Research article

Delving into the loss of heterostyly in Rubiaceae: Is there a similar trend in tropical and non-tropical climate zones?

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ABSTRACT

Heterostyly is a specialised floral polymorphism consisting in the presence within the populations of two or three morphs that differ reciprocally in sexual organ position. The function of heterostyly has usually been related to the promotion of cross-pollination fostered by the perfect adjustment between pollinators and flower morphologies. Rubiaceae is the largest family in which this polymorphism is present. Nevertheless, just a few studies on the evolution of heterostyly have been carried out in this family.

To investigate the appearance and maintenance of heterostyly we select the subfamily Rubioideae as study group. Rubioideae occur in both tropical and temperate regions and since the tropics are known to contain higher biodiversity and greater ecological specialisation than temperate areas, we characterise the taxa as tropical, non-tropical or mixed distributed (when they are present in tropical and non-tropical areas) and explored whether the heterostyly, as a specialised system, is more stable in tropical regions than in other climates of the world.

Ancestral nodes in Rubioideae present heterostyly, which also is maintained along most evolutionary lineages of this group. Although we do not find a significant correlation between the presence of heterostyly and the climate zones along the whole subfamily, our results show that two of the main clades in the Spermacoceae alliance where heterostyly is lost are distributed in non-tropical areas or, at least, they are not restricted to tropical distributions.

These results partially support the hypothesis that plant lineages when exposed to different pollination scenarios may evolve towards divergent pollination systems and different degrees of specialisation. However, a more detailed analysis at the species level is suggested for future studies.

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Introduction

Heterostyly is a genetically controlled floral polymorphism primarily characterised by the presence of two or three intra-population morphs whose flowers differ reciprocally in the position of the stigmas and anthers (Barrett, 2002). The function of heterostyly has been seen as a safeguard against self-fertilisation and inbreeding depression on one hand, and as a mechanism of promotion of cross-pollination between morphs on the other (Barrett, 1992). Some models explaining the evolution of heterostyly consider pollinators as the selective force driving the appearance of reciprocal herkogamy (Lloyd and Webb, 1992). This hypothesis is supported by different pollination systems (e.g., Stone and Thomson, 1994; Nishihiro et al., 2000; Lau and Bosque, 2003; Ferrero et al., 2011). Still, most of the groups in which the origin and evolution of heterostyly have been studied occupy temperate ecosystems (e.g., Primulaceae, Mast et al., 2006; Lithospermeae, Ferrero et al., 2009; *Narcissus*, Graham and Barrett, 2004; Pérez-Barrales et al., 2006).

Following the seminal work of Darwin (1877) and the hypothesis of Lloyd and Webb (1992), heterostyly is considered as a specialised system because of the perfect adjustment between pollinators and flowers required for efficient pollen transfer and thereby, for the origin and maintenance of stylar polymorphism in the plants. One of the most common hypotheses in this topic is the increasing specialisation from temperate to tropical regions (Johnson and Steiner, 2000). Comparisons of plant-pollinator networks from different latitudes support this idea, at least for flowering plants (Olesen and Jordano, 2002). However, contrary results have been reported in plant pollinator systems (Ollerton and Cranmer, 2002; Corlett, 2004), as well as in other mutualistic systems, suggesting that this hypothesis may vary depending on

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the category of interactions and on the group of taxa under study (Ollerton and Cranmer, 2002).

In this study we propose, for the first time, heterostyly as a scenario where to test the above-mentioned prediction. Tropical forests are known to contain a higher diversity of species than any other terrestrial ecosystem (Whitmore, 1998), since resources split more finely among a greater number of species (McArthur, 1972; Janzen, 1973). The predictable climate in the tropics led to a greater importance of biotic interactions (Dobzhansky, 1950). In these ecosystems we thus could expect to find more obligate mutualisms than in temperate forests because tropical forests are stable environments where environmental perturbations to population sizes are hypothesised to be minimal (May, 1973; Farnworth and Golley, 1974). In temperate zones, oscillations in ecological and environmental conditions are more frequent and drastic than in tropical areas (Fischer, 1960). For this reason, we can expect that greater possible variations in pollinator arrays in temperate environments could affect the maintenance of the heterostylous condition (e.g., *Narcissus papyraceus*, Pérez-Barrales et al., 2009) to a greater extent than in tropical systems.

When testing this kind of hypothesis, a proper phylogenetic framework is required together with an adequate characterisation of the floral morphology. Rubiaceae are one of the largest families of flowering plants with more than 13,100 species, 611 genera and 3 subfamilies (Robbrecht and Manen, 2006; Govaerts et al., 2006; Bremer and Eriksson, 2009). Rubioideae and, particularly, the Spermacoceae alliance (*sensu* Bremer and Manen, 2000), are distributed worldwide and possess high diversity of life forms and flower morphology (Bremer and Manen, 2000). Heterostyly is well known to be present especially in this subfamily (e.g., Keegan et al., 1979; Sobrevila et al., 1983; Ree, 1997; Faivre and McDade, 2001). Rubioideae have been subjected to numerous phylogenetic studies in the last decades (reviewed in Bremer, 2009a,b), resulting in a good knowledge of the relationships within the subfamily (e.g., Andersson and Rova, 1999; Nepokroeff et al., 1999; Malcomber, 2002; Backlund et al., 2007). Moreover, the extraordinary diversity of morphologies in reproductive and vegetative traits in the subfamily has attracted the interest of researchers examining character evolution (e.g., Huysmans et al., 2003). In particular, in this study the Spermacoceae alliance (Bremer and Manen, 2000) is given more attention because it is well-represented in number by temperate taxa (Bremer and Eriksson, 2009) and there is extensive information on types of stylar polymorphism in the group (e.g., Wyatt and Hellwig, 1979; Naiki and Nagamasu, 2003; Consolaro, 2008).

The main aim of this work was to analyse the evolution of heterostyly in Rubioideae with particular attention to the Spermacoceae alliance, and to discuss whether this polymorphism is more likely to be lost in non-tropical than in tropical environments. To examine whether heterostyly is an ancestral character we reconstructed the evolution of the polymorphism in a molecular phylogenetic framework. We also characterised the taxa as tropical, non-tropical, or distributed in both climate zones (henceforth "mixed") and then we analysed the evolution of this character. Afterwards, we tested whether the presence of the polymorphism was correlated to the fact of being tropical. Finally, we discussed possible differences in the evolutionary loss of heterostyly between tropical, non-tropical and mixed areas.

Materials and methods

Data set

We downloaded from GenBank the sequences of species from 85 genera of Rubioideae (Rubiaceae) and one outgroup (following Rydin et al., 2009). We randomly selected one species per genus among the set of species for which most sequences were available in GenBank. Taxa names were assigned following the International Plant Names Index (http://www.ipni.org/index.html). We used the taxonomic treatment of Psychotrieae and Spermacoceae alliances following Bremer and Manen (2000). In the case of paraphyletic genera, we specified the group considered in the footnotes (Appendix S1). As the evolution of the heterostyly was assessed at the genus level, we characterised a genus as heterostylous if at least one of its species presented heterostyly. Such characterisation was based on Bremekamp (1952), Ganders (1979), Keegan et al. (1979), Wyatt and Hellwig (1979), Steyermark (1988), Robbrecht (1988, 1993), Tange (1997), Rutishauser et al. (1998), Western Australian Herbarium (1998–), Delprete and Boom (1999), Taylor and Clark (2001), Malcomber (2002), Naiki and Nagamasu (2003), Kårehed and Bremer (2007), Puff (2007), eFloras (2008), Aluka database (2000–2010), Consolaro (2008), García-Robledo and Mora (2008), Sonké et al. (2008), Groeninckx et al. (2009), Neupane et al. (2009) and Rydin et al. (2009). To characterise the genera as tropical, non-tropical or mixed, we used the world checklist of selected plant families: Royal Botanic Gardens, Kew (available at: http://apps.kew.org/wcsp/home.do) and the updated World Map of the Köppen–Geiger climate classification (Kottek et al., 2006). The climate classification combines temperature and precipitation conditions. Main climates are (A) Equatorial (minimum temperature $\geq +18\,°C$), (B) Arid (accumulated annual precipitation <10 mm), (C) Warm temperate (minimum temperature above $-3\,°C$ and below $+18\,°C$), (D) Snow (minimum temperature $<-3\,°C$) and (E) Polar (maximum temperature $<+10\,°C$). We defined the taxa as tropical when they were distributed in regions classified as (A); non-tropical when distributed in (B), (C) and/or (D); and, as mixed when distributed in (A) and other areas. We did not find any Rubiaceae species with polar (E) distribution.

Phylogenetic analysis

Sequences for three loci, *ndhF*, *rps16* and nrITS, were downloaded from GenBank (Appendix S1). We aligned the sequences with MUSCLE (Edgar, 2004) as implemented in the Seaview program (Galtier et al., 1996). The combined matrix comprised 3774 aligned characters. Characters between position 70–260 and 487–461 in the nrITS sequences were discarded because of difficulty in the alignment. We generated the following partitioning strategies to be assessed according to Brown and Lemmon (2007): (1) no partitioning; (2) mitochondrial and nuclear loci separately; and (3) each locus separately. We runned MrBayes for each of them and then we compared values of Bayes Factor (BF) among the three approaches (Pagel and Meade, 2006). BF was calculated as $2 \times (\ln margL_a - \ln margL_n)$, where margL refers to the marginal likelihood of the null ($margL_n$, no partitioning) and alternative ($margL_a$) models, respectively. The lnmargL values are well approximated by the harmonic mean likelihood values from the posterior distribution in the analysis. A BF value greater than 2 reveals a positive evidence, values greater than 5 a strong evidence, and greater than 10 a very strong support of the alternative model (Pagel and Meade, 2006). After finding no differences between these strategies we considered all the loci as independent. To determine the best fit model of sequence evolution for each gene, three substitution schemes were selected in jModelTest 0.1.1 (Posada, 2008), including invariable sites and rate variation among sites, for a total of 24 models. Thus, the evolutionary model used in the Bayesian reconstruction was the general time reversible model of sequence evolution, with gamma (GTR + Γ) for each partition.

The Bayesian phylogenetic reconstruction was conducted with BayesPhylogenies (Pagel and Meade, 2004, available from www.evolution.rdg.ac.uk). We used *Colletoecema dewevrei* as outgroup in the analysis. Two independent runs of Markov chain Monte Carlo (MCMC) simulations over the partitioned data set were run for six million generations each, with trees sampled every 600 generations. We used TRACER 1.5 (Rambaut and Drummond, 2007) to assess for convergence in the analyses. Afterwards, we selected 2500 trees from each of the runs, which were used to compute the consensus tree in the BayesTrees software (available at http://www.evolution.rdg.ac.uk/BayesTrees.html). At least 1800 trees were discarded as burn-in in each analysis.

In addition, a Maximum-likelihood analysis (ML) with a rapid hill-climbing algorithm (Stamatakis, 2006) was conducted in RAxML 7.0.3. Support values were obtained through a rapid bootstrap algorithm (Stamatakis et al., 2008). We used the GTR+Γ model with 4 rate categories. Support values were obtained through a rapid bootstrap algorithm (Stamatakis et al., 2008) with 5000 iterations.

Character reconstruction

For the reconstruction of heterostyly, we used a reversible-jump MCMC run as implemented in BayesTraits (Pagel et al., 2004; Pagel and Meade, 2006). We randomly selected 1000 trees from the 2500 trees used to infer the consensus and BayesTraits was applied to account for the phylogenetic uncertainty. Heterostyly was coded as present (1) or absent (0) and taxa were defined as non-tropical (0), tropical (1) or mixed (2) according to the climate zones they occupy. In the analyses we used a uniform hyperprior (Pagel and Meade, 2006) and the amount of change in rate coefficients among generations in the MCMC (i.e., the ratedev parameter) was set to achieve acceptance rates in the range 20–40%. Markov chains were run for 10 million generations sampled each 1000 steps, with a burn-in of 10,000. The inference of ancestral character states depends on the model of character evolution that includes the direction, order and reversibility of state changes. For this reason, we compared several models of evolution. The simplest model, the Brownian motion model (H0), which assumes equally likely changes in character states in any direction, was compared to another (H1) with different rates of change between each state (Lewis, 2001). Moreover, we also tested a third hypothesis in which the rate of change from no-heterostyly to heterostyly was set to 0 (H2). To compare among models we calculated the Bayes Factor (BF) as for the partitioning strategy (see 'Phylogenetic reconstruction' section) where margL refers to the marginal likelihood of the null (margL$_n$, H0) and alternative (margL$_a$) models (H1 and H2) respectively. In this case, the values were approximated by the harmonic mean likelihood values from the posterior distribution in the BayesMultiStates analysis. BayesMultiState is used for reconstructing ancestral states when they adopt a finite number of discrete states and for testing models of trait evolution. The criteria to select the best model were the same as for the type of partitions (Pagel and Meade, 2006). We performed the state reconstruction using the model with the best support.

Finally, the phylogenetic association of heterostyly and climate zone was tested by means of a generalised estimating equation (GEE) procedure that uses a generalised linear model (GLM) approach incorporating the phylogenetic relatedness among species as a correlation matrix in the model. GEE analyses were done with the "compar.gee" function within the APE software package in R (Paradis and Claude, 2002).

Results

Phylogenetic analysis

The topology of the phylogeny with three partitions (*rps16*, *ndhF* and nrITS) was consistent between the Bayesian and ML analyses (Fig. 1). In the consensus tree recovered, three tribes were retrieved with high support (PP: Bayes posterior probability values, BS: bootstrap support): Ophiorrhizeae (1.00 PP/100 BS), Urophylleae (1.00 PP/100 BS), and Lasiantheae (1.00 PP/100 BS), although the relation among them was not clear. Coussareeae were recovered as sister to the clade of the Psychotrieae and Spermacoceae alliances. The relationships among taxa were well resolved in the Spermacoceae alliance but not in the Psychotrieae alliance. Several tribes were found within the Psychotrieae alliance (Morindeae, Craterispermeae, Psychotrieae, Schradereae, Gaertnereae) but relations among them were unclear, as indicated by the low PP and BS values (Fig. 1).

Within the Spermacoceae alliance, *Schismatoclada* and *Danais* form an independent group corresponding to Danaideae (1.00 PP/100 BS). The rest of genera form two groups with strong support: one includes Knoxieae and Spermacoceae (1.00 PP/93 BS); and the other, Anthospermeae, Argostemmateae, Dunnieae, Paederieae, Putorieae, Theligoneae and Rubieae (1.00 PP/90 BS) (Fig. 1).

Character reconstruction

For the reconstruction of heterostyly the best model of character evolution according to the BF was the one with independent rates of evolution between states (H1; q01 = 22.93, q10 = 17.93) [margL(H0) = −53.16; margL(H1) = −54.36; margL(H2) = −70.07]. For the climate zones, the best model according to the BF was the one considering independent rates of evolution (H1; q01 = 64.95, q02 = 58.90, q10 = 41.24, q12 = 65.12, q20 = 20.50, q21 = 54.64), [margL(H0) = −92.29; margL(H1) = −88.48].

The reconstruction of heterostyly (Fig. 2) suggested that the most recent common ancestor of Rubioideae was heterostylous (posterior probability of no heterostyly (0) = 0.26, posterior probability of heterostyly (1) = 0.74). Heterostyly evolved in Coussareeae (node 16: 0 = 0.09, 1 = 0.91) and at some point in Ophiorrhizeae (node 10: 0 = 0.14, 1 = 0.86).

The state of the most recent common ancestor of the clade consisting of the Psychotrieae and Spermacoceae alliances was heterostylous (node 20: 0 = 0.12, 1 = 0.88). The ancestor of the Psychotrieae alliance was also apparently heterostylous (node 21, 0: 0.16; 1: 0.84) and within the Psychotrieae alliance a reversion to non-heterostyly was reconstructed in Morindeae. The ancestor of the Spermacoceae alliance was also probably heterostylous (node 37: 0 = 0.09, 1 = 0.91). Loss of heterostyly in this group appeared to occur late and punctually, in Anthospermeae (node 63: 0 = 0.92, 1 = 0.08), Argostemmateae (node 72: 0 = 0.65, 1 = 0.34) and Rubieae (node 81: 0 = 0.91, 1 = 0.09) (Fig. 2).

With regard to the climate zones, the ancestral condition of the subfamily was not well resolved when considering three states [non-tropical (0), tropical (1), and mixed (2)]. Tropical distribution appeared to be the most probable state in Urophylleae and Ophiorrhizeae but only supported at the tip of the clades (node 6: 0 = 0.19, 1 = 0.64, 2 = 0.17 and node 10: 0 = 0.30, 1 = 0.52, 2 = 0.18). Mixed distribution is the most common state in the tribes Psychotrieae (e.g., node 26: 0 = 0.27, 1 = 0.29, 2 = 0.44), Morindeae (nodes 34: 0 = 0.19, 1 = 0.22, 2 = 0.57 and 36: 0 = 0.11, 1 = 0.12, 2 = 0.77) and Danaideae (nodes 38: 0 = 0.10, 1 = 0.11, 2 = 0.79). Ancestors of Paederieae and Rubieae probably occurred in a non-tropical environment (nodes 77: 0 = 0.60; 1 = 0.24, 2 = 0.16 and nodes 81: 0 = 0.53, 1 = 0.26, 2 = 0.21) as well as the ancestor of Dunnieae and Argostemmateae
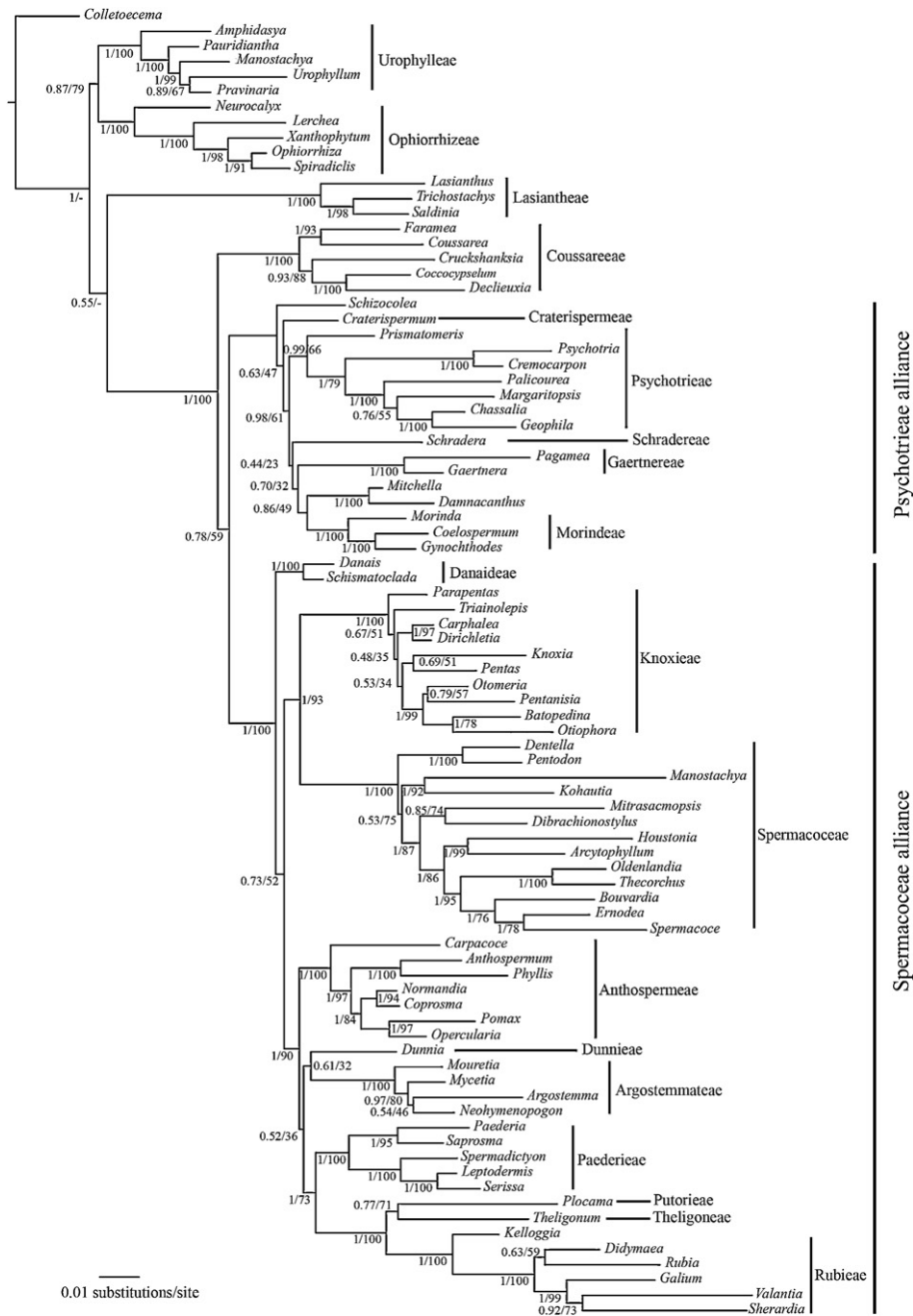
Fig. 1. Consensus tree of Rubioideae recovered from the Bayesian analysis of the combined *rps16*, *ndhF* and nrITS datasets. Node numbers show, in this order, posterior probabilities from the Bayesian analysis and bootstrap support values from the maximum likelihood analysis. Tribe names are indicated on the right side of genus names.

(nodes 70: $0 = 0.42$, $1 = 0.31$, $2 = 0.28$). We did not obtain a resolved reconstruction of climate zones for the rest of the tribes (Fig. 2).

The GEE approach showed no correlation between heterostyly and climate zones ($\beta_1 = 0.41 \pm 0.31$, $t = 1.32$, $P = 0.20$, significance level $= 0.05$).

Discussion

In this paper we propose a new perspective about the differential occurrence of heterostyly throughout the world, taking the cosmopolitan family Rubiaceae as a touchstone. Our results show that heterostyly is a character apparently ancestral to the clade consisting of the Psychotrieae and Spermacoceae alliances (see

Fig. 2, node 20). Reversals to monomorphism probably occurred more frequently within Spermacoceae (see Anthospermeae and Spermacoceae) than in any other group of Rubiaceae. Within the Spermacoceae alliance, heterostyly largely prevailed through Danaideae, Knoxieae, Spermacoceae, Dunnieae and Paederieae. Contrarily, loss of heterostyly occurred mostly in Anthospermeae and in the group that includes Rubieae, Putorieae and Theligoneae.

On the other hand, the ancestral reconstruction of the climate zones was not conclusive for these alliances. Climate zones where these groups evolved were not well resolved and did not appear to follow any particular pattern according to our results. Tropical distribution is the most probable state in the late evolution of Urophylleae and Ophiorrhizeae (Fig. 2, nodes 6, 7, 10 and 11), and other punctual nodes (Fig. 2, nodes 51, 67). The mixed
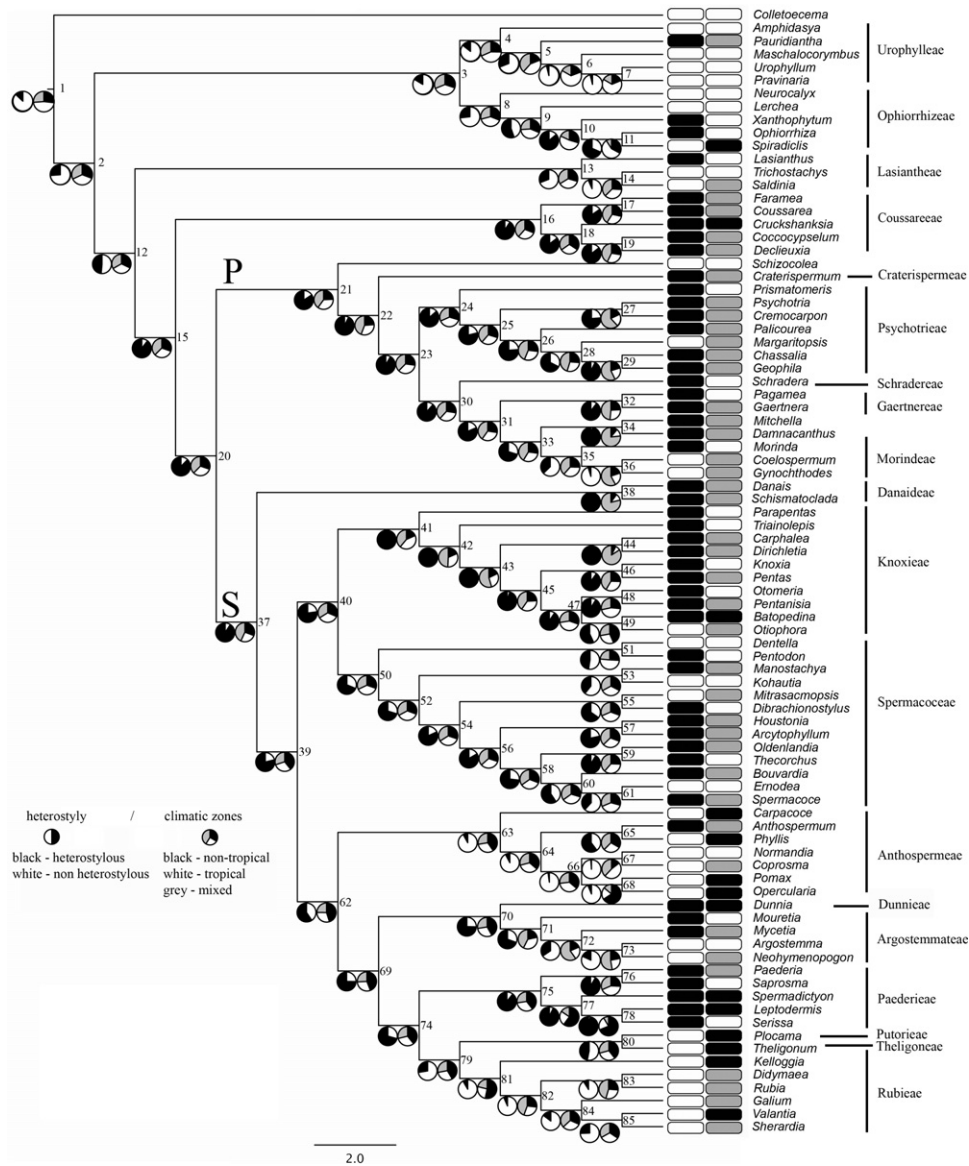
Fig. 2. Evolution of heterostyly and climate zones occupied by taxa in the Rubioideae on the consensus tree recovered from the Bayesian analysis of the combined *rps16*, *ndhF* and nrITS datasets. Node charts show the relative probabilities of presence of heterostyly (left) and climate zone (right) in which the taxa are distributed. Probabilities for the states in each node are summarised in Supporting Information (Appendix S2). S: Spermacoceae alliance; P: Psychotrieae alliance. Tribes are indicated to the right.

distribution appears to follow the general pattern in genera of the Psychotrieae alliance (12 from 17 genera) where it is also the most plausible state in several nodes (Fig. 2, nodes 26–29, 32, 34, 36). The same type of distribution is found for the ancestors of Knoxieae (Fig. 2, nodes 42, 43) and at some point in the Argostemmateae (Fig. 2, nodes 72, 73). The non-tropical distribution is only resolved for the Paederieae (Fig. 2, nodes 75–78) and it is relatively abundant in Anthospermeae (4 out of 7 genera) and the group including Rubieae, Putorieae and Theligoneae (4 out of 8 genera, whereas the rest of genera have mixed distribution, Fig. 2).

The hypothesis of this study was that heterostyly, which is considered a specialised mutualistic system, would be much more stable in tropical than in temperate climates. The relation between tropical/non-tropical areas and specialisation had already been questioned several times (Ollerton and Cranmer, 2002; Corlett, 2004). An increase in specialisation in the tropics has been found in larval host plants (Scriber, 1973) or in parasitoids of phytophagous insects (Hawkins, 1990). However, contrary results were found for

bark and ambrosia beetles host specificity (Beaver, 1978). In the present study, the correlation between presence of heterostyly and the climate zones occupied was not significant at the subfamily level; however, when considering just the resolved nodes for the climate zones, we found partial support to our hypothesis in specific groups. The loss of heterostyly appeared to be related to a non-tropical distribution in Rubieae (Fig. 2, node 81) and in Anthospermeae (Fig. 2, node 68; but see also node 67). In the case of Argostemmateae, a mixed distribution was more probable.

Our results indicate lack of correlation between the two characters, which could be attributed to the fact that specialisation, as here proposed, would not be a necessary condition for the maintenance of the polymorphism. However, similar functional groups of pollination could be acting in cases when strong oscillations in the environment occur. As our results show, an absence of a coevolutionary pattern for these characters at the genus level may exist in the studied group, although the failure of ancestral reconstruction of the climate zones could be fostering this result.

In any case, facing a total absence of correlation between these traits, we suggest that future studies should perform a more fine-grained scale analysis and explore the relationship at the species level. For the moment this idea has not been feasible because the stylar polymorphism is rarely reported or unknown for many species of each genus, and also because of the lack of molecular information at the species level. In addition, characterisation of heterostyly at the genus level can be difficult since this polymorphism may be variable among species of the same genus and even within the same species (e.g., Nakamura et al., 2007; Sakai and Wright, 2008). In addition, we consider Rubieae and Anthospermeae as the most appropriate groups for carrying out such analyses in the future.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ppees.2011.11.005.

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