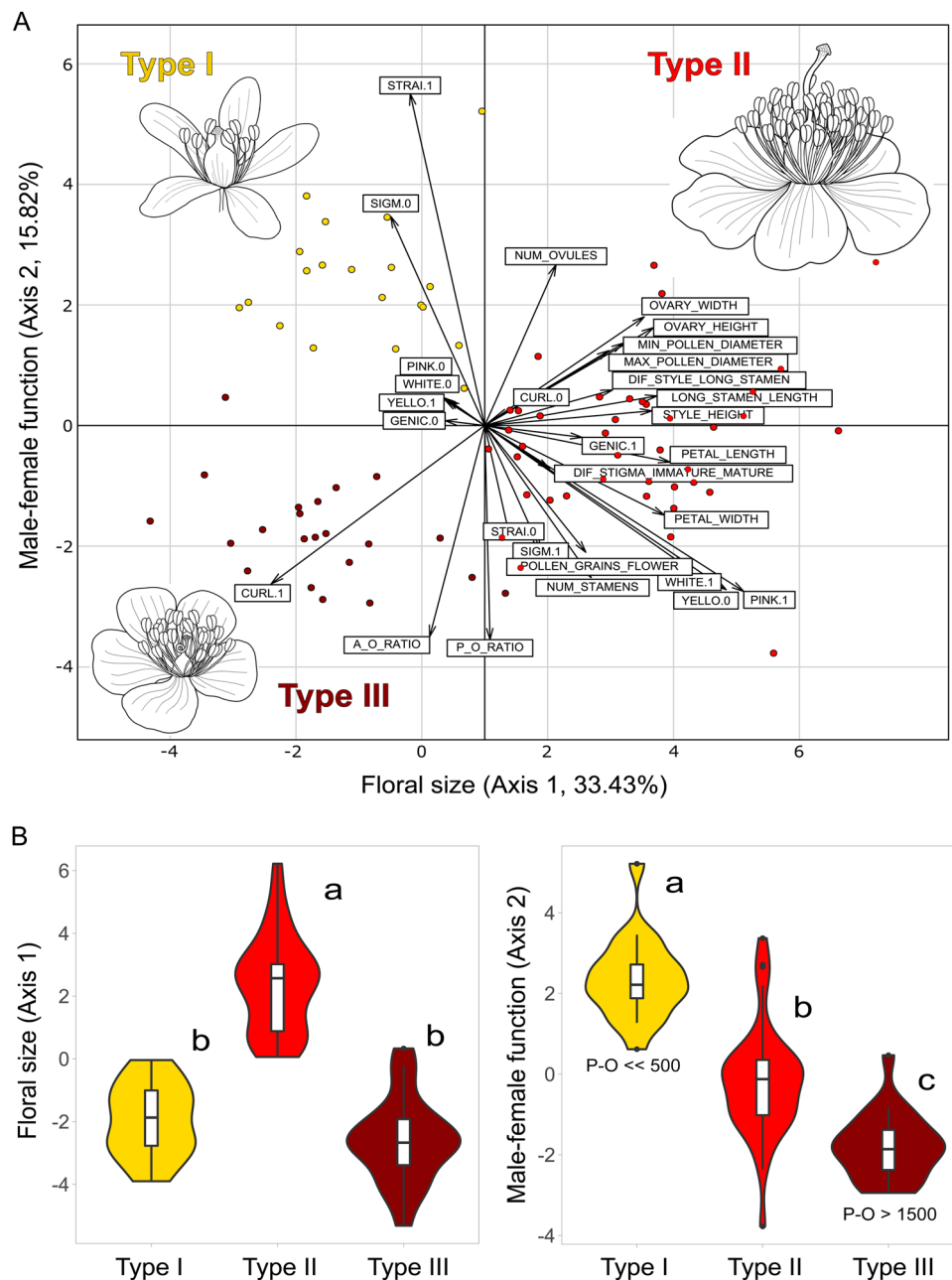


FIGURE 2 Characterization of the three floral morphologies (type I, type II, type III) defined in *Helianthemum*. (A) Scatterplot of the first two components of the PCA–Hill Smith showing their relationship with the measured floral traits for 83 taxa of the genus (dots). Dots are colored according to the type of floral morphology identified in the clustering analyses (see main text for more details). (B) Violin plots of the first two axes extracted from the PCA–Hill Smith for the 83 taxa of the genus *Helianthemum* grouped by the three floral morphologies identified in the cluster analyses. Different letters indicate significant differences among floral morphologies inferred by PGLS and post hoc tests.

Both the elbow method and the dendrogram suggested three main floral morphologies in *Helianthemum* (Appendix S5): (1) type I characterized by small yellow flowers and no herkogamy, low ratios of anther to ovule and pollen to ovule (most taxa with pollen to ovule ratio $\ll 500$; Appendix S3), and straight styles (Figures 1 and 2A); (2) type II characterized by large flowers with approach herkogamy (i.e., stamens below the stigma), intermediate or high ratios of anther to ovule and pollen to ovule (mostly between 500 and 1500; Appendix S6), geniculate or sigmoid styles, and yellow, pink, or white petals (Figures 1 and 2A); and (3) type III characterized by small flowers with reverse herkogamy (i.e., stigma below the stamens), the highest ratios of anther to ovule and pollen to ovule (mostly >1500 ; Appendix S6), and curly styles (Figures 1 and 2A).

The PGLS results showed that the values of the first two axes were strongly and significantly different between the three floral morphologies ($F = 37.052$, $df = 2$, $P < 0.001$ in axis 1, and $F = 14.975$, $df = 2$, $P < 0.001$ in axis 2). Post hoc tests showed that floral size (axis 1) of type I and type III were not significantly different, but all three types were strongly differentiated by relative allocation to male–female functions (axis 2) (Figure 2B). Accordingly, most quantitative floral traits were significantly different between the three floral morphologies (Appendix S6).

Comparative analysis of floral evolution

Phylogenetic signal

Our estimates of Pagel's λ provided evidence of moderate, but significant, values of phylogenetic signal ($\lambda = 0.69$; $P \ll 0.001$) for floral size (axis 1) and strong phylogenetic signal ($\lambda = 0.97$; $P \ll 0.001$) for the male–female function (axis 2). We also found evidence of strong phylogenetic signal in the floral morphology ($\lambda = 1$), indicating that closely related species tend to show the same type of floral morphology (see Figure 3A).

Evolution of floral morphology

The best-fitted evolutionary model identified for floral morphology was the “SYM” model. The ancestral state reconstruction analysis provided relative probabilities (rp) above 0.75 as optimal reconstruction in most of the shallow nodes, while a high uncertainty was retrieved in the deepest nodes (Figure 3A). The most likely ancestral floral morphology of *Helianthemum* was the type I (rp = 0.46), followed by type II (rp = 0.29) and type III (rp = 0.25) (Figure 3A). This relative ambiguity was also retrieved in the ancestral floral morphology recovered for the divergence between clades I and III (type I: rp = 0.50; type III: rp = 0.28; type II: rp = 0.22), and the crown node of clade II (type I: rp = 0.49; type II: rp = 0.37; type III: rp = 0.14). The three floral morphologies may have arisen early in the evolutionary history of

Helianthemum since the common ancestor of each of the three largest sections (i.e., *Eriocarpum*, *Helianthemum*, and *Pseudocistus*) displayed high support for a different one. Furthermore, diversification within these clades (taxonomical sections) mostly occurred under one different floral morphology, i.e., type I in sect. *Eriocarpum* (clade II), type II in sect. *Helianthemum* (clade III) and type III in sect. *Pseudocistus* (clade I).

Likewise, the analysis of stochastic character mapping inferred that transition rates between the different floral morphologies were low, indicating that changes between states were infrequent (Figure 3A), as suggested also by the strong estimated phylogenetic signal of floral morphology. The few shifts of floral morphology in *Helianthemum* mainly occurred between type I and type II, less frequently between type I and type III, and very rarely between type II and type III (cf. Figure 3 for estimated transition rates). In fact, the estimated transition events and rates suggest that reversal from type III to type II virtually never happened during the evolution of *Helianthemum*. Lastly, the relative time spent in type II was the highest (proportion of total time = 43%) compared to type I and type III (27% and 30%, respectively).

Diversification rates

The best-fitted model of the trait-dependent species diversification analysis inferred by MuSSE indicated that speciation rates did not significantly differ between the three floral morphologies but type II was associated with lower extinction rates and, consequently, with the highest net diversification rates (Figure 3B; Appendices S7 and S8).

Diversification rates of floral traits was strongly decoupled from species and environmental niche diversification (Figure 4; Appendices S9 and S10). Firstly, floral trait shifts did not coincide with the accelerations of species diversification at the base of the three largest sections of the genus *Helianthemum* (Appendix S9). Secondly, diversification rates of both floral traits and environmental niches increased toward the present, particularly in the first axes of floral and environmental data (i.e., floral size and aridity) but in different times (Figure 4; Appendices S9 and S10). Our rate estimates through time illustrate that shifts in species diversification were phylogenetically deeper than shifts in diversification rates of floral traits (i.e., floral size) and environmental niches (i.e., aridity) (Figure 4; Appendix S10). Similarly, shifts in diversification of floral size underwent a lag after environmental niche shifts in clades II and III (Figure 4). No shift in the diversification rates of floral size was detected in clade I.

Phenotype-environment correlations

The independent models of trait evolution using Pagel's method received higher support than the dependent models

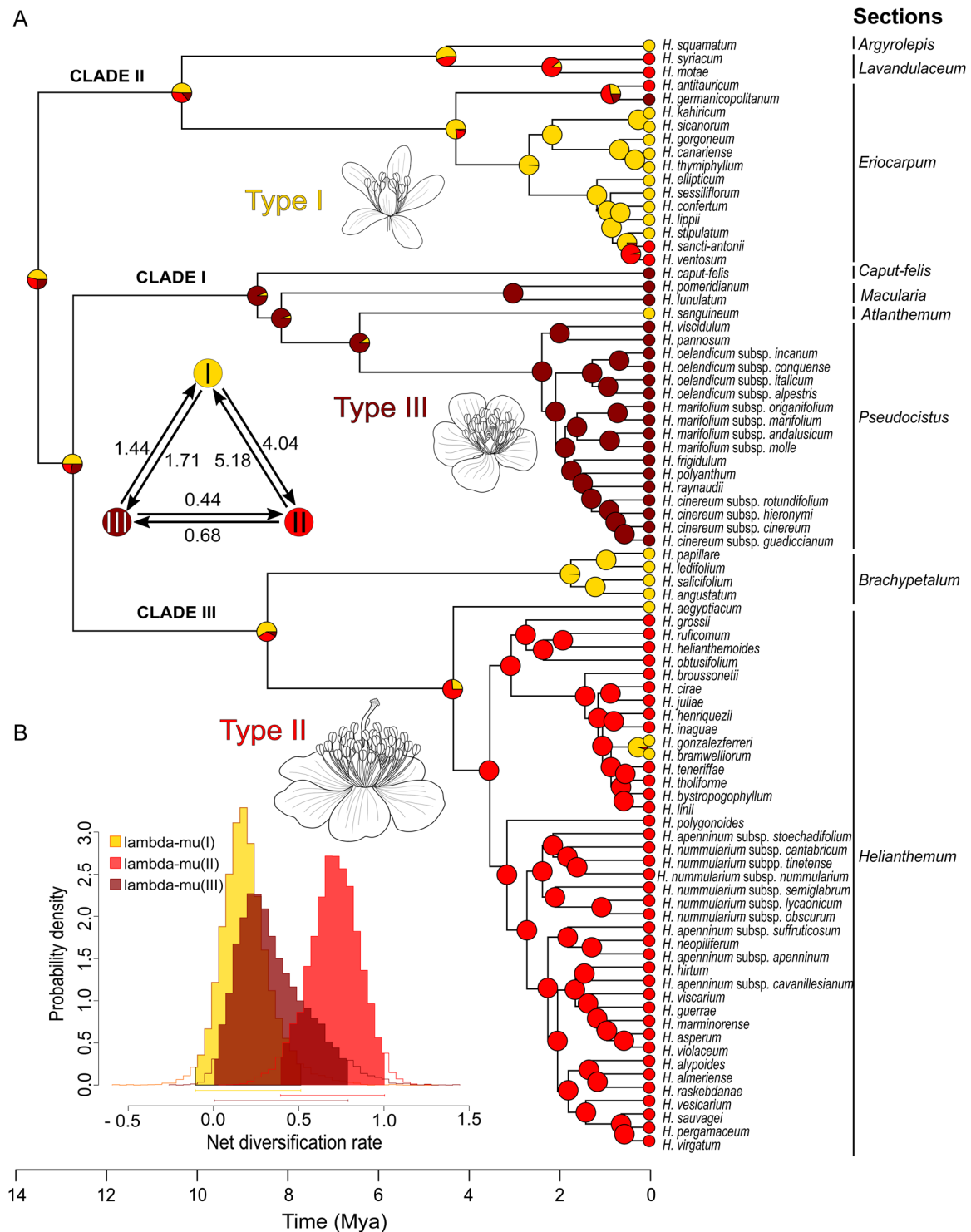


FIGURE 3 Floral morphology evolution of the genus *Helianthemum*. (A) Reconstruction of ancestral states of the three characterized floral morphologies in *Helianthemum* (type I, type II, and type III) by applying stochastic character mapping analysis on the RD GBS-trees, and the transition diagram inferred under the SYM model (see Materials and Methods, *Comparative analyses of floral evolution*). Pie charts in each node report the relative probabilities for each floral morphology. (B) Marginal distribution of the net diversification rates inferred for the best-fitting model resulting from the multi-state trait-based analyses inferred by Multi-State Speciation and Extinction (MuSSE) analysis.

for all pairs of correlations analyzed (Table 1). Congruently, results from PGLS indicated that environmental aridity had no significant effect on floral size ($t = 1.21$, $P = 0.23$) or male–female function ($t = -0.47$, $P = 0.64$) and that

environmental continentality had no significant effect on floral size ($t = 0.04$, $P = 0.98$) or male–female function ($t = -0.10$, $P = 0.92$). These results suggest that floral trait variation in *Helianthemum* is not linked to environmental

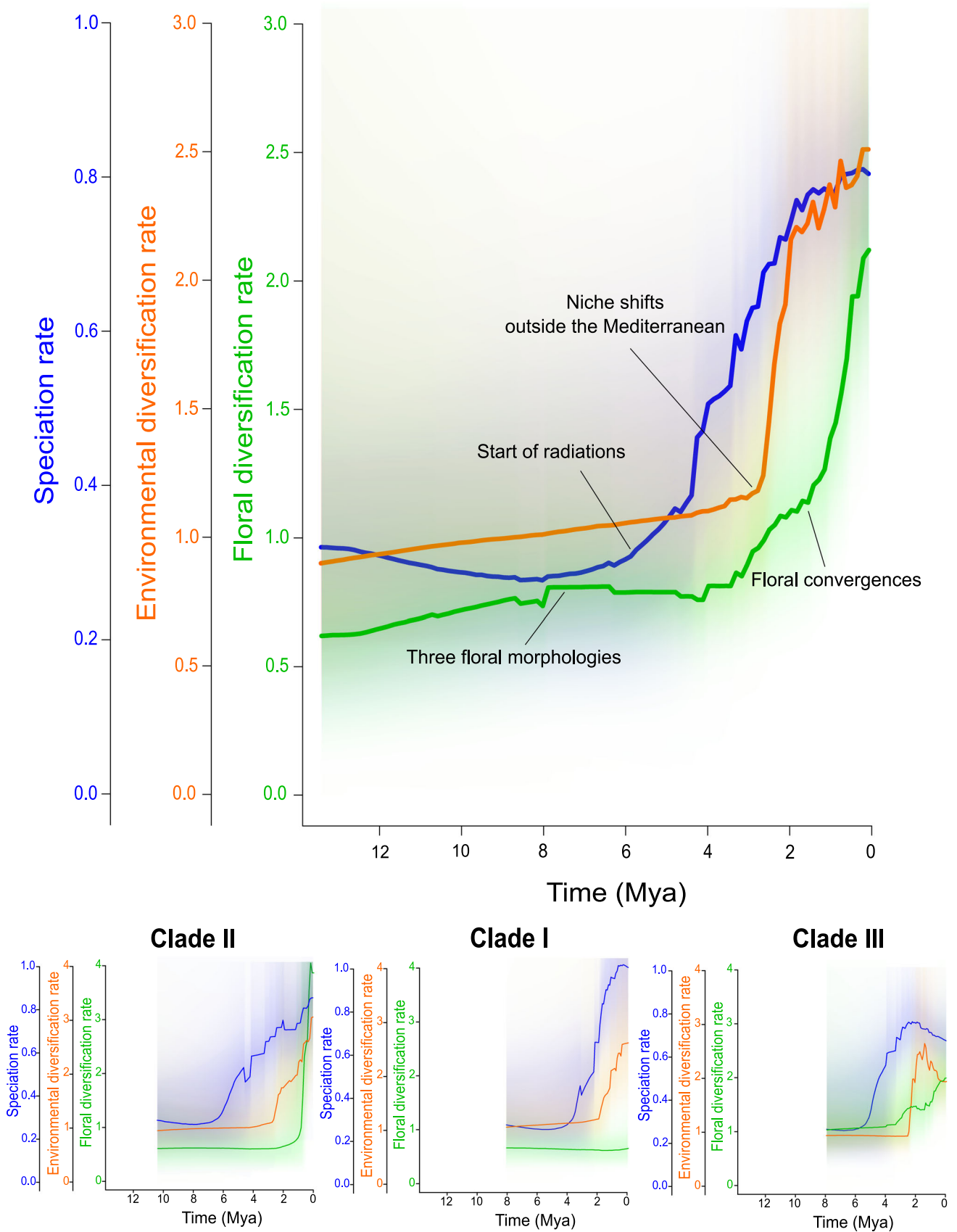


FIGURE 4 Species, floral, and environmental diversification rates over time for the genus *Helianthemum* and its three main clades with 95% confidence interval indicated by shaded areas obtained from BAMM. The unit of speciation rates is speciation events per million years; floral and environmental rates are unitless.

TABLE 1 Results of the comparisons between pairs of binary traits using Pagel's method for correlated evolution between environmental niches and floral morphologies in *Helianthemum*. Trait codes: A, Mediterranean; B, humid-montane; C, subdesert; D, subtropical-insular; 1, small flowers without herkogamy and low pollen to ovule ratio (type I); 2, large flowers with approach herkogamy and intermediate pollen to ovule ratio (type II); 3, small flowers with reverse herkogamy and the highest pollen to ovule ratio (type III). AIC, Akaike information criterion.

Traits	AIC independent	AIC dependent	Δ AIC	P
A – 1	149.964	152.682	2.717	0.260
A – 2	156.346	161.358	5.012	0.560
A – 3	128.293	132.057	3.763	0.375
B – 1	105.428	109.919	4.490	0.476
B – 2	111.810	117.900	6.090	0.752
B – 3	83.757	86.709	2.952	0.282
C – 1	134.071	137.736	3.665	0.363
C – 2	140.453	140.071	0.381	0.079
C – 3	112.400	117.625	5.225	0.596
D – 1	84.185	84.439	0.253	0.101
D – 2	90.567	94.123	3.556	0.349
D – 3	62.514	67.871	5.357	0.619

conditions and consequently do not support a phenotype–environment correlation.

DISCUSSION

Floral morphology and putative breeding systems

Our analyses, based on a comprehensive data set of floral traits, a robust phylogenetic framework, and several comparative methods, revealed three well-defined floral morphologies in *Helianthemum* (Figures 1 and 2A). Our results also provide compelling evidence for a strong pattern of trait conservatism within subclades of *Helianthemum*, meaning that these three floral morphologies appeared in *Helianthemum* before any radiations and have been largely maintained over time (Figure 3A).

The three floral morphologies (i.e., type I, type II, and type III) may likely be related to three breeding systems in *Helianthemum* (i.e., autogamy, facultative xenogamy, and xenogamy) based on empirical evidence (Tébar et al., 1997; Rodríguez-Pérez, 2005; Aragón and Escudero, 2008; Agulló et al., 2015) and our own observations. This potential relationship arises from the analysis and comparison of the floral size, pollen to ovule ratios and type of herkogamy (i.e., no herkogamy, approach herkogamy, and reverse herkogamy; Appendix S2) characteristic of each floral morphology (Cruden, 1977; Olmstead, 1989; Armbruster, 1993; de Vos et al., 2013; Jiménez-Lobato et al., 2019).

The usefulness of Cruden's (1977) categories for pollen to ovule ratio have been questioned because they can be affected by the pollination system itself, the life form of the plant, or phylogenetic constraints (Chouteau et al., 2006; Michalski and Durka, 2009). However, it is still considered of great value for indirectly inferring the prevalent breeding system of a species or group of species (Herrera, 1992; Jürgens et al., 2002; Mazer et al., 2009; Scalone et al., 2013; Astuti et al., 2018; Pellmyr et al., 2020).

The first floral morphology (type I) is characterized by small non-herkogamous, frequently cleistogamous flowers with very low pollen to ovule ratios (Figure 2A; Appendix S6) and may be putatively related to an autogamous breeding system. Although flowers with this morphology can also be chasmogamous, we have observed that in many instances anthers dehisce in the bud and that pollination takes place before anthesis (for the existence of cleistogamous flowers in species of the genus, see Grosser, 1903; Nandi, 1998; Arrington and Kubitzki, 2003; Brullo et al., 2007). This type of floral morphology is almost restricted to the therophyte species of *Helianthemum* (irrespective of the taxonomic section and the phylogenetic position) and most desert shrubs and subshrubs in sect. *Eriocarpum* (Figure 3A). However, this floral morphology was also assigned to the true gypsophile shrub *H. squamatum*, which was partially self-compatible in previous experimental pollination studies (Aragón and Escudero, 2008). This species has unique vegetative, karyological, and environmental characteristics within the genus (Aparicio et al., 2019), so it may also have peculiarities in its floral traits and reproductive strategies. In any case, experimental studies are required to evaluate the autogamous character of the species that have this floral morphology.

The second floral morphology (type II) is featured by rather large chasmogamous flowers with intermediate pollen to ovule ratios (500–1500) and approach herkogamy, with the style protruding beyond the stamens (Figure 2A; Appendix S6). This floral morphology may be putatively related to a facultative xenogamous breeding system based on the scarce available empirical data on the reproductive biology in this group of species (e.g., *H. apenninum*; Tébar et al., 1997). It is almost restricted to the fruticose and suffruticose species of sect. *Helianthemum* (Figure 3A), mostly occurring in the Mediterranean. Interestingly, this floral morphology is also associated with the highest net diversification rates in the genus (Figure 3B), although caution must be taken with the inference of extinction from models depicting trait-dependent variation in diversification rates (Pyron and Burbrink, 2013).

Lastly, the third floral morphology (type III) is defined by small flowers with large pollen to ovule ratios (>1500) and reverse herkogamy, in which the style is shorter than the stamens (Figure 2A; Appendix S6). The scarce empirical data available (i.e., *H. marifolium*, Rodríguez-Pérez, 2005; *H. caput-felis*, Agulló et al., 2015) agree with our experimental observations, showing a breeding system with complete or partial self-incompatibility to

avoid self-fertilization. This floral morphology is restricted to subgen. *Plectolobum* (Figure 3A), a group of suffruticose plants distributed around the Mediterranean and most of Northern, Central, and Eastern Europe.

Has floral morphology been subjected to strong divergent evolution during the process of speciation?

Although we found greater net diversification rates in one of the three floral morphologies of *Helianthemum* (Figure 3B), they seem to have been acquired early in its evolutionary history and then maintained within the three radiating lineages. We have found that the common ancestor of each of the three evolutionary radiations already displayed a different floral morphology, each one currently prevailing within one of the three main subclades (Figure 3A). Under this scenario, different floral morphologies could have exhibited certain advantages triggering the evolutionary radiation even in the same geographical context and under similar environmental conditions. In particular, types I and II (putatively related to autogamy and facultative xenogamy, respectively) may have boosted the establishment of new sexually reproducing populations from a single or few individuals, setting seeds even in the absence of pollinators (Crawford et al., 2008). On the other hand, type III (putatively related to xenogamy) may have boosted diversification by enhancing the genetic diversity of the progeny and then allowing local adaptation to emerge quickly (Martín-Hernanz et al., 2019b).

The early origin of the three floral morphologies in *Helianthemum* generated a high phylogenetic signal in most floral traits, which is congruent with the strong floral trait conservatism also reported for the whole family Cistaceae (Teixido et al., 2017). Indeed, the overall low variation in basic floral architecture at family level may have hampered the mode of evolution within the genus. A strong phylogenetic conservatism has been likewise detected for the environmental niche in the genus (Martín-Hernanz et al., 2021a), stressing that the three evolutionary radiations have taken place in the absence of remarkable floral or climatic niche diversification. Therefore, the assumption that clades with higher evolutionary rates have more divergent traits and niches than clades with lower evolutionary rates (Liu et al., 2015) is not applicable to the traits and niches analyzed to date in *Helianthemum*. Although previously documented in unrelated evolutionary groups such as ferns (Testo and Sundue, 2018), Saxifragales (Folk et al., 2019) or *Myrcia* (Myrtaceae; Vasconcelos et al., 2019), rapid diversification among lineages exhibiting long-term maintenance of their environmental niche and low morphological diversification such as in *Helianthemum* is an under-appreciated phenomenon driving the buildup of species richness (Kozak et al., 2006).

Is the evolution of floral morphology associated with shifts in the environmental niche?

In *Helianthemum*, shifts in floral morphology were not associated with major shifts in the environmental niche. Our analyses did not support a phenotype–environment correlation (Table 1), and patterns of diversification in floral morphology and environmental niche appeared strongly decoupled (Figure 4). Surprisingly, the ancestors of the three radiations may have acquired the three floral morphologies in the same geographical area (i.e., northern Africa) and under similar environmental conditions (i.e., Mediterranean; Martín-Hernanz et al., 2021a). Furthermore, lineages with a given floral morphology have successfully colonized several environments (Figure 5). These results show that the three floral morphologies perform efficiently all along the aridity gradient. For example, type I floral morphology (putatively related to autogamy) is characteristic of ecologically generalist, therophytic species of *Helianthemum* with a broad distribution around the Mediterranean Basin, but also acquired by a wealth of desert-specialist shrubs growing under severe aridity mainly in northern Africa and the Middle East (Aparicio et al., 2017; Martín-Hernanz et al., 2021a). The presence of type I floral morphology in therophytes and shrubs from desert areas suggests this morphology may have arisen in response to different selective forces such as transitions to short-life forms or subsistence in extreme environmental conditions.

Have shifts in floral divergence triggered the rapid diversification of *Helianthemum*?

The evolutionary radiations that occurred in *Helianthemum* were not triggered by floral morphology. Firstly, none of the three floral morphologies that we define here is per se a floral key innovation because each one arose before the onset of evolutionary radiations and can be found in both radiated and poorly diversified lineages (Figure 3A). Furthermore, recent transitions in floral morphology, interpreted as evolutionary convergences or homoplasy events, have not resulted in changes in diversification rates. Such is the case of the type I floral morphology independently acquired in therophyte lineages, or the type II floral morphology found in *H. ventosum* and *H. sancti-antonii* among the desert-specialist shrubs (Figures 1 and 3A). Also, we have found that floral divergence is quite decoupled from net diversification rates in *Helianthemum* (Figure 4), showing that floral morphology did not have a relevant role in its diversification (Martín-Hernanz et al., 2021a). Hypothetically, floral divergence was a driver of speciation only during the early stages of evolution of the genus when the three major clades diverged (Skeels et al., 2021) (Figure 3A). Therefore, other biological or environmental factors triggered the rapid diversification of the three evolutionary radiations, which certainly deserves further examination.

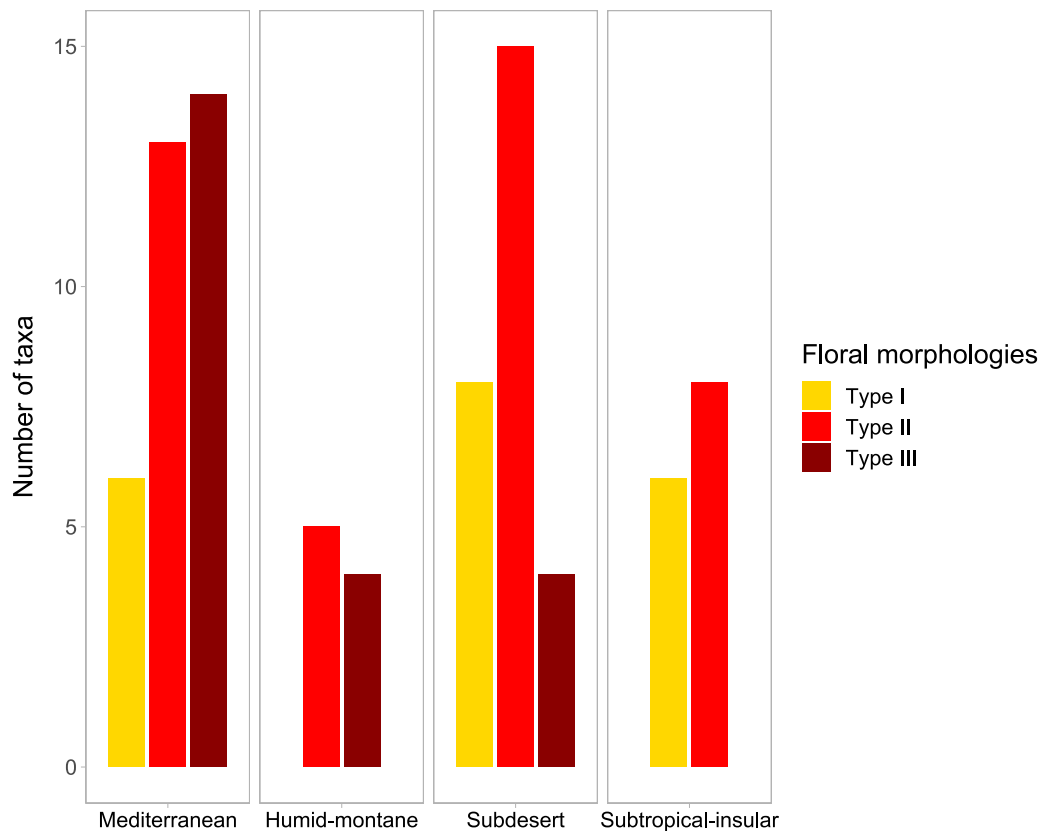


FIGURE 5 Histogram showing the number of taxa by floral morphology (types I, II, and III) in each of the environmental niches (Mediterranean, humid-montane, subdesert, and subtropical-insular) inhabited by species of *Helianthemum*. Notice that we have not found taxa with type I floral morphology in humid-montane environments or taxa with type III floral morphology in subtropical-insular environments.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this study, we provide compelling data showing the existence of three floral morphologies likely associated with distinct breeding systems in *Helianthemum*. Further, we reconstructed an early origin of the three morphologies, which were conserved during the diversification of each evolutionary radiation (Martín-Hernanz et al., 2019a). We suggest that floral divergence may not have been directly involved in recent speciation events or in driving environmental niche diversification in *Helianthemum*. Other factors such as negative interactions between species in sympatry (e.g., competition for pollinators), environmental variables not considered here (e.g., soil conditions) or other stochastic effects (e.g., founder effect, drift) may have influenced the evolution of floral morphology and convergence events (Figure 3A; Skeels et al., 2021). Future microevolutionary studies about the reproductive biology at species or population levels are necessary to address which of these factors can explain floral divergence in recent times.

The strong floral morphology conservatism along the evolution of *Helianthemum* goes against the long-standing idea that shifts in floral traits are associated with rapid

diversification in angiosperms (Skeels et al., 2021). Furthermore, the acquisition of floral key innovations or the colonization of a new environments does not seem to have triggered the three acceleration of diversification rates previously reported in *Helianthemum* (Figure 4; Martín-Hernanz et al., 2019a). Thus, the diversification patterns of floral traits do not correspond to those expected under an adaptive radiation (Gittenberger, 1991; Schluter, 2000). In contrast, our results suggest that paleoenvironmental events occurred in the Mediterranean Basin and surrounding areas in the last 6 Myr might have had a greater impact on species diversification. In particular, the strong trait and niche phylogenetic conservatism may have contributed to the geographical expansion during the period of prevailing aridity that characterized the Upper Miocene and to the rapid accumulation of lineages by allopatric speciation during the climatic oscillations from the Pliocene (Albaladejo et al., 2021; Martín-Hernanz et al., 2021a) as occurred in other Mediterranean plant groups (e.g., *Dianthus*, Caryophyllaceae: Valente et al., 2010; *Centaurium*, Gentianaceae: Jiménez-Lobato et al., 2019; *Limonium*, Plumbaginaceae: Koutroumpa et al., 2021). The geographical expansion during the Upper Miocene could also have been reinforced by the high phenotypic plasticity of vegetative traits, facilitating local

adaptation and diversification of other phenotypic features not explored here (Widén et al., 2023). Understanding how different abiotic, biotic, and historical factors have sequentially driven the rapid diversification of *Helianthemum* is thus important to understand explosive evolutionary radiations and the origins of uneven biodiversity patterns between different regions or clades.

AUTHOR CONTRIBUTIONS

The idea and design of the study were developed by S.M.-H., R.G.A., and A.A. Plant material was mostly collected by S.M.-H., R.G.A., E.R., and A.A. Buds and flowers were dissected and floral traits quantified by S.M.-H., E.R., and M.M.-R. The analyses and interpretation of the data were carried out by S.M.-H., R.G.A., S.L., J.A., and A.A. The manuscript was written and discussed by all authors, but writing was led by S.M.-H. and A.A.

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DATA AVAILABILITY STATEMENT

Data of floral traits are accessible in the public repository Depósito de Investigación Universidad de Sevilla: <https://idus.us.es/handle/11441/142850>.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

APPENDIX S1. Details on geographic origin, collection information, and number of samples for the 83 *Helianthemum* taxa analyzed in this study.

APPENDIX S2. Light micrographs of floral structures from species of *Helianthemum*. (A–C) Types of stigma–anther

separation (i.e., herkogamy). (A) No stigma–anther separation (no herkogamy) in *H. angustatum*. (B) Stigma above stamens (i.e., approach herkogamy) in *H. almeriense*. (C) Stigma below stamens (i.e., reverse herkogamy) in *H. oelandicum* subsp. *conquense*. (D–G) Style shapes in *Helianthemum*. (D) Straight style in *H. brammwelliorum*, (E) Sigmoid style in *H. almeriense*. (F) Geniculate style in *H. alypoides*. (G) Curly style in *H. cinereum* subsp. *rotundifolium*.

APPENDIX S3. Phylogenetic heat map for standardized mean values calculated for the 18 quantitative floral traits from the 83 taxa of *Helianthemum* analyzed in this study. Blue tones: taxa with the lowest values for the measured traits; green: intermediate values; yellow: highest values.

APPENDIX S4. Summary of the first two axes extracted from the PCA–Hill and Smith using 18 quantitative floral traits and two discrete traits (i.e., petal color, style type). Column coordinates associated with each variable, reflecting the contribution of the variables to the principal component, the eigenvalue, and the explained variance are included. Values in bold (<or> than |0.5|) represent a high contribution of the variable to the component.

APPENDIX S5. Cluster analyses to determine the optimal number of morphological groups. (A) Result of the elbow method analysis derived from the PCA–Hill Smith. (B) Dendrogram constructed with 18 floral traits for 83 taxa of the genus *Helianthemum*. Colors indicate the three major morphological floral groups detected in the PCA–Hill Smith.

APPENDIX S6. Violin plots of the 18 quantitative floral traits analyzed for the 83 taxa of the genus *Helianthemum* by the three floral morphologies identified from the PCA–Hill Smith. Different letters indicate significant differences among floral morphologies inferred using phylogenetic generalized least squares regressions and post hoc tests. Plots are numbered and colored according to the three floral morphologies (i.e., type I, type II, and type III).

APPENDIX S7. Results of the multistate trait-based analyses inferred by Multi-State Speciation and Extinction (MuSSE) analysis. λ , speciation rates; μ , extinction rate; q , transition rate; NP, number of parameters; logL, log Likelihood; AIC, Akaike information criterion. Best-fit model is indicated in red and bold; the second best-fit model is in black and bold.

APPENDIX S8. Representation of the estimated parameters (speciation rate, extinction rate, and net diversification rates) resulting from applying multistate trait-based analyses inferred by MuSSE over the three floral morphologies of *Helianthemum*.

APPENDIX S9. Best configurations for diversification rate shifts (i.e., with the highest posterior probability) of the genus *Helianthemum* based on floral traits (i.e., floral size and male–female function) and environmental data (i.e., aridity and continentality; Martín-Hernanz et al., 2021a) obtained using BAMM.

APPENDIX S10. Floral and environmental diversification rates over time obtained from BAMM for the genus *Helianthemum*. The 95% confidence intervals are indicated by shaded areas. Floral diversification is based on floral traits (i.e., floral size and male–female function) and environmental diversification on environmental data (i.e., aridity and continentality; Martín-Hernanz et al., 2021a). Floral and environmental rates are unitless.

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