

Quaternary diversification in European alpine plants: pattern and process

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Molecular clock approaches applied previously to European alpine plants suggest that *Primula* sect. *Auricula*, *Gentiana* sect. *Ciminalis* and *Soldanella* diversified at the beginning of the Quaternary or well within this period, whereas *Globularia* had already started diversifying in the (Late-)Tertiary. In the first part of this paper we present evidence that, in contrast to *Globularia* and *Soldanella*, the branching patterns of the molecular internal transcribed spacer phylogenies of both *Primula* and *Gentiana* are incompatible with a constant-rates birth–death model. In both of these last two taxa, speciation probably decreased through Quaternary times, perhaps because of some niche–filling process and/or a decrease in specific range size. In the second part, we apply nonlinear regression analyses to the lineage-through-time plots of *P.* sect. *Auricula* to test a range of capacity-dependent models of diversification, and the effect of Quaternary climatic oscillations on diversification and extinction. At least for one major clade of sect. *Auricula* there is firm evidence that both diversification and extinction are a function of temperature. Intriguingly, temperature appears to be correlated positively with extinction, but negatively with diversification. This suggests that diversification did not take place, as previously assumed, in geographical isolation in high-altitude interglacial refugia, but rather at low altitudes in geographically isolated glacial refugia.

Keywords: alpine flora; climate change; diversification; extinction; lineage-through-time plots; Quaternary

1. INTRODUCTION

The Quaternary is a period of geologically rapid climatic oscillations resulting in the frequent recurrence of similar abiotic conditions within a rather short (2.75 Ma or less) time period. It is well documented that these climatic changes resulted in equally rapid changes of geographical distribution of species (Lang 1994) leading to extinction, geographical isolation and the breakdown of geographical isolation. Considering the large role of geographical isolation for speciation (Mayr 1963; Grant 1971; White 1978), it is conceivable that the Quaternary is a period of rapid evolutionary change. It is also conceivable, however, that the time available was too short for evolutionary divergence in geographical isolation to stabilize, and that diversification achieved in one period was wiped out (through extinction) or levelled (through secondary contact of divergent populations) in another period, effectively resulting in stasis.

Accordingly, opinions on the magnitude of Quaternary evolution differ strongly. For example, Bennett (1997), mainly on the basis of fossil evidence, claimed that no or next to no extinction and speciation took place in this period. By contrast, proponents of the ‘Late Pleistocene origins’ hypothesis (e.g. Klicka & Zink 1999) have advocated

accelerated evolution in the latter half of the Quaternary. Recently, strong evidence for the Quaternary as a period of very active evolution has come from molecular phylogenetic work in plants and animals by using a molecular clock approach (e.g. McCune 1997; Avise *et al.* 1998; Hungerer & Kadereit 1998; Hewitt 2000; Knowles 2000; Comes & Abbott 2001; Mummenhoff *et al.* 2001; von Hagen & Kadereit 2001; Zhang *et al.* 2001).

(a) *Diversification as the sum of speciation and extinction has a spatial and temporal component*

Spatial patterns of diversification can be deduced from the geographical interpretation of phylogenetic trees both at the intra- and interspecific level. The combined consideration of genealogical evidence and genetic diversity/distance data particularly at the intraspecific level further allows the interpretation of extant distribution patterns as the result of different processes of range formation such as contiguous expansion, long distance colonization, allopatric fragmentation, restricted gene flow, etc. (Templeton 1998).

A large body of literature has recently been published on the description and analysis of diversification rates through time (Nee *et al.* 1992, 1994a,b; Purvis *et al.* 1995; for reviews see Mooers & Heard 1997; Barraclough & Nee 2001). In these methods LTT plots are constructed by plotting the cumulative number of lineages of a reconstructed phylogeny against absolute time or a genetic distance measure. These LTTs illustrate diversification

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through time across the lineage under investigation and allow the investigation of whether diversification rates have been constant through time or not (Pybus & Harvey 2000; Nee 2001; Pybus *et al.* 2002). Non-constancy may result from evolutionary phenomena such as rapid radiations, origin of key innovations, density-dependent cladogenesis or from environmental changes. Because LTTs rely exclusively on extant taxa, these plots illustrate the net diversification of a lineage and do not easily allow one to separate speciation from extinction rate as components of diversification. However, by interpreting the final and recent upturn of LTT plots as a direct measure of speciation rate (Nee *et al.* 1994*a,b*; Barraclough & Nee 2001), and considering the relative positions of branching events in a phylogeny (Pybus & Harvey 2000; Pybus *et al.* 2002), efforts have been made to overcome this.

After briefly introducing our study systems and establishing that diversification of the plant groups investigated to a large part took place in the Quaternary, we will use molecular phylogenies to answer two questions. First, we will investigate whether rates of net diversification were constant through time or not. Second, we will investigate in one of our studied groups whether LTT plots, which show no apparent pattern of oscillation, are compatible with the assumption that Quaternary climatic oscillations (and their implications for extinction and geographical isolation) have influenced diversification and extinction rates. Essentially, this poses the question as to what pattern can tell about process. For this, we use a simulation approach to investigate if net diversification observed can also be obtained with temperature-dependent oscillations of diversification and extinction rates as well as temperature-dependent oscillations of limited capacity of geographical/ecological space for number of lineages. The consideration of limited capacity is based on the finding of decreasing diversification through time in two of the four lineages investigated here (see below).

(b) Study system

In a series of publications we have investigated the phylogeny and phylogeography of seven plant taxa of wide distribution in the European high mountains. These are *Gentiana* sect. *Ciminalis* (seven species; Gentianaceae (Hungerer & Kadereit 1998)), *Soldanella* (16 species; Primulaceae (Zhang *et al.* 2001)), *Anthyllis montana* (Fabaceae (Kropf *et al.* 2002)), *Papaver alpinum* (Papaveraceae (Bittkau & Kadereit 2002)), *Pritzelago alpina* (Brassicaceae (Kropf *et al.* 2003)), *Primula* sect. *Auricula* (25 species; Primulaceae (Zhang *et al.* 2004)) and *Globularia* (23 species; Globulariaceae (H. P. Comes, unpublished data)). Of these, *Globularia* is also widely distributed outside the European high mountains, particularly in the Mediterranean area. *Primula* sect. *Auricula* consists of two monophyletic subclades with a predominantly western (15 species) and eastern (10 species) distribution in the European high mountains, but with some overlap particularly in the Alps (Zhang *et al.* 2004).

Except for *Pa. alpinum*, in which no sequence variation could be detected (Bittkau & Kadereit 2002), phylogenetic trees of all other taxa were inferred from sequence variation in the ITS gene region of nuclear ribosomal DNA, thus allowing comparison of times of divergence and relative rates of diversification across taxa. ITS genetic

distances and either calibration with fossil evidence or comparison with ITS rates from other plant taxa were used to determine stem group (where possible) and crown group ages. Except for *Globularia* with a crown group age of *ca.* 7.6 Ma (H. P. Comes, unpublished data; Kadereit & Comes 2004), all taxa started diversifying from near the beginning to well into the Quaternary. The crown group ages found were: *G.* sect. *Ciminalis*: 0.76 Ma, *A. montana*: 0.80 Ma, *Pr. alpina*: 0.88 Ma, *Soldanella*: 0.98 Ma (Kadereit & Comes 2004); *P.* sect. *Auricula*: 2.4 Ma (Zhang *et al.* 2004)); *P.* sect. *Auricula* western clade: 1.8 Ma, and *P.* sect. *Auricula* eastern clade: 2.0 Ma (this paper).

Summaries of the above studies concentrating on the problem of spatial and temporal congruency among taxa are presented by Comes & Kadereit (2003) and Kadereit & Comes (2004).

2. MATERIAL AND METHODS

(a) Diversification rates

To investigate temporal variations in diversification rates, we re-analysed the ITS data of the following supraspecific taxa (plus appropriate outgroup or probable sister taxa) also considered by Kadereit & Comes (2004): *P.* sect. *Auricula* (all 25 species (Zhang *et al.* 2004)); *Globularia* (22 out of 23 species (H. P. Comes, unpublished data)); *G.* sect. *Ciminalis* (all seven species (Hungerer & Kadereit 1998)); and *Soldanella* (all 16 species (Zhang *et al.* 2001)). To avoid bias caused by uneven intraspecific sampling, and to evaluate patterns of intra- and inter-specific diversification versus speciation, two new datasets were generated with surplus intraspecific accessions either included or excluded from the original sequence matrices (hereafter referred to as data 1 and 2, respectively; only for *Primula*, *Globularia* and *Gentiana*). Pruning of intraspecific sequences was done arbitrarily except in those rare instances where paraphyletic relationships were involved (e.g. *P. hirsuta*/*P. daonensis*). Owing to very low levels of interspecific sequence divergence in *Soldanella* (Zhang *et al.* 2001), analysis was restricted to 10 accessions representing 'major' ITS lineages (see also Kadereit & Comes 2004).

For *Globularia*, *Gentiana* and *Soldanella*, the assumption of fairly constant rates of ITS sequence evolution could be verified in the above studies using tree-based LRTs (Felsenstein 1988) or relative rate tests (Wu & Li 1985). Hence, variations in diversification rates were assessed by using LTT plots derived from ML trees with a molecular clock enforced (Kadereit & Comes 2004). Because the branch lengths in such ultrametric phylogenies are proportional to time, and all root-to-tip distances are equal, internal node heights were used as proxies for relative divergence times.

By contrast, the ITS data of *P.* sect. *Auricula* were significantly non-clocklike (Zhang *et al.* 2004), i.e. each of the two reciprocally monophyletic clades (hereafter 'western' and 'eastern') showed different rates of molecular evolution. Thus, a neighbour-joining tree based on ML genetic distances was generated and corrected for among-lineage rate variation using Sanderson's (1997) NPRS method (see also Zhang *et al.* 2004). The NPRS method estimates a local rate of molecular evolution for each node in the tree, and then seeks to minimize the difference in these rates across the whole tree. Branch lengths in the rate-smoothed tree of sect. *Auricula* were then used as proxies for

relative time, and LTT plots were calculated for each of the two major clades separately.

All LTT plots were evaluated in two ways.

- (i) We used the CR method developed by Pybus & Harvey (2000) and Pybus *et al.* (2002) for testing whether the splitting events in a phylogeny are randomly distributed through time. Based on a statistical analysis of trends (Cox & Lewis 1966), the CR method uses internode-distance information for calculating a summary statistic, γ , of the relative locations of internal nodes within a phylogeny. Significant departures towards negative values indicate that the internal nodes of a phylogeny are closer to the root than expected under the null hypothesis that the diversification rate (b) and extinction rate (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 (cf. Pybus & Harvey 2000; Pybus *et al.* 2002). All calculations of γ were performed using a program written by E. M. Griebeler (available from <http://perdix.biologie.uni-mainz.de/people/evi/download/gammastatistics.exe>).
- (ii) As an alternative, we used the method developed by Paradis (1997) based on survival analysis and implemented in his program DIVERSI, v. 020.0 (Paradis 2000) to detect shifts in *net* diversification rate ($b-d$) within each study group. The program 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 increases through time. Maximum log-likelihood ($\log L$) scores and the AIC (Akaike 1973) were computed for each model to evaluate which model best explained the data. Because LRTs can only be used to compare nested models, such an approach is restricted to evaluating models A versus B and A versus C (Paradis 1997). We calculated LRTs using the statistic: $-2(\log L_1 - \log L_2)$, where $\log L_2$ refers to the value of the least parameter-rich model, i.e. A (constant rates), and $\log L_1$ to the alternative model. This statistic follows a χ^2 distribution with one degree of freedom (Paradis 1997). By contrast, the AIC is appropriate to evaluate any two models, whatever their relationship of nestedness. In general, the model with the smallest AIC value is selected as the one that best describes the data (Paradis 1997). However, we followed this rationale only for further differentiating between models B and C once model A had clearly been falsified by the LRT.

(b) Simulations

In the context of Quaternary climatic change, we used a non-linear regression analysis to examine whether *temperature-dependent*

oscillations of diversification and extinction rates could have given rise to the LTT plots obtained for the two clades of *P. sect. Auricula* (data 1; figure 1a,b). Given the convex shape of these LTTs, and following the failure of a CR birth-death model (see § 3), we used a parametric growth model of *capacity dependence*, called the 'expansion growth model', as our basic hypothesis testing framework. This simple and general model, which has also been used to reconstruct the demographic history of populations (Strimmer & Pybus 2001), was then further modified to accommodate temperature dependence of parameters. Altogether four nested models, with three to six estimated parameters (k), were considered.

(i) Diversification models

For the simplest model (our null hypothesis) we assumed temperature-independent rates of diversification and a constant capacity, α . Accordingly, the expected number of lineages at some arbitrary time t , $N(t)$, i.e. from the first bifurcation in the clade ($t=0$) up to the present, was derived from the formula:

$$N(t) = N_0 [\alpha + (1 - \alpha) e^{-rt}], \quad (2.1)$$

where N_0 is the initial number of lineages at the first bifurcation in the clade, and r is the net diversification rate, equalling the difference between a constant (intrinsic) diversification rate (b) and a constant (intrinsic) extinction rate (d).

Given a specified course of temperature fluctuations through time (see below), the assumption of temperature-dependent oscillations of b and d only requires a slight modification of equation (2.1):

$$N(t) = N_0 [\alpha + (1 - \alpha) e^{-r(T(t))}], \quad (2.2)$$

where $r(T(t))$ is the net diversification rate depending on temperature T at time $t \geq 0$, and equalling the difference between diversification rate $b(T(t))$ and extinction rate $d(T(t))$.

In our third model, we assumed temperature independence of net diversification and temperature dependence of capacity α , which then leads to the growth function:

$$N(t) = N_0 [\alpha(T(t)) + (1 - \alpha(T(t))) e^{-r}]. \quad (2.3)$$

Finally, both net diversification and capacity may depend on a given temperature course:

$$N(t) = N_0 [\alpha(T(t)) + (1 - \alpha(T(t))) e^{-r(T(t))}]. \quad (2.4)$$

(ii) Model for temperature dependence

To model temperature dependence of parameters, we used estimates of the average July temperature over the past 2.4 Myr published by Lang (1994) based on Zagwijn (1985). From this source a new database of 240 temperature values was generated (one entry per 10 kyr), and the minimum, maximum and mean temperature across the entire period was calculated. Temperature dependence of b , d and α was modelled by assuming a mean value for each of these parameters and a respective \pm range (here defined as ρ), i.e. ρ_b , ρ_d and ρ_α . The mean temperature was mapped to the mean of the respective parameter, the minimum temperature to the difference between this mean minus ρ , and the maximum temperature to the mean plus ρ . Temperature values between the minimum and the maximum were mapped according to a linear interpolation defined by these upper and lower bounds. As a consequence, positive values of ρ_b , ρ_d or ρ_α imply that the respective values of b , d or α increase linearly with

increasing temperature, whereas negative values correspond to a decrease with increasing temperature.

(iii) Nonlinear regression analyses

To find the best model explaining the LTT plots of the two clades of *Primula*, we performed a series of nonlinear regression analyses pertaining to models 2.1 through to 2.4. Specifically, we searched for: (i) constant values of α , b and d in the case of model 2.1 ($k = 3$); (ii) constant α , mean b , mean d and ranges ρ_b and ρ_d in the case of model 2.2 ($k = 5$); (iii) mean α , constant b , constant d , and a range ρ_α in the case of model 2.3 ($k = 4$); and (iv) mean α , mean b , mean d , and ranges ρ_α , ρ_b and ρ_d in the case of model 2.4 ($k = 6$).

Curve fitting (i.e. estimation of parameters) was performed by the 'DS method' described in Press *et al.* (1992) (see also Nelder & Mead 1965) and implemented in DELPHI v. 6.0 (Borland Software Corporation, CA, USA). The DS optimization algorithm seeks to minimize the SS of the vertical distances of the observed LTT data points to those of the fitted plot. Plots generated during the finding of a minimization of distances for models 2.1 to 2.4 were obtained from the respective finite difference equations. Plots were calculated iteratively in steps of 10 kyr, and were started with each of two lineages (N_0) extant at time $t = 0.6$ Ma and $t = 0.4$ Ma in the western and eastern clades, respectively. The last two values correspond to molecular clock estimates of crown group ages 1.8 and 2.0 Ma, respectively, based on the assumption that the two clades last shared a common ancestor *ca.* 2.4 Ma (for details see Zhang *et al.* 2004). For models 2.2 through to 2.4, temperature values corresponding to each iteration step were read from the database (see above), and those parameters assumed to be temperature dependent were calculated accordingly. Although robustness of each fit was also explored by using different initial starting values of parameters, this resulted in estimates very similar to those reported (data not shown).

(iv) Comparison of models

To evaluate which model best explained the data, the SS derived from the DS algorithm were used as a first approximation. However, the SS of a more complex (i.e. parameter-rich) model will almost always be lower (i.e. 'better') than those of a simpler model. Therefore, we also employed the AIC probability method as outlined by Motulsky (1995) as an alternative goodness-of-fit procedure. Specifically, we computed the relative probability of the null model compared with an alternative model based on the difference between their corrected AIC_c values, the latter of which are valid for small sample sizes (Motulsky 1995; Strimmer & Pybus 2001).

For comparative purposes, we also calculated the number of lineages expected over time under a CR birth–death process according to the equation:

$$N(t) = N_0 e^{Rt}, \quad (2.5)$$

where R is the *average* net diversification rate per million years. Where possible, this rate was computed for each model (2.1 to 2.4) by averaging the changes in the number of lineages between two successive time steps during iteration of the respective finite difference equation. The R -value corresponding to the best-fit model was then used for the generation of LTT plots expected under the CR birth–death process.

3. RESULTS

(a) Diversification rates

The results of the CR test (Pybus & Harvey 2000) are indicated in table 1. Based on the γ -values calculated, the null hypothesis of constant diversification (b) and extinction (d) rates was clearly rejected in both the western and eastern clade of *P. sect. Auricula* (data 1 and 2), in agreement with the apparent convex shape of their observed LTT plots (see the curves marked with filled circles in figure 1*a,b*). Moreover, the CR model was also rejected in *G. sect. Ciminalis* when intraspecific accessions were excluded (data 2). Because the relevant γ -values are significantly negative ($p < 0.05$, one-tailed test), these results imply a decrease of diversification and/or speciation rate in *Primula* and *Gentiana* as the present time is approached. Although there is some indication that a slowdown of diversification may have also occurred in the more recent history of both *Globularia* (data 2: $\gamma = -0.309$) and *Soldanella* ($\gamma = -0.399$) these values are not significant.

The analysis based on the method of Paradis (1997) (table 2) agreed with the CR test and also identified non-constancy of diversification in both *Primula* and *Gentiana*. As indicated by the LRTs and/or the AIC, either of the two rate-variable models, B or C, best explained the data for each of the two *Primula* clades (depending on which datasets are analysed), and model B provided the best fit for *G. sect. Ciminalis* (only data 2). In those instances, β -values of more than 1 were consistently observed with model B and $\delta_1 < \delta_2$ with model C (data not shown), further suggesting a decrease in diversification through time. By contrast, model A was clearly superior (in terms of LRT) in explaining the data of both *Globularia* and *Soldanella*, implying a constant rate of diversification in both taxa.

(b) Simulations

The results of the nonlinear regression analysis for each clade of *P. sect. Auricula* are summarized in table 3, including parameters estimated under each model (2.1 to 2.4) and the evaluation of these models in terms of SS and AIC_c probability. For each *Primula* clade, the corresponding LTT plots generated under these models are shown in figure 1, together with the curve expected under a CR birth–death process.

For the western clade, model 2.2 was the best one, suggesting that temperature-dependent oscillations of b and d (in conjunction with a constant α) better explain the observed LTT plot than temperature-independent rates (table 3; figure 1*a*). Compared with the null model 2.1, model 2.2 not only had a lower SS but also a much higher AIC_c probability. It thus appears unlikely that the lower SS of model 2.2 is simply owing to the fact that there are more parameters to fit (i.e. 5 versus 3). Model 2.3 did not provide any improvement of fit compared with models 2.2 and 2.1, as indicated by its particularly high SS. Model 2.4 either diverged, i.e. the DS method did not find a minimum after 10 000 iteration steps, or converged to biologically unreasonable parameter estimates, e.g. diversification rates (b), suggesting more than 10 000 splitting events per 1 Myr. The estimated range parameters according to the best-fit model 2.2 are: $\rho_b = -1.164$ and $\rho_d = 0.153$. As $\rho_b < 0$ and $\rho_d > 0$, this result provides evidence for a linear decrease of diversification or increase of

Table 1. Plant groups examined for shifts in diversification rates using the CR test of Pybus & Harvey (2000). (Analyses were conducted separately with surplus conspecific accessions (lineages) either included (data 1) or excluded (data 2). Negative or positive γ -values indicate a decrease or increase, respectively, in diversification as the present time is approached. The CR model is rejected if $\gamma < -1.645$ (one-tailed test). * $p < 0.05$.)

| | taxon | data 1 | | data 2 | | reference |
|---|--|----------|--------------------|----------|-------------------|--------------------------------|
| | | γ | number of lineages | γ | number of species | |
| 1 | <i>Primula</i> sect. <i>Auricula</i> (western clade) | -2.955* | 25 | -3.999* | 15 | Zhang <i>et al.</i> (2004) |
| 2 | <i>Primula</i> sect. <i>Auricula</i> (eastern clade) | -1.871* | 13 | -3.293* | 10 | Zhang <i>et al.</i> (2004) |
| 3 | <i>Globularia</i> | 0.788 | 28 | -0.309 | 22 | H. P. Comes (unpublished data) |
| 4 | <i>Gentiana</i> sect. <i>Ciminalis</i> | -0.908 | 12 | -1.939* | 7 | Hungerer & Kadereit (1998) |
| 5 | <i>Soldanella</i> | -0.399 | 10 ^a | — | — | Zhang <i>et al.</i> (2001) |

^a Only 'major' ITS lineages were considered owing to very low levels of interspecific sequence divergence.

Table 2. Results of fitting three diversification models (Paradis 1997, 2000) to datasets 1 and 2 of each group. (Models are described in § 2a(ii). Significant differences between maximum log-likelihood (log L) scores of nested models A versus B and A versus C are indicated based on LRTs: * $0.05 > p > 0.01$; ** $0.01 > p > 0.001$; *** $p < 0.001$. The AIC was used to further differentiate between non-nested models B and C, i.e. after the most general model, A, had been falsified by the LRT. AIC values being decisive for model selection are bold.)

| | taxon/model | data 1 | | data 2 | |
|---|--|-----------|-----------------|-----------|-----------------|
| | | log L | AIC | log L | AIC |
| 1 | <i>Primula</i> sect. <i>Auricula</i> (western clade) | | | | |
| | A | 68.587 | -135.173 | 41.303 | -80.605 |
| | B | 75.265*** | -146.529 | 52.693*** | -101.386 |
| | C | 74.311*** | -144.623 | 53.243*** | -102.486 |
| 2 | <i>Primula</i> sect. <i>Auricula</i> (eastern clade) | | | | |
| | A | 33.356 | -64.712 | 25.106 | -48.212 |
| | B | 35.103 | -66.207 | 34.763*** | -65.526 |
| | C | 38.646** | -73.293 | 28.489** | -52.979 |
| 3 | <i>Globularia</i> | | | | |
| | A | 98.910 | -195.820 | 72.304 | -142.609 |
| | B | 98.930 | -193.860 | 72.320 | -140.639 |
| | C | 100.262 | -196.523 | 72.701 | -141.403 |
| 4 | <i>Gentiana</i> sect. <i>Ciminalis</i> | | | | |
| | A | 46.000 | -90.000 | 23.009 | -44.018 |
| | B | 47.596 | -91.192 | 27.545** | -51.091 |
| | C | 47.474 | -90.949 | 23.890 | -43.780 |
| 5 | <i>Soldanella</i> (major lineages) | | | | |
| | A | 41.081 | -80.162 | — | — |
| | B | 41.850 | -79.700 | — | — |
| | C | 41.600 | -79.201 | — | — |

extinction with increasing temperature for the western clade of sect. *Auricula*.

In terms of AIC_c probability, the LTT plot of the eastern clade was best explained by the null model 2.1 with temperature-independent parameters of b , d and α (table 3; figure 1b). However, the SS of all four models tested were rather similar, and preference of model 2.1 by the AIC_c probability method should be taken with care, especially because the number of lineages in the eastern clade ($n = 13$) is small compared with the number of estimated parameters (k) in the models considered (Motulsky 1995). The LTT plots generated under models 2.1 and 2.4 were identical (see figure 1b).

4. DISCUSSION

(a) Diversification pattern

Both the CR test (Pybus & Harvey 2000) and the method by Paradis (1997) show that both clades of *P. sect. Auricula* deviate from rate constancy irrespective of inclusion or exclusion of intraspecific accessions. Of the diversification models tested, gradual decrease of diversification (model B), fits the eastern clade best (data 2), whereas abrupt change in diversification with decline (model C), best fits the western clade (data 2). Model B fits the western, and model C the eastern clade best when intraspecific accessions are included in the dataset

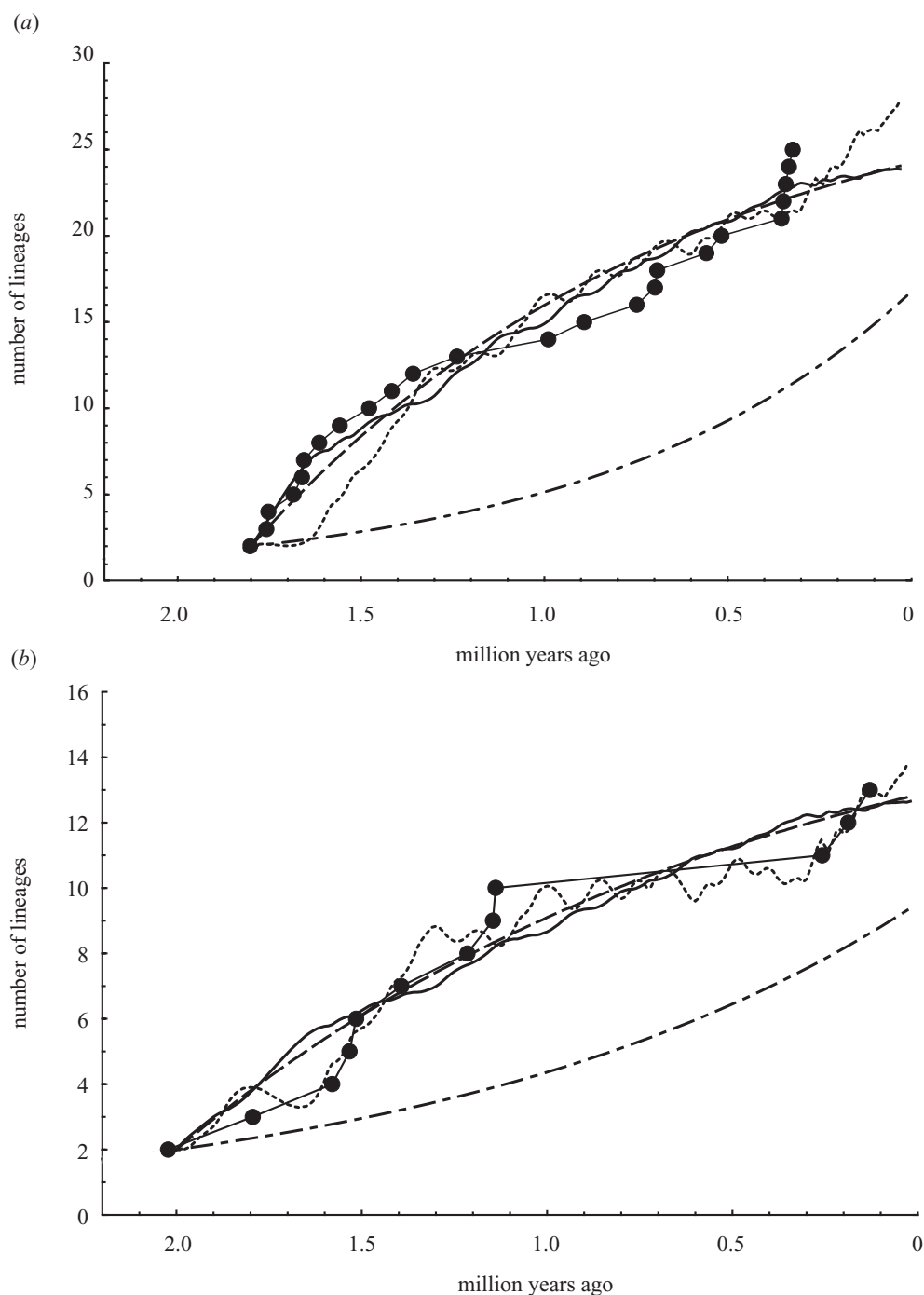


Figure 1. Observed and expected LTT plots for the (a) western and (b) eastern clade of *Primula* sect. *Auricula*. The black thin curves marked with filled circles represent the observed ('reconstructed') number of lineages through time (in Ma). The LTT curves generated under diversification models 2.1, 2.2 and 2.3 are marked with dashed, solid and dotted lines, respectively. Note that model 2.4, initially fitted to the western clade, either diverged, or converged to biologically unreasonable parameter values (LTT thus not shown). For the eastern clade, the LTTs expected under models 2.1 and 2.4 were identical. For comparison, the curves marked with long and short dashes indicate the LTTs expected under a CR birth–death process. Models are described in § 2b, (i)–(iv).

analysed. Non-constancy of diversification rate could be shown by the CR test for *G.* sect. *Ciminalis* without intra-specific accessions, and the method of Paradis again revealed model B as the best model for the same dataset. For *Globularia* and *Soldanella* the null hypothesis of rate constancy could not be rejected.

Following Pybus & Harvey (2000) and Pybus *et al.* (2002), rejection of the null hypothesis of constant rates of diversification can only result from a decrease in diversi-

fication rate and not from an increase in extinction rate, barring incomplete sampling which tends to underestimate the number of nodes towards the present (Nee *et al.* 1994a). Possible causes for the inferred decrease of speciation rate in *P.* sect. *Auricula* and *G.* sect. *Ciminalis* may be filling of geographical and/or ecological space (Schluter 2000), and/or the decrease of specific range size decreasing the likelihood of geographical speciation through range subdivision (Rosenzweig 1996). A choice between these

Table 3. Results of fitting diversification models 2.1 to 2.4 to the observed LTT plots of the western and eastern clade of *Primula* sect. *Auricula* (see figure 1*a,b*). (Models are described in § 2*b(i)-(iv)*). Parameters estimated under each model were obtained by nonlinear regression analysis. The SS of each model is indicated. The AIC_c probability method (Akaike 1973; Motulsky 1995) was used to calculate the relative probability of the null model 2.1 compared to an alternative model (2.2, 2.3 or 2.4). Based on these comparisons, the model considered the best one is marked bold. *K*, number of model parameters estimated. *R*, average net diversification rate per million years.)

| group/model | <i>k</i> | α | <i>b</i> | <i>d</i> | parameter estimates ^a | | | | goodness-of-fit | | | |
|------------------|----------|---------------|------------------|------------------|-----------------------------------|----------|----------|----------|-----------------|---------------|---|---|
| | | | | | $r = b - d$ | ρ_a | ρ_b | ρ_d | <i>R</i> | SS | AIC _c probability ^b | |
| western clade | | | | | | | | | | | | |
| 2.1 | 3 | 30.037 | 0.101 779 | 0.101 778 | 8.450 34 × 10 ⁻⁷ | — | — | — | 1.426 | 55.748 | — | — |
| 2.2 | 5 | 43.215 | 0.100 458 | 0.100 457 | 4.379 32 × 10⁻⁷ | — | — | — | 1.432 | 34.639 | 0.934 | — |
| 2.3 | 4 | 29.892 | 0.104 687 | 0.104 686 | 9.410 79 × 10 ⁻⁷ | 0.008 | — | — | 1.520 | 169.726 | 0.8 × 10 ⁻⁸ | — |
| 2.4 ^c | 6 | — | — | — | — | — | — | — | — | — | — | — |
| eastern clade | | | | | | | | | | | | |
| 2.1 | 3 | 17.076 | 0.094 261 | 0.094 260 | 6.341 18 × 10⁻⁷ | — | — | — | 0.946 | 12.536 | — | — |
| 2.2 | 5 | 27.764 | 0.096 675 | 0.096 672 | 2.675 30 × 10 ⁻⁷ | — | — | — | 0.942 | 15.247 | 0.001 | — |
| 2.3 | 4 | 11.506 | 0.023 688 | 0.023 687 | 1.862 46 × 10 ⁻⁶ | -4.046 | — | — | 0.999 | 9.937 | 0.276 | — |
| 2.4 | 6 | 17.076 | 0.100 010 | 0.100 009 | 6.341 26 × 10 ⁻⁷ | -0.428 | 1.265 | 0.363 | 0.946 | 12.536 | 0.001 | — |

^a α , capacity; *b*, diversification rate; *d*, extinction rate; *r*, net diversification rate; ρ_a , ρ_b , ρ_d , range of respective parameter.

^b Relative to the null model.

^c For the western clade, model 2.4 either diverged, or converged to biologically unreasonable parameter values (see § 3*b*).

alternatives cannot be made. Possibly a careful reconstruction of the geographical and ecological contexts of diversification based on fully resolved phylogenies could help to determine the relative importance of limited space or habitat for the decrease of speciation rate.

A comparison of age and species number of the four taxa investigated does not help explain why changes in diversification rate were found in *P. sect. Auricula* and *G. sect. Ciminalis* but not in *Globularia* and *Soldanella*. Comparatively high age and comparatively high species number of *P. sect. Auricula* might suggest that geographical/ecological saturation is approached only in a late stage of the diversification of a taxon. Such a hypothesis, however, is clearly falsified by the remaining three taxa. Thus, *G. sect. Ciminalis* is both much younger and has fewer species than *P. sect. Auricula* and shows a decrease in speciation; *Globularia* is substantially older than *P. sect. Auricula* and has a comparably high number of species but shows no decrease in diversification; and *Soldanella* is as young as *G. sect. Ciminalis*, has more species, but shows no decrease in diversification.

Our finding of decreasing speciation rates in two out of four taxa investigated has important implications for the choice of diversification models in the development of methods to estimate speciation or extinction rates from molecular phylogenetic trees. For example, a CR birth-death model has been widely used as an 'appropriate null model' for diversification both for complete phylogenies including fossil data (Raup *et al.* 1973; Gould *et al.* 1977) as well as for molecular phylogenies of contemporary taxa (e.g., Nee *et al.* 1994a; Purvis *et al.* 1995; see also Magallón & Sanderson 2001). In this model the probabilities of speciation and extinction are assumed to remain constant over time, with the expected number of lineages showing an exponential increase. Our data, to our knowledge the first plant examples tracing changes in diversification rates through time, question this null hypothesis, as do previous and comparable studies of several animal taxa (see Zink & Slowinski 1995; Paradis 1997; Pybus & Harvey 2000). Clearly, before using such CR models, e.g. for estimating speciation (and extinction) rates from reconstructed phylogenies (Nee *et al.* 1994b; McCune 1997; Baldwin & Sanderson 1998; Magallón & Sanderson 2001), it is essential to test whether rates have remained constant through time or not (Pybus & Harvey 2000; Nee 2001).

(b) *Diversification process*

The statistically supported decline of diversification rates through time in *P. sect. Auricula* (see above) made the use of diversification models with capacity dependence appropriate for our simulation studies. These, however, produced different results for the two major clades of the section. Whereas the observed pattern of intra- and inter-specific diversification of the western clade is clearly best explained by model 2.2 which assumes temperature-dependent diversification and extinction rates but temperature-independent lineage capacity, the eastern clade, following the AIC_c probability, is best explained by the null model 2.1 assuming temperature-independent diversification, extinction and capacity. However, the number of lineages in the eastern clade is small in relation to the number of estimated parameters in the models investigated. Therefore, it could be argued that the reliability of

the results obtained in the analysis of the western clade is higher than that of the eastern clade, and that, considering the analysis of both clades together, evidence in favour of model 2.2 is stronger than evidence in favour of model 2.1, the null model. This reasoning implies that the results obtained for the eastern clade are an artefact. The different results obtained for the two clades, however, might also reflect their different geographical distribution, assuming that climatic changes in the geographical range of the western clade (Pyrenees, western and central Alps, Apennines) were stronger than in the range of the eastern clade (central and eastern Alps, Balkans).

Although model 2.2 is more complicated than the null model, we believe that it rests on biologically more realistic assumptions, and that the simplicity of the null model fails to take account of the climatic peculiarities of the Quaternary. It is an established fact that the Quaternary climatic oscillations had a strong influence on plant geographical distribution (e.g. Comes & Kadereit 1998; Taberlet *et al.* 1998; Hewitt 1999, 2000). In turn, changes in geographical distribution by range contraction and range expansion during different climatic episodes may have resulted in: (i) extinction of populations in formerly populated areas; (ii) geographical isolation, e.g. by retreat into different refugia; or (iii) breakdown of isolation, e.g. when formerly isolated populations retreated into the same refuge, or came into contact after newly available areas were colonized from different source regions. The population genetic correlates of the above processes, i.e. reduction of genetic diversity, genetic subdivision and genetic homogenization are well accepted and important components of evolutionary diversification. In consequence, it is reasonable to suppose that temperature-dependent changes in geographical distribution did indeed influence rates of net diversification.

It could be argued that the preference of different models of diversification in the two clades of *P. sect. Auricula* should caution against over-interpretation. Even so, we believe we have demonstrated that a given pattern of diversification as depicted in an LTT plot cannot easily and unambiguously be interpreted in terms of process, and that very different processes can lead to similar courses of temporal diversification.

Our simulation study allows us to infer the influence of climatic changes on rates of diversification and extinction. We initially assumed that in an alpine taxon such as *P. sect. Auricula* the temperature-dependent oscillations of diversification and extinction rates would be parallel. High temperatures would result in high diversification rates because of geographical isolation at high altitudes, and in high extinction rates because of extinction in comparatively large low altitude areas. In turn, low temperatures would decrease speciation rates because of the breakdown of isolation in low altitude areas, and decrease extinction rates because extinction took place in comparatively small high-altitude areas. Such parallel oscillation of speciation and extinction was believed to add up to non-oscillating net diversification as seen in the LTT plots. Much to our surprise, we found for the western clade of *sect. Auricula* that although temperature is correlated positively with extinction ($\rho_d = 0.153$), it is correlated negatively with diversification ($\rho_b = -1.164$; see table 3, model 2.2). This implies that diversification did not take place, as originally

assumed, in geographical isolation in high-altitude interglacial refugia, but rather at low altitudes in geographically isolated glacial refugia. Geographically isolated glacial refugia of alpine species had already been postulated for two largely sympatric species of *Soldanella* (Zhang *et al.* 2001). In contrast to this, low levels of genetic differentiation in the alpine *Pritzelago alpina* were believed to have resulted from contact during glacial periods between populations from different mountain ranges resulting in genetic homogenization (Kropf *et al.* 2003). Probably the absence of ecological differentiation in this latter species did not allow the retreat into geographically isolated refugia. The hypothesis of predominantly glacial rather than interglacial diversification in the western clade of *P. sect. Auricula* is further supported by the relative length of glacial and interglacial periods. Whereas the glacial periods of the mid-to-late Quaternary (1.5 Ma to the present) lasted between 70–90 kyr, interglacials were only 10–26 kyr long (Skelton 1993).

5. CONCLUSIONS

We here were able to show mainly two things. First, in two (*P. sect. Auricula*, *G. sect. Ciminalis*) out of the four lineages investigated we found non-constant net diversification rates, which are best interpreted as decreases in diversification (*P. sect. Auricula*) or speciation (*G. sect. Ciminalis*). Although we could neither identify the likely causes of this behaviour nor explain the difference among the lineages with non-constant and those (*Globularia*, *Soldanella*) with constant rates, our findings clearly show that it may be inappropriate to use a CR model of diversification for, e.g. the estimation of speciation rates. Second, we could demonstrate in our simulation study that the pattern of diversification at least in the western clade of *P. sect. Auricula* is best explained by temperature-dependent rather than temperature-independent diversification and extinction rates. The simulation study resulted in the hypothesis that speciation events were concentrated in glacial rather than interglacial periods.

Explanation of the decrease in speciation rates and further confirmation of oscillating speciation and extinction rates associated with glacials or interglacials will require fully resolved and well-dated phylogenies as well as the investigation of further examples. In a next step these phylogenies should be employed to reconstruct mechanisms of speciation throughout the history of lineages using appropriate methods of character optimization and models of the past distribution of taxa.

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GLOSSARY

- AIC: Akaike information criterion
 CR: constant-rates
 DS: Downhill Simplex
 ITS: internal transcribed spacer
 LRT: likelihood ratio test
 LTT: lineage-through-time
 ML: maximum likelihood
 NPRS: non-parametric rate smoothing
 SS: sums of squares