

Habitat transitions and patterns of diversification of the genus *Aquilegia* in the Mediterranean context.

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Abstract

Genus *Aquilegia* in North America has become a recurrent example of adaptive radiation driven by pollinator specialization and floral syndromes differentiation. However, Eurasian columbines show similar taxonomic diversity despite to be mainly pollinated by bumblebees. This situation makes Eurasian columbines an ideal counterpoint to explore diversification process under low pollinator diversity but high environmental heterogeneity scenarios. In this paper we explore diversification rates and patterns of habitat (habitat and altitudinal range) transitions underlying the European columbines evolution. A density-dependent lineal model of diversification best fitted our data, which implies a lineal decrease of diversification rate through radiation. Our results point to an important role of habitat (14.3-15.8 habitat transitions) in driving European columbines diversification. Habitat and altitudinal range reconstruction showed a pattern from wide altitudinal range (encompassing 1000-1200 m) and generalist ancestors to narrow altitudinal ranges (\approx 800 m) and open-rocky habitats in more recent taxa (i.e. those species inhabiting mountains regions of South Europe). Ecological opportunity has been the preferred explanation for density-dependence diversification during a radiation. Glacial impacts on European flora and the coincidence of the initiation of the radiation with the onset of Mediterranean climate probably did generate ecological opportunities for niche expansion and habitat specialization and evolution. However, we suggest that some biological features of *Aquilegia* (low seed dispersal, small population sizes and frequent inbreeding depression) unrelated to ecological opportunity together with the orography might also be involved in density-dependent speciation and habitat evolution in this genus. Specifically, we propose that high geographical isolation between and within distinct South European peninsulas, restricted geographical range size and specialization on open-rocky habitats combined with low seed dispersal might have contributed to the density-dependence mode of diversification in European columbines.

Keywords: *Aquilegia*, altitudinal range, density-dependent diversification, European columbines, glaciations, habitat specialization, South European peninsulas.

Riassunto

Il genere *Aquilegia* nel Nord America costituisce un esempio di radiazione adattativa favorita da impollinatori specializzati e differenziazioni floreali specifiche. Tuttavia le aquilegie eurasiatiche mostrano una comparabile diversità tassonomica, nonostante siano principalmente impollinate da bombi. Questa situazione rende le aquilegie eurasiatiche un contrappunto ideale per esplorare il processo di diversificazione in condizioni di bassa diversità impollinatoria, ma con uno scenario di elevata eterogeneità ambientale. In questo articolo esploriamo i tassi di diversificazione e modelli di habitat (habitat ed estensione altimetrica) che stanno alla base della evoluzione delle aquilegie europee. Un modello lineare di diversificazione densità-dipendente si adatta meglio ai nostri dati, il che implica una riduzione lineare del tasso di diversificazione per irraggiamento. I nostri risultati indicano un ruolo importante dell'habitat (14.3-15.8 habitat di transizioni) nella guida alla diversificazione delle aquilegie europee. Habitat e ricostruzione dell'estensione altimetrica hanno mostrato un modello di ampia estensione altimetrica (che comprende 1000-1200 m) e gli antenati generalisti a bassa estensione altitudinale (\approx 800 m) e habitat aperti rocciosi in taxa più recenti (cioè quelle specie che abitano le regioni montagnose del Sud Europa). L'opportunità ecologica è stata la spiegazione preferita per la diversificazione densità-dipendente nel corso di una radiazione. Impatti sulla flora glaciale europea e la coincidenza della iniziazione della radiazione con l'inizio del clima mediterraneo, probabilmente hanno generato l'opportunità ecologica per l'espansione delle nicchie e per la specializzazione ed evoluzione degli habitat. Tuttavia, suggeriamo che alcune caratteristiche biologiche del genere *Aquilegia* (bassa dispersione dei semi, piccole dimensioni delle popolazioni e frequenti depressioni di consanguineità), estranee a opportunità ecologiche insieme con l'orografia, potrebbero anche essere coinvolti nella specializzazione densità-dipendente ed evoluzione dell'habitat in questo genere. In particolare, proponiamo che l'alto isolamento geografico tra e all'interno delle singole penisole sud europee, abbia ristretto la dimensione geografica e la specializzazione in habitat rocciosi aperti in combinazione con la bassa dispersione dei semi che potrebbero aver contribuito alla modalità densità-dipendente di diversificazione nelle aquilegie europee.

Parole chiave: *Aquilegia*, estensione altimetrica, diversificazione densità-dipendente, aquilegie europee, glaciazioni, specializzazione dell'habitat, penisole Sud europea.

Introduction

Adaptive radiation refers to those evolutionary groups that have exhibited an exceptional extent of adaptive diversification into a variety of ecological niches (Schluter, 2000), with such divergence often occurring extremely rapidly (Givnish *et al.*, 1997). In recent years, the application of molecular techniques

to the study of widespread plant groups has served to highlight that recent radiations could have played an important role in the exceptional species richness of several continental regions of the world. Examples include the genera *Lupinus* in South America (Hughes & Eastwood, 2006), *Penstemon* in North America (Beardsly *et al.*, 2004; Wolfe *et al.*, 2006), *Cistus* in the Mediterranean basin (Guzmán *et al.*, 2009).

The canonical view of adaptive radiation in animal-pollinated plants (Grant & Grant, 1965; Stebbins, 1970) relies on the hypothesis that speciation proceeds through reproductive isolation resulting from pollinator specialisation and differentiation of floral traits. Among the most cited examples of animal-pollinated plants' adaptive radiations is the *G. Aquilegia* (columbines) in North America (Hodges & Arnold, 1994; Whittall & Hodges, 2007). North American columbines strongly support the hypothesis that flower morphology (particularly spur length and floral pigments) has diversified in association with changes in the type of pollinator, which shifted from bees and bumblebees to hummingbirds and finally to hawkmoth (Whittall *et al.*, 2006; Whittall & Hodges, 2007). Furthermore, North American columbines also showed a considerable habitat diversification (Whittall & Hodges, 2007; Bastida *et al.* in press). In contrast to the well-stated diversification process of New World columbines the knowledge on reproductive biology, ecology and the mechanisms driven the diversification of Eurasian columbines is scarce and scattered (but see Lavergne *et al.*, 2005; Gafta *et al.*, 2006; Medrano *et al.*, 2006; Tang *et al.*, 2007). What is known about Eurasian columbines also challenges the canonical view. *Aquilegia* in Europe (≈ 21 spp.) and Asia (≈ 23 spp.) shows similar taxonomic diversity to North America (≈ 25 spp.), despite of less pollinator diversity. Concretely, Eurasian species are quite similar in floral traits and differ mainly in vegetative traits in close association with habitats occupied (Bastida *et al.* unpublished). Besides, mode of geographical speciation within the European species is predominantly allopatric, due to the insular character of distinct South European peninsulas (Bastida *et al.* in press). In fact, Balkans, Italian (plus Corsica and Sardinia) and Iberian peninsulas constitute the main centres of diversification, where columbines are distributed through mountainous regions, showing high degree of endemism restricted spatially and altitudinally within each peninsula. These geographical peculiarities and habitat specificity makes interesting explore habitat specialization and diversification patterns of European columbines. Additionally, the diversification of European columbines should be affected by the quaternary glaciations (2.54 mya, Bastida *et al.* in press).

These features are ideal to test one of the most striking patterns of adaptive radiations, a tendency for species-level diversification rates to decline through time. We explored the diversification process of *Aquilegia* in Europe in relation to habitat specialization by

testing: 1- whether European columbines diversified following a constant or non-constant rate, which can be due to phenomena such as rapid radiations, phenotypic innovations, environmental changes or by density-dependent cladogenesis. 2- whether European columbines followed a habitat diversification pattern consistent with the "Ancestor-generalist vs. Derived-specialist" hypothesis.

Material and methods

PHYLOGENY OF EUROPEAN COLUMBINES

To explore habitat transitions and diversification patterns of European columbines we used the recently published phylogeny of Genus *Aquilegia* (see Bastida *et al.* in press), which involved nineteen Eurasian species of which thirteen were from Europe. Our phylogeny came from the combined Bayesian analysis of three molecular markers (ITS, trnK-matK and trnS-trnG). Concretely, we employed the 50% majority rule consensus tree and random samples of trees from the post-burn-in tree file, in which we pruned all species not belonging to the Euroasiatic lineages (Tab. 1).

DIVERSIFICATION PATTERNS

We explored if European columbines diversification followed a constant or non-constant rate, which can be due to phenomena such as rapid radiations, phenotypic innovations, environmental changes or by density-dependent cladogenesis.

We used a pruned consensus tree involving thirteen European species of columbines plus the Asiatic *A. olympica*. To add a temporal axis (relative time units) to the phylogeny the NPRS method (Sanderson, 1997), as is implemented in APE package (Paradis *et al.*, 2004), was used. All analyses were carried out on ultrametric trees. Firstly, we analyzed the consensus tree, in which politomies were randomly resolved using the function "multi2di" of APE (Paradis *et al.*, 2004). Secondly, to incorporate topological and branch length uncertainty we analyzed a random sample of 100 trees from post-burn-in tree file.

To explore the diversification we plotted the cumulative number of lineages of the phylogeny against time (lineages through time, LTT). LTT plot of European columbines was evaluated in three ways:

i) We used the CR method (Constant Rate, Pybus & Harvey, 2000; Pybus *et al.*, 2002) for testing whether the splitting events in a phylogeny are randomly distributed through time. The CR method uses internode-distance information for calculating a

Taxa	Habitat	Altitudinal range
<i>A.pyrenaica</i> DC.	Saxicolous-meadows	1200-2250
<i>A.vulgaris</i> L.	Forest-meadows-saxicolous	0-2100
<i>A.aurea</i> Janka	Saxicolous-meadows	1800-2500
<i>A.fragrans</i> Benth.	Saxicolous-meadows	2400-3700
<i>A.ecalcarata</i> Maxim. Fl. Tangut.	Forest-meadows	1800-3500
<i>A.yabeana</i> Kitakawa	Forest-meadows	1000-2200
<i>A.viscosa</i> Gouan	Saxicolous	1900-2350
<i>A.oxysepala</i> Trautv. & Mey.	Forest-meadows	400-2700
<i>A.sibirica</i> Lamarck	Forest-meadows	1600-2000
<i>A.thalictrifolia</i> Schott & Kotschy	Saxicolous	500-1600
<i>A.olymptica</i> Boiss.	Forest-meadows	1250-2600
<i>A.nigricans</i> Baumg.	Forest-meadows	1000-1800
<i>A.glandulosa</i> Fisher ex Link	Forest-meadows	1900-2700
<i>A.einseleana</i> F.W. Schultz	Saxicolous-meadows	600-2100
<i>A.bernardii</i> Gren. & Gord.	Saxicolous	1500-2300
<i>A.atrata</i> Koch	Forest-meadows-saxicolous	400-2000
<i>A.alpina</i> L.	Saxicolous-meadows	1600-2600
<i>A.flabellata</i> Sieb. & Zucc.	Saxicolous-meadows	¿?-2950
<i>A.ottonis</i> Orph.	Saxicolous	1000-1500
<i>A.nugorensis</i> Arrigoni & Nardi	Forest	900-¿?
<i>A.barbaricina</i> Arrigoni & Nardi	Forest	1300-1400

Tab. 1 - List of species, habitat states and altitudinal ranges reconstructed.

summary statistic, γ -statistic, of the relative locations of the internal nodes within a phylogeny. Significant departures towards negative values indicated that the internal nodes are closer to the root than expected under the null hypothesis that the diversification rate (b) and extinction (d) have remained constant among lineages and through time (“CR birth–death model”). Departures towards positive values suggest that internal nodes are closer to the tips. Observed γ -values below the critical value of -1.645 are sufficient to reject the null hypothesis at the 5% level (one-tailed test). In practice, γ can only reject the null hypothesis if b has decreased (rather than d increased) through time. We corrected γ -statistic for incomplete taxon sampling by the *mccrTests* (Pybus *et al.*, 2002) implemented in LASER (Rabosky, 2006a)

ii) Alternatively, we used the method developed by Paradis (1997) based on survival analysis and implemented in APE package (Paradis *et al.*, 2004) to detect shifts in net diversification rate ($b - d$). This method takes divergence times (node heights) in a reconstructed phylogeny as the data, and performs likelihood analysis of three survival models. Model A assumes a CR of diversification (i.e. the expected number of lineages follows an exponential increase), model B assumes a gradually changing rate of diversification, and model C assumes an abrupt change in diversification before and after some break-point in the past, but with constant rates through time otherwise. The relevant parameters in models B and C are β and δ_1/δ_2 , respectively: if $\beta > 1$ or $\delta_1 < \delta_2$, diversification declines whereas if $\beta < 1$ or $\delta_1 > \delta_2$, diversification increase through time. Maximum log-likelihood (LH)

scores and the AIC (Akaike, 1973) were computed for each model to evaluate which model best explained the data. In general the model with smallest AIC value is selected as the one that best fits the data (Paradis, 1997). However, we followed this rationale only for further differentiating between models B and C once model A had clearly been rejected.

iii) Approaches such as the γ -statistic (Pybus & Harvey, 2000; Pybus *et al.*, 2002) and survival analysis (Paradis, 1997), test for departures from the pure birth-death model of cladogenesis, and can only be used to infer temporal decreases in diversification rates (Nee, 2001). These methods are thus unable to address many questions of interest to evolutionary biologists. Furthermore, existing methods suffer reduced power to detect temporal decreases in diversification rates when clades have diversified under high background extinction rates (Rabosky, 2006b).

We used “Likelihood Analysis of Speciation and Extinction Rates” LASER package (Rabosky, 2006a), which fits a candidate set of five rate-variable diversification models to phylogenetic data and contrasts the likelihood of the data under these models to alternatives where speciation and extinction rates have remained constant over time. The test statistic for constancy of diversification rates is computed as: $\Delta AIC_{Crc} = AIC_{Crc} - AIC_{Crv}$

Where AIC_{Crc} is the Akaike Information Criterion (AIC) score for the best-fit rate-constant model of diversification, and AIC_{Crv} is the AIC score for the best-fit rate-variable model under consideration. Thus, a positive ΔAIC_{Crc} value suggests that a rate-variable model of diversification best approximates the data.

Phylogenies with incomplete taxon sampling can result in spurious decline in diversification rates over time (Pybus *et al.*, 2002). To address this problem in LASER, one can generate rate-constant phylogenies with distinct taxon sampling and tabulated the null distribution of the ΔAIC_{Crc} test statistic. A call to the function “*fitdAICrc.batch*” will then generate the null distribution of the test statistic and return the probability of the observed ΔAIC_{Crc} index under the null hypothesis.

LASER solve some of above mentioned problems by rather than explicitly testing a specific null hypothesis of diversification, such as the pure-birth process, likelihood methods do not need to assume a particular parameterization of the birth-death null model. Finally, such model-based approaches can simultaneously assess rate variation over time and provide estimates of relevant diversification parameters (Barraclough & Vogler, 2002).

ANCESTRAL HABITAT RECONSTRUCTION

We classified each species into one of five combinations of three habitats use (forest, saxicolous, forest-meadows, saxicolous and meadows and forest-saxicolous-meadows, see Bastida *et al.* in press). We also recorded the maximum and minimum altitude of each species. The adscription of a species to a single habitat (forest or saxicolous) was indicative of habitat specialist while the last category was indicative of generalist. Habitat and altitudinal limits were gathered from our own field observations, specimen label data and the literature (Munz, 1946; Pignatti, 1983; Díaz Gonzalez, 1986; Fu Dezhi & Robinson, 2001; Nold, 2003; Bacchetta *et al.*, 2004; Arrigoni, 2006; Polyakova *et al.*, 2008).

We applied the Bayesian mutational mapping approach (BMM) (Nielsen, 2002; Huelsenbeck *et al.*, 2003) for mapping habitat state. We used a stochastic substitution model as implemented in SIMMAP (Bollback, 2006) to calculate the posterior probability of each ancestral state reconstruction (SIMMAP considers only those trees possessing the node in question). We also used SIMMAP to explore the posterior expectation of the number of transitions for the habitat (SIMMAP re-scales the branch lengths of the tree in memory such that the overall length of the tree is one).

We used different parameter values for the priors to test the effect on the results of using a low-rate or high-rate prior on the tree length: ($\alpha:1 \beta:1$), ($\alpha:3 \beta:2$), ($\alpha:10 \beta:1$) with 100 samples or realisations for each tree, of the 2,000 last trees from the post-burn-in tree file.

For continuous eco-characters (e.g. a rainfall gradient or altitude where individual species occur in ranges) it has been proposed several coding methods that preserve both range and order information (e.g. GFC “generalized frequency coding” and MaxMin coding) (Graham *et al.*, 2004; Hardy & Linder, 2005). We used the MaxMin Coding for altitudinal range reconstruction, which codes ranges for a terminal by two characters: one giving the maximum value and the second the minimum value for that parameter in the species. The range for the terminal is then the set of values between the maximum and the minimum. These two characters are then optimized independently to the internal nodes using Squared-Change parsimony. The results of these independent optimizations of maxima and minima are then combined, and the ancestral ranges inferred to lie between them. Altitudinal range reconstruction was carried out in Mesquite 2.7.1 (Maddison & Maddison, 2006)

All analyses were performed on the post-burn-in

tree file (2,000 full-resolved trees) generated in the Bayesian analysis for the three combined data sets, which were pruned retaining only the Euroasiatic lineage.

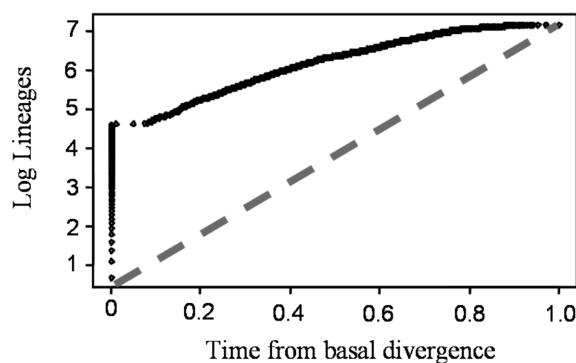
Results

PATTERNS OF DIVERSIFICATION

As a preliminary step we estimated the γ -statistic for one randomly resolved tree from our consensus tree ($\gamma = -0.74$, $P > 0.05$; $\gamma_{\text{corrected}} = -1.54$, $P = 0.29$). Based on this statistic, the null hypothesis of constant diversification (b) and extinction (d) rates was not possible to reject for European columbines. However, when we incorporated topological and branch length uncertainty to the estimated γ -statistic (over a random sample of 100 trees), we found strong support to the hypothesis that diversification rates of European columbines declined through time. A lineage-through-time plot (LTT) apparently indicates an excess of lineages relatively early in the history of the European columbines relative to the expected rate of lineage accumulation under a constant-rate model of diversification (Fig. 1), with calculated γ -statistic for the random samples of trees taking value of -26.97 ($P < 0.00001$) and corrected γ -statistic for incomplete sampled species of -1.22 ($P < 0.001$).

Survival analysis shed some light to the diversification process, since model A (constant diversification rate) fitted worst than model B (Model A vs. Model B: $\chi^2 = 854.232$ $df = 1$, $P < 0.00001$) and model C (Model A vs. Model C: $\chi^2 = 373.317$ $df = 1$, $P < 0.00001$), where model B follows a Weibull distribution and model C assumes that diversification changes with a breakpoint

Fig. 1 - Lineage-through-time plot (LTT) of European columbines based on 100 random sampled trees. The dashed line indicates expected rate of lineage accumulation under constant-rate diversification with no extinction. Note that compared to the constant-rate diversification model, lineages accumulate more quickly in the early phases of the radiation.



at time ($t = 0.5$). Among model B and C the best model was the model B with the lowest AIC (Tab. 2), which assumes a progressive decrease of diversification ($\beta > 1$).

The results for one randomly resolved tree from our consensus tree under “Likelihood Analysis of Speciation and Extinction Rates” (LASER) pointed to the same direction. The best rate constant model was “pure-birth” (AICrc = -7.31) and the best rate variable model was DDX (AICrc = -9.74). Thus, Δ AICrc resulted in a positive value (Δ AICrc = 2.43, $P = 0.08$), which suggests that the data are best approximated by a rate variable-model of diversification, the Density-Dependent Exponential model (DDX). However, when we estimated the best fitted model over a random sample of 100 trees the best rate constant model was “pure-birth” (AICrc = -30.66) and the best rate variable model was DDL (AICrv = -36.24) (Tab. 3), for 93 out of the 100 trees. These results strongly indicated that diversification of European columbines is best approximated by a rate variable-model of diversification (Δ AICrc = 5.58, $P = 0.02$). Concretely, our results are consistent with a density-dependent pattern of cladogenesis, the density-dependent linear model where the speciation rate (λ_0) was $r1 = 5.74$ and with the parameter $k = 14.77$, analogous to the carrying capacity parameter of population biology.

HABITAT RECONSTRUCTION

Because the posterior probabilities of the ancestral habitat states obtained using different parameter values were not different, we show only the posterior probabilities for the ancestral habitat states for the default parameter values of SIMMAP ($\alpha: 3$ and $\beta: 2$; Fig. 2, 3).

The Euroasian lineage showed between 14.3 and 15.8 habitat transitions. Most probable ancestral habitat states for the basal node of the Euroasian lineages was saxicolous and meadows. This ancestral state shifted to forest and meadows in the two successive nodes

	Model A	Model B	Model C
LH	-393.62	33.49	-206.96
AIC	789.24	-62.99	417.92
δ	2.00 ± 0.05	-	-
α	-	1.77 ± 0.02	-
β	-	2.13 ± 0.04	-
δ_1	-	-	1.04 ± 0.05
δ_2	-	-	4.31 ± 0.17

Tab. 2 - Results from the Survival Analysis of diversification over a random sample of 100 trees.

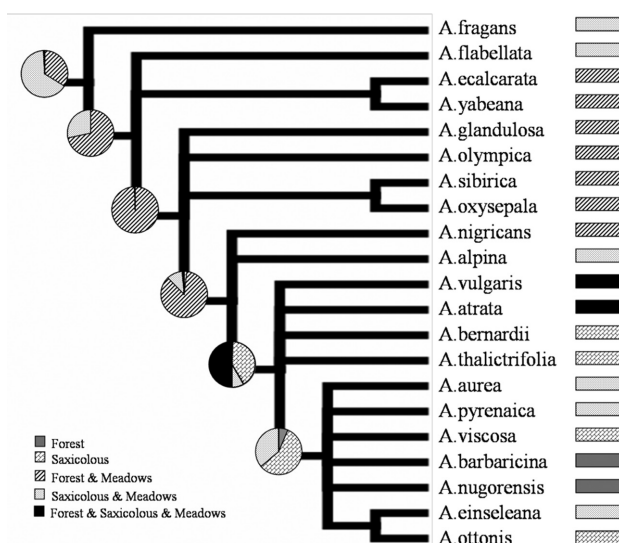


Fig. 2 - Ancestral habitat reconstructions of European columbines over a set of 2000 trees. Characters optimized by stochastic substitution model, as it is implemented in SIMMAP (Bollback 2006). (Figure modified from Bastida *et al.* in press).

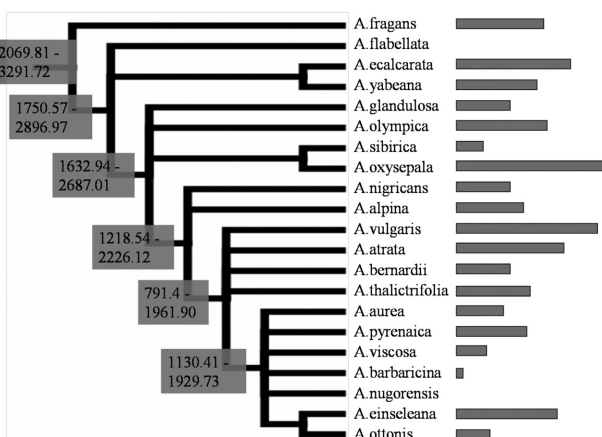


Fig. 3 - Altitudinal range reconstructions following Max Min Coding (Hardy & Linder 2005). Each altitudinal boundary was optimized by Squared-Change Parsimony, as is implemented in Mesquite v2.7.1 (Maddison & Maddison 2006), over a set of 2000 trees. The approximated current altitudinal range is indicated in the right side of the picture.

leading to the rest of the Asiatic species, and remained as the most probable ancestral state of the European species. Within the European species, there was a shift from the Asiatic forest and meadows ancestor to a forest, meadows and saxicolous ancestral state (a generalist ancestor). In the following node leading to the South European columbines, the ancestral state shifted to saxicolous or saxicolous and meadows (restricted and marginal habitats).

Respect to altitudinal range evolution, the internal nodes of Eurasian lineages showed mainly wide ancestral altitudinal ranges (range of around 1000-

1200 m). These ancestral nodes were altitudinally-distributed upper than most terminal European nodes (Fig. 3). Apparently, within European columbines seems to have undergone a narrowing of its altitudinal range from the ancestral nodes to the most recent node (800 m), which contain mainly species inhabiting marginal open and/or rocky places from South European peninsulas (Fig. 2).

Discussion

In contrast to North American columbines, the knowledge of diversification history of European species is scant. In this paper we have shown the temporal pattern of diversification and of niche differentiation of the genus in Europe. We suggest a temporal decline in diversification through the radiation that we found to be explained by a density-dependent linear model of speciation. Regarding the niche diversification we have extended the habitat diversification exploration (see Bastida *et al.* in press) to the altitudinal range evolution. Interestingly, in consonance with the pattern for habitat evolution, altitudinal range reconstruction followed a consistent trend toward narrowing altitudinal ranges in more recent lineages and coinciding with those clades containing eminently species from open and/or rocky habitats.

Taking in consideration the contingencies and geographical complexity inherent to Europe history, this pattern of ecological niche diversification could be related to the density-dependent linear model of diversification detected. However, much process (niche filling, ecological opportunity, interspecific competition or demographic features) can result in temporally declining diversification. In the following sections we discussed in depth these possibilities for European columbines.

ECOLOGICAL CAUSES OF DENSITY-DEPENDENT SPECIATION IN EUROPEAN COLUMBINES: ECOLOGICAL OPPORTUNITY AND HABITAT EVOLUTION.

We found a temporal decline in diversification through radiation consistent with a density-dependent linear pattern of speciation. One alternative to density-dependent speciation is that temporal declines in diversification are attributable to increasing extinction rates during evolutionary radiation. In fact, a limit on total resource availability implies that increasing species diversity will result in lower mean population sizes per species (Hubbell, 2000). Since population

	PureBirth	bd	DDL	DDX	yule2rate
LH	16.29	16.29	20.08	18.09	19.58
AIC	-30.65	-28.59	-36.24	-32.19	-33.17
ΔAIC^a	5.58	7.65	0.00	4.05	3.06
r1	1.62	1.62	5.74	9.77	3.18
a	-	0.001	-	-	-
k	-	-	14.77	-	-
x	-	-	-	0.82	-
r2	-	-	-	-	0.64
ts	-	-	-	-	0.35

^a Difference in AIC scores between each model and the overall best-fit model.

Tab. 3 - Results from the analysis of diversification with LASER over a random sample of 100 trees.

size is a determinant of extinction probability, extinction rates may increase with number of species. However, Rabosky & Lovette (2008) have shown that temporally declining speciation is the only process that can leave a signature of early rapid diversification in a molecular phylogeny of extant taxa.

Ecological opportunity has been the favoured explanation for a temporal declining of speciation rate within an adaptive radiation (Hughes & Eastwood, 2006; Rieseberg & Willis, 2007; Gavrillets & Losos, 2009). Ecological opportunity arises from relaxation of interspecific competition in conjunction with the availability of diverse resources (Schluter, 2000), and would drive both lineage and phenotype diversification during adaptive radiations (Erwin *et al.*, 1987; Foote, 1996). From a mechanistic point of view, ecological opportunity can facilitate higher per lineage rates of speciation by increasing the likelihood that a population will split and successfully occupy multiple adaptive peaks (Gavrillets & Losos, 2009). It is widely accepted that ecological opportunity might be responsible for explosive ecological diversification of many insular clades (Baldwin & Sanderson, 1998; Garcia-Maroto *et al.*, 2009). Species colonizing these insular environments would encounter a combination of high resource availability and a paucity of competing species. However, continental radiations occur against a complex ecological and geological background that differs from the majority of island systems, and it is unclear whether the processes and conditions that facilitate adaptive radiations on islands are also important during continental radiations (Barraclough *et al.*, 1999). Therefore, is it possible that conditions of ecological opportunity present in early stages of island radiations usually do not occur in continental systems.

Diversification of European columbines might be

moulded by a complex geography and by historical events (quaternary glaciations and the onset of the Mediterranean climate) that occurred through the origin and diversification of the genus in Europe. The origin of European columbines has been placed around 2.54 mya (Bastida *et al.* in press), what site its diversification under the recurrent impact of glaciations. Furthermore, this temporal window coincides with the onset of Mediterranean climate (2 - 3 mya; Thompson, 2005). Both events would have contributed to the emergence of newly and underutilized niches, such as alpine and montane habitats, consequently, increasing ecological opportunity.

Regarding habitat evolution, habitat seems to have played an important role in the diversification of Eurasian columbines (a minimum of 14.3 transition, in contrast to a minimum of 10.13 in the Asiatic-North American lineages, see Bastida *et al.* in press). Patterns of habitat and altitudinal range evolution point to the direction of increasing specialization to open and rocky places and a narrowing of altitudinal ranges from past to present. These trends would have occurred within each southern European peninsulas (plus Corsica and Sardinia) and in isolation from each other, due to the barrier effect of Pyrenees, Alps and Balkans. Thus, the geographical mode of speciation of columbines in Europe would have been eminently allopatric (Bastida *et al.* in press), as a consequence of insular character of distinct peninsulas and islands where columbines diversified. In North America, in contrast, mountain systems orientation, land mass continuity plus a higher pollinator diversity would have created ecological opportunity for *Aquilegia* diversification and rendered in a highly partitioned ecological niches within the same area, driving to a sympatric mode of speciation (Whittall & Hodges, 2007; Bastida *et al.* in press)

ALTERNATIVES TO ECOLOGICAL OPPORTUNITY IN EUROPEAN COLUMBINES DIVERSIFICATION.

Other processes, not directly related to ecological opportunity, may explain density-dependent diversification. Historical contingencies (glaciations and absence of hummingbirds) and orography of European continent, as well as some biological features of *Aquilegia* species may be involved in diversification.

Among the biological features of *Aquilegia*, low seed dispersal capacity, low populations densities or frequent high inbreeding depression (Strand *et al.*, 1998; Eckert & Herlihy, 2004) might influence density-dependent diversification in the European columbines. For example, there are numerous columbines species

restricted to marginal habitats (frequently open/rocky) through the distinct European peninsulas. A possibility would be a progressive extinction of central populations of these species once ice sheet advanced, surviving only those populations from marginal and peripheral portions of their distributions. Indeed, adaptation to these marginal habitats (mainly open and rocky places) would have enhanced the probability of survival and differentiation of these species in their southern refugia during the glacial-interglacial cycles. This type of habitat, usually located at middle or high altitude, with a steep terrain, shallow soils, and frequent exposure to harsh weather, likely remained scarcely forested through the Holocene, providing open habitats for the persistence of alpine species after the Last Glacial Maximum (Birks & Willis, 2008). However, when ice sheet went back and Mediterranean climate was established these species would have been isolated, and without the possibility of recolonizing their ancestral ranges because of specialization (increasing genetic constraints, i.e. less plastic). This possible link between the use of rocky habitats and endemism has been described as a general trend for the plant species of Western Europe (Lavergne *et al.*, 2004) and agrees with the allopatric mode of recent speciation we found for the clade. However, this possibility does not explain the current geographical and ecological ranges of forest species like *A. vulgaris*, *A. atrata* or *A. nigricans*.

Other possibility is that apparent density-dependence arises as an artefact of phylogeny reconstruction and branch length estimation. Also, incomplete taxon sampling and inclusion of misclassified taxa could generate a pattern of lineage accumulation through time that can mimic temporally declining diversification.

Conclusions

European columbines diversification has followed a density-dependent linear mode. The recurrence of the advance and retreat of ice sheet and the starting of Mediterranean climate would have promoted an increment in ecological opportunity. The existence of new suitable habitats would have increased diversification rate early in the radiation. This kind of ecological opportunity would have allowed adaptation and specialization to open and rocky environments, from a forest or generalist ancestors, within mountain-alpine zones of south Europe. As suitable niches were filled and species become more specialized (increasing genetic constraints) species diversity

decline and then reached a plateau (“overshooting pattern” Gravilets & Losos 2009). Nevertheless, due to some biological peculiarities of *Aquilegia* other complementary possibilities to ecological opportunity seem to be involved. One possibility, not mutually exclusive, would have been that central populations of ancestral columbines were extinct during the glaciations, surviving only in peripheral and marginal areas through the south European mountain areas. The spatial restriction and the specialized phenotypes required for these open and rocky habitats would have avoided their spread through their ancestral areas, although no explain wide geographical and ecological ranges of more generalist species.

However, there are no phylogeographic evidences supporting this possibility but merit further exploration by extensive populations sampling and employing phylogeographic tools.

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