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Appendix

Estimating Ancestors with Discrete 1.01b

Discrete, version 1.01b (Pagel, 1997), is a DOS-based program that can be used to estimate individual ancestral values. Discrete was written to test for correlated evolution between two discrete characters, and producing ancestor reconstructions in such a way requires several steps. First, Discrete deals with two characters at a time; therefore, for the purposes of estimating ancestors for a single character, the states must be entered twice. For example, for a tip (tipA) in state 1 on a branch 2 units long and whose immediate ancestor is nodeA, the entry would be "tipA, nodeA, 2, 1,1." Because two characters are input, Discrete estimates two (or four) rates: α_1 and β_1 refer to $\hat{q}_{01}$ and $\hat{q}_{10}$ for the first character, and α_2 and β_2 refer to $\hat{q}_{01}$ and $\hat{q}_{10}$ for the second character. With this in mind:

1. Estimate $\hat{q}$ (or $\hat{q}_{01}$ and $\hat{q}_{10}$), using the command "testi." To estimate one rate, use the "restrict $\alpha_1 = \beta_1$" option. The program will return the ML estimates for $\hat{q}$. Leaving out this restriction yields two rates ($\hat{q}_{01}$ and $\hat{q}_{10}$) for each trait. Record the ML rate estimates.

2. Revise the input file by modifying the data corresponding to the node of interest. Place either :0,0 or :1,1, respectively, after the branch-length data for that node. This triggers the "fossil" option in Discrete. For example, the row entry for a focal nodeA that is separated from its immediate ancestor nodeB by a branch of length 2 would be "nodeA, nodeB, 2:0,0" or "nodeA, nodeB, 2:1,1".

3. Run "testi" on the revised input file; repeat for the "0,0" and "1,1" alternatives. In both cases, restrict all α's and β's to the ML values estimated in step (1). Use the "restrict" command (e.g., restrict $\alpha_1 = 0.08$) to set α_1, α_2, β_1, and β_2 to $\hat{q}$, or to set both α's to $\hat{q}_{01}$ and both β's to $\hat{q}_{10}$;

4. Obtain the likelihood for the two trees you have made. Discrete returns negative ln(likelihoods), so the ML estimate at the node (0 or 1) will be that associated with the smaller of the two output values. The relative support for the ML state at the node will be e raised to a power equal to half the difference in the returned –ln(likelihoods) for the two trees (we must halve the difference because Discrete deals with both characters simultaneously) .

Inferring Rates of Change in Flower Symmetry in Asterid Angiosperms

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The question of whether evolutionary change in a character is more likely to occur in one direction than another is of general interest to evolutionary biologists, and the development of quantitative comparative methods has allowed hypotheses of bias in character gain/loss in phylogenies to be tested with increasing rigor. Parsimony algorithms are commonly used to infer ancestral character states, and thereby the location and direction of character changes in phylogenetic trees (e.g., Maddison and Maddison, 1992; Swofford and Maddison, 1992).

Ancestral states can be used in statistical tests of gain/loss bias, e.g., using the method of Sanderson (1993). One drawback to using parsimony to derive ancestral state estimates is that the estimates themselves are contingent on a particular set of transformation costs (step matrix), and parsimony itself does not provide any criterion to optimize such costs (Ree and Donoghue, 1998).

Maximum likelihood methods of estimating instantaneous rates of evolution between categorical character states avoid this issue, by optimizing the rate parameters of a continuous-time Markov model over all possible ancestral state reconstructions

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(e.g., Pagel, 1994, 1997; Milligan, 1994). Hypotheses of directional bias in character evolution can then be evaluated by using likelihood ratio tests for differences in rates of gain and loss of a character state. Maximum likelihood has also been applied to ancestral state estimation (Schluter, 1995; Schluter et al., 1997; Pagel, 1997, 1999). Schluter et al. (1997) used rates of change estimated over all ancestral state combinations to calculate the likelihood of the data given that a node of interest is fixed for each state. These "partial" likelihoods measure the level of support for the inference of ancestral states, with the state corresponding to the highest partial likelihood taken to be the best ancestral state estimate for that node (Schluter et al., 1997). Other approaches to obtaining such partial likelihoods are also possible (e.g., Schluter, 1995; see Pagel, 1999, for a detailed discussion).

In this paper, we examine the use of maximum likelihood to detect asymmetric rates of evolution in flower symmetry in the angiosperm clade Asteridae (s.l.; see Chase et al., 1992; Olmstead et al., 1992). We also use this example to compare parsimony- and maximum likelihood–based inferences of ancestral states, particularly with respect to the root of the Asteridae. Our emphasis here is not on the details of flower symmetry or asterid phylogeny. Instead, we use this example to explore the factors that influence the estimation of evolutionary rates. This is motivated in part by Schluter et al. (1997; see also Mooers and Schluter, 1999), who observed that for most phylogenies, models that utilize independent rates of evolution in different directions are not warranted because of insufficient data (i.e., not enough terminal taxa). We felt that a larger data set might provide insight into the properties of phylogenies that are critical to maximum likelihood inferences of character evolution, particularly the detection of asymmetric rates of change.

Flower Symmetry in Asteridae

The most common forms of flower symmetry are actinomorphy (radial symmetry, polysymmetry), in which multiple axes of symmetry bisect the flower, and zygomor-

phy (bilateral symmetry, monosymmetry), in which the abaxial side of the flower is differentiated from the adaxial side, with a single vertical axis of symmetry bisecting the flower. The evolution of flower symmetry is a subject of great interest to many botanists and has been studied from a variety of perspectives, including developmental morphology (e.g., Endress, 1994), molecular genetics (e.g., Coen and Nugent, 1994), and phylogeny (e.g., Donoghue et al., 1998; Reeves and Olmstead, 1998). It is an appropriate example with which to test hypotheses of directional bias, because a range of opinion exists as to the relative likelihood of change between zygomorphy and actinomorphy. The traditional view (e.g., Stebbins, 1974; Takhtajan, 1991) is that zygomorphy is the derived condition in angiosperms, because factors such as pollinator selection would favor the repeated evolution and maintenance of zygomorphy; on the other hand, developmental genetic data (Coen and Nugent, 1994) suggest that the loss of zygomorphy can result from simple mutations in relatively few genes. If losses of zygomorphy are indeed more probable than gains, it is possible that zygomorphy evolved earlier in angiosperm evolution than is generally supposed and has subsequently been repeatedly lost in actinomorphic lineages. The Asteridae, an angiosperm clade of $\sim$ 65,000 species containing most of the sympetalous angiosperms, is an appropriate focal group in which to detect unequal rates of gain and loss of zygomorphy: A large proportion of its species ($\sim$ 50% as estimated from Thorne, 1992) are zygomorphic; it has been the focus of previous discussion on the evolution and development of flower symmetry; and its phylogeny has been relatively well-studied, both in terms of deep divergences as well as on a finer scale, in more-detailed studies of smaller groups.

METHODS

Our analyses were conducted with use of a composite tree of asterid angiosperms (Donoghue et al., 1998). This was constructed by using a backbone phylogeny based on *rbc*L sequences (Rice et al., 1997;

Olmstead et al., 1992) that we expanded with published phylogenies from more-detailed studies of particular groups that were substituted for "placeholder" taxa on the *rbc*L tree (see Sanderson et al., 1998, for alternative approaches to supertree construction). In a few cases, clades were resolved arbitrarily to obtain a fully resolved phylogeny (see Donoghue and Ackerly, 1996, for a discussion of sensitivity analyses in large trees). The resulting focal tree had 379 taxa, of which 113 were scored as having zygomorphic corollas, and 266 as having actinomorphic corollas. Figure 1 shows alternative reconstructions of flower symmetry evolution on this tree. (The tree is also available at http://www. herbaria. harvard. edu/~mdonoghu/zygo, where the NEXUS file containing the tree and character data, and references to literature used to construct the tree, can also be obtained.)

Most-parsimonious ancestral character states were reconstructed over a range of transformation costs by using MacClade version 3.0 (Maddison and Maddison, 1992) and a program developed independently (Ree and Donoghue, 1998). Maximum likelihood estimates of rates of evolution between actinomorphy and zygomorphy on the tree, and "partial" likelihoods for the root node of the Asteridae, were calculated by using Discrete (Pagel, 1994, 1997). Differences in the rates of gain and loss of zygomorphy were tested statistically by comparing the likelihood score of the data under a model with independent rates of gain and loss (2 free parameters) with the likelihood score under a model in which rates of gains and losses are constrained to be equal (1 free parameter). We used the likelihood ratio (LR) statistic:

$$LR = -2 \ln(H_0/H_1),$$

where H_0 is the likelihood of the data given the one-rate model and H_1 is the likelihood of the data given the two-rate model; under these conditions, this statistic is χ^2–distributed with 1 df (following Pagel, 1997).

Because we lacked branch-length information for the entire tree, and because the maximum likelihood methods require that branch lengths be specified, all branch lengths were initially set equal to one. We then assessed the sensitivity of the maximum likelihood rate estimates to two factors—branch lengths and taxon sampling—by performing four experimental manipulations of the data. The first two manipulations involved scaling the lengths of branches such that the terminal taxa became contemporaneous, i.e., making the sum of branch lengths from any terminal taxon to the root of the tree the same for all taxa. These differed in assuming "early" or "recent" speciation events. Under the "early speciation" procedure, we took the phylogeny with equal branch lengths and simply extended the lengths of terminal branches to bring all terminal taxa up to the same level. Under the "recent speciation" procedure, terminal branches were extended only if no internal branch could be lengthened to render terminal taxa contemporaneous; otherwise, the internal branch nearest the root was lengthened.

The issue of taxon sampling is relevant because the proportion of zygomorphic taxa in our tree (30%) is less than our estimate of the actual proportion in the Asteridae (~ 50%). Although we are reasonably confident that the tree is not missing any critical zygomorphic lineages, particularly near the base of the tree, most zygomorphic clades in the Asteridae are in reality much larger than is represented in our tree. To address this, we transformed our data in two ways: (1) we replaced each zygomorphic taxon in our tree with two taxa, raising the proportion of zygomorphic taxa in the tree to 46%; and (2) we doubled the length of all zygomorphic branches, including terminal branches, internal branches, and the stem branch, of clades that were monomorphic for zygomorphy. Each of these procedures had the effect of more or less equalizing the overall length sum of actinomorphic and zygomorphic branches on the tree.

RESULTS

The results of all maximum likelihood rate estimates are summarized in Table 1. Under the assumption of equal branch lengths, the rate of transformation from zygomorphy
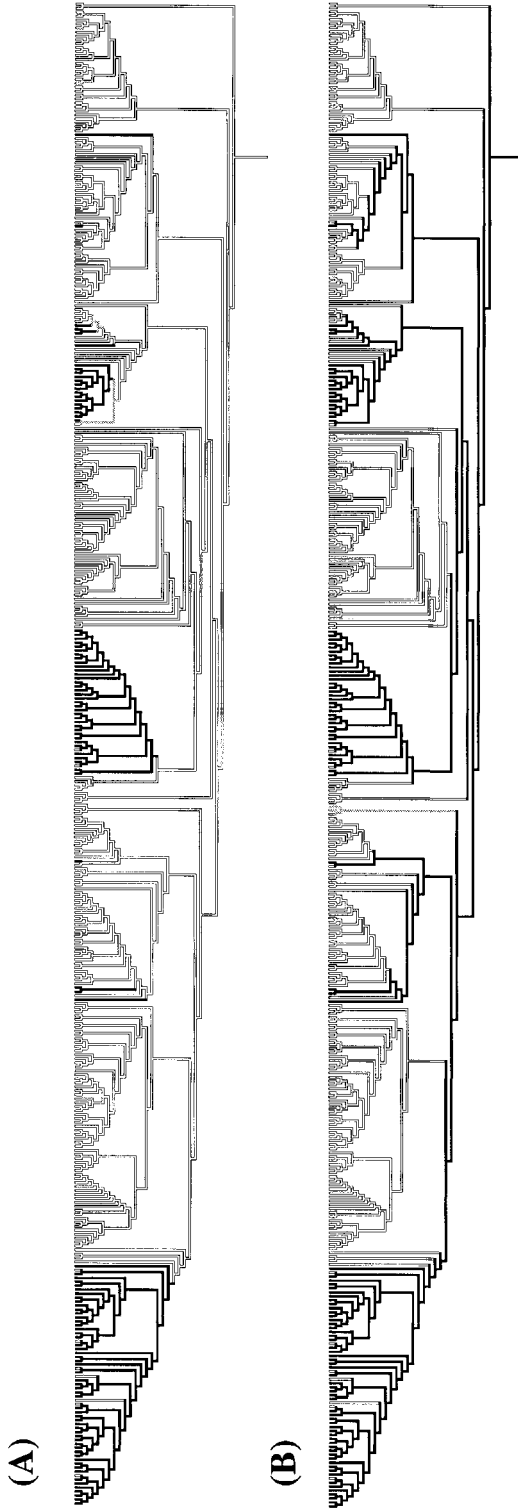
(A)

(B)

FIGURE 1. Maximum parsimony reconstructions of flower symmetry on a phylogeny of asterid angiosperms. Black branches = zygomorphic; white branches = actinomorphic. (A) With equal transformation costs, there are a minimum of 8 gains and 9 losses of zygomorphy. (B) If the cost of transformations from actinomorphy to zygomorphy is > 10 times the cost of transformations in the opposite direction, it becomes most-parsimonious to suppose that all occurrences of zygomorphy among the terminal taxa have been retained from a common ancestor.

TABLE 1. Maximum likelihood estimates of rates of evolution in flower symmetry in the Asteridae. Likelihoods are reported as negative logarithms.

Experimental manipulation	Two-rate model		One-rate model		Two-rate model favored?
	Estimated rates of evolution (gain, loss of of zygomorphy)	Likelihood	Estimated rate of evolution	Likelihood	
All branch lengths equal	0.021, 0.057	189.60	0.031	194.32	Yes ($P = 0.002$)
Contemporaneous taxa with "recent speciation"	0.003, 0.010	197.06	0.004	205.15	Yes ($P < 0.001$)
Contemporaneous taxa with "early speciation"	0.003, 0.009	187.26	0.004	193.46	Yes ($P < 0.001$)
Zygomorphic taxa doubled in number	0.022, 0.026	206.04	0.024	206.15	No ($P = 0.639$)
Zygomorphic branches doubled in length	0.025, 0.027	191.52	0.026	191.55	No ($P = 0.806$)

to actinomorphy is significantly higher than the rate of transformation from actinomorphy to zygomorphy (Fig. 2). In other words, losses of zygomorphy are more likely than gains. If branch lengths are scaled such that terminal taxa are contemporaneous, losses are still favored over gains, and the relative difference in rates actually increases (Fig. 3). In contrast, the effect of doubling the number of zygomorphic terminal taxa was to dramatically decrease the difference in rates of gain and loss of zygomorphy. Doubling the lengths of zygomorphic branches in the tree had the same effect. In both cases, the differences in rates became nonsignificant (Fig. 4).

As we reported elsewhere (Donoghue et al., 1998), if the costs of gaining and losing zygomorphy are weighted equally, there are a minimum of eight gains and nine losses of zygomorphy on the phylogeny under parsimony (Fig. 1A), and the ancestor of the Asteridae is inferred to be actinomorphic. For zygomorphy to be the most-parsimonious ancestral state for the Asteridae, the cost of gains must be weighted > 3 times the cost of losses. To reconstruct all occurrences of zygomorphy on the tree as being retained from a common zygomorphic ancestor, the cost of gains must be weighted > 10 times the cost of losses (Fig. 1B). Maximum likelihood estimates also overwhelmingly favor an actinomorphic ancestor at the ancestral node.

Following Schluter et al. (1997), we interpret the ratio of partial likelihoods for each state at the root node as a simple measure of relative weight of evidence: In each analysis, the actinomorphy:zygomorphy likelihood ratio greatly exceeds the minimum threshold of 7.4 (Edwards, 1972) (Table 2).

DISCUSSION

Inferring Unequal Rates of Change

Given that the numbers of transformations in each direction inferred under parsimony are about the same, it is perhaps counterintuitive that the rates of change between actinomorphy and zygomorphy estimated by maximum likelihood are significantly different, favoring losses of zygomorphy. Taking this initial result at face value for the moment, it appears to corroborate developmental genetic evidence (Coen and Nugent, 1994) implying that losses of zygomorphy, possible via mutations in genes such as *CYCLOIDEA*, should occur at a higher rate. No simple genetic pathways from actinomorphy to zygomorphy have been found, suggesting that transformations in this direction are genetically more complex and should occur at a lower rate. However, before concluding that our data provide a link between inferences of character evolution from phylogenies and underlying genetic mechanisms of change, we should
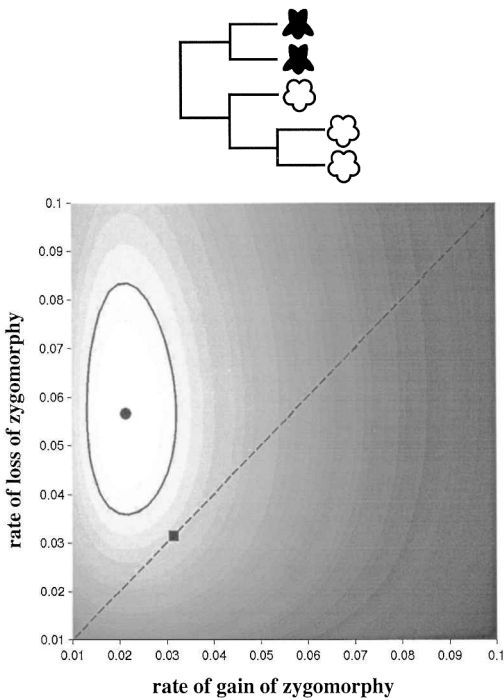
FIGURE 2.   Plot of the likelihood surface for rates of gain (x-axis) and loss (y-axis) of zygomorphy in the phylogeny of Asteridae shown in Figure 1. All branches were assumed to be of equal length (shown diagrammatically above the surface plot). Lighter shading indicates regions of higher likelihood. The optimum given two rates (black circle) is significantly higher than the optimum given only a single rate (black square, on diagonal dotted line). The contour line encloses the region that is not significantly different from the unrestricted optimum (χ^2 distribution, df = 1, $\alpha = 0.05$).
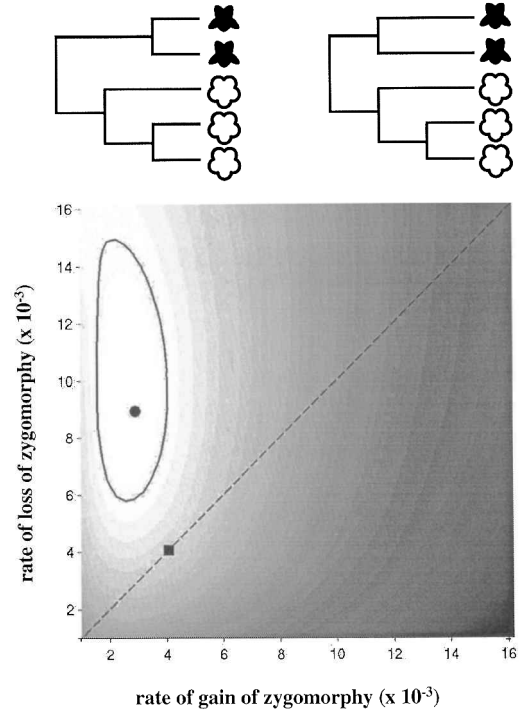
FIGURE 3.   Effect of rendering terminal taxa contemporaneous on rate estimates of gain and loss of zygomorphy in the Asteridae. Both methods of scaling branches ("recent speciation," left, and "early speciation," right) are illustrated. The likelihood surface shown (see Fig. 2) is from the "recent speciation" analysis; the plot from the "early speciation" analysis is almost identical. In each case, branch-length scaling increases the relative difference between rates of gain and loss under a two-rate model (see Table 1).

consider the possibility that the difference between rates of gain and loss of zygomorphy is an artifact related to sampling issues.

Our experimental manipulations of branch lengths and taxon sampling provide insight into the cause of the observed asymmetry in rates. Because evolutionary rates are by definition time-dependent (here represented by branch length), differentially changing the lengths of branches occupied by each state will change the relative magnitudes of the rate estimates. This point can be made clearer with reference to the most-parsimonious ancestral-state reconstruction and to the idea of evolutionary rate as change per unit branch length. An estimate

of the rate of change in a particular direction, e.g., losses of zygomorphy, could be obtained by dividing the number of inferred losses by the length sum of those branches that are reconstructed as zygomorphic (see Sanderson, 1993). The proportion of the tree mapped as zygomorphic thus represents the overall opportunity for zygomorphy to be lost. If we take the relative proportion of zygomorphic taxa as a proxy for coverage of the tree by zygomorphy, it becomes clear why the rate of loss exceeds the rate of gain: Zygomorphic taxa comprise only about one-third of the taxa in our tree, yet the number of inferred gains and losses of zygomorphy are about equal. Because evolutionary rate
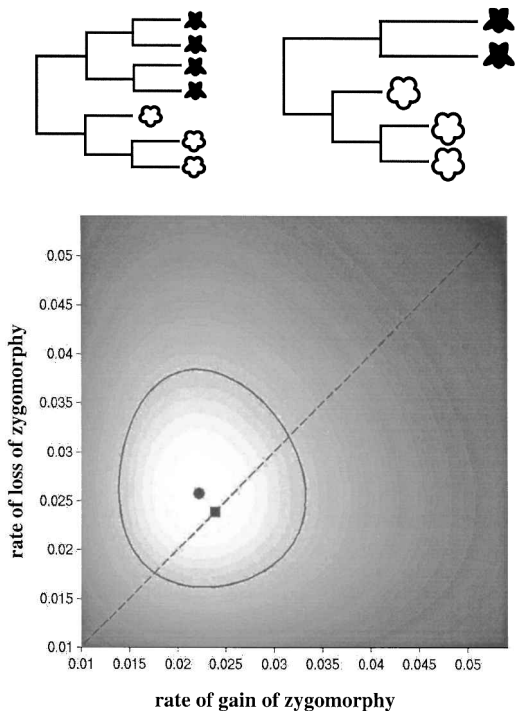
FIGURE 4. Effect of equalizing the representation of actinomorphy and zygomorphy on rates of gain and loss of zygomorphy in the Asteridae. Both methods (doubling each zygomorphic terminal taxon [left] and doubling the length of zygomorphic branches [right]; see text) are illustrated. The likelihood surface shown (see Fig. 2) is from the taxon-doubling analysis; the plot from the branch-length-doubling analysis is almost identical (see cover). In each case, equalizing the representation of zygomorphy on the tree removes support for preferring a two-rate model of evolution over a one-rate model (see Table 1).

is an increasing function of the number of transformations, but a decreasing function of the opportunity for change, the rate of loss of zygomorphy is therefore estimated to be higher than the rate of gains. This is why increasing the zygomorphic proportion of the tree by doubling the number of zygomorphic taxa, or doubling the lengths of zygomorphic branches on the tree, significantly lowers the rate of loss. This is also why scaling branch lengths to make terminal taxa contemporaneous accentuates the rate disparity: actinomorphic lineages tend to be more basal in the phylogeny and

are therefore lengthened disproportionately more relative to zygomorphic lineages.

This raises the question: What is an appropriate measure of the opportunity for evolutionary change? We have shown that measuring opportunity for change can be influenced by both taxon sampling and assumptions of branch length. Our experiment that increased the proportion of zygomorphic taxa in the tree was motivated by the concern that the size of zygomorphic lineages relative to actinomorphic lineages was underrepresented in our data. One could object to this on the grounds that the proportion of zygomorphic species alone is not an appropriate proxy for the "true" opportunity for losses of zygomorphy, because this implies a correlation between change in flower symmetry and speciation events, but the same result is achieved by keeping the taxon proportions unchanged and differentially scaling the lengths of zygomorphic branches. Branch-length information from other sources can be incorporated if such information is available, and evolutionary inferences about the character of interest would then be conditioned on the branch-length units of measurement.

Ultimately, what constitutes an appropriate measure of evolutionary opportunity will depend on the particular character under consideration. Here we have attempted to estimate rates of change in flower symmetry over a broad range of plausible conditions of our data. Because our initial finding of a significantly higher rate of loss of zygomorphy in the Asteridae does not hold up if the representation of each state is equalized (either by branch-length scaling or taxon sampling) on the tree, we conclude that whether there indeed is such a bias remains unclear. This means that Coen and Nugent's (1994) suggestion of an evolutionary trend towards losses of zygomorphy, apparently contradicted by parsimony-based inferences of equal numbers of gains and losses (Donoghue et al., 1998), remains an open hypothesis.

Flower Symmetry in the Ancestor of Asteridae

In contrast to the uncertainty associated with inferring differential evolutionary rates

TABLE 2. Actinomorphy:zygomorphy likelihood ratios at the root of the Asteridae. Values > 7.4 are interpreted as significant support for inferring an actinomorphic asterid ancestor.

	Single rate of gain and loss of zygomorphy	Independent rates of gain and loss of zygomorphy
All branch lengths equal	944.65	286.06
Contemporaneous taxa with "recent speciation" events	146.69	26.11
Contemporaneous taxa with "early speciation" events	11637.78	1764.52
Zygomorphic taxa doubled in number	1664.79	1412.01
Zygomorphic branches doubled in length	1452.11	1333.66

in flower symmetry, inferences about the ancestor of the Asteridae are more definite. Both parsimony (with equal costs of change) and maximum likelihood agree that the ancestor of the Asteridae had actinomorphic flowers. Although the parsimony estimate is robust if the assumed cost of gaining zygomorphy is < 3 times the cost of losses, it is more difficult to quantify the robustness of the ancestral state estimate under maximum likelihood to different rates, because node probabilities are affected by both the relative and absolute magnitude of rates. However, the maximum likelihood ancestral state estimate is stable over a wide range of conditions: In all manipulations of the data (branch lengths and taxon sampling, with two-rate or one-rate models), actinomorphy is always overwhelmingly favored at the root node (Table 2). Note that this does not mean that all ancestral states under parsimony that are robust to differential cost-weighting by a factor of three or more are indeed the most likely states; indeed, the relationship between the robustness of ancestral state inferences under parsimony and under likelihood is not clear and deserves further attention. For our data, with equal branch lengths and equal rates of gain and loss, nodes that were equivocal under parsimony were also equivocal under maximum likelihood (i.e., the actinomorphy:zygomorphy likelihood ratios were much less than 7.4). The partial likelihoods at these nodes were also the most

sensitive to rates, in that under two-rate models, support for one state or the other seemed to depend on the difference between the rates of gain and loss. This suggests a positive relationship between the stability of ancestral states under parsimony and under likelihood, but more studies are needed to draw firm conclusions (see also Mooers and Schluter, 1999).

Future Prospects

The benefits of larger trees are that more-precise estimates of evolutionary patterns, e.g., asymmetric rates of change, are possible from larger data sets. As phylogenetic studies accumulate in the literature, it will become more and more feasible to assemble supertrees and analyze supersets of comparative data (Sanderson et al., 1998), and databases like Tree-BASE (http://www.herbaria.harvard.edu/treebase) will facilitate such studies. However, it is important to keep in mind that such trees will tend to have a higher margin of error, and uncertainty over taxon sampling and branch lengths will necessitate sensitivity analyses of the results to changes in these factors (cf. Donoghue and Ackerly, 1996). Moreover, the analysis of large trees requires a more critical assessment of the assumption that rates of change are uniform across the entire phylogeny, which, for the sake of simplicity, is a standard assumption of models of character evolution that have been developed to date. It is more likely that there

have been shifts in rate on large phylogenies that encompass more distantly related clades and deeper divergences. A promising extension of maximum likelihood methods of inferring character evolution would be to relax the constraint of having fixed rates, along the lines of models to detect the location and direction of shifts in rates of diversification (Sanderson and Donoghue, 1995; see also Mooers and Schluter, 1999). The ability to detect the location and direction of shifts in evolutionary rate in phylogenies would allow much deeper analyses of constraint and burden in character evolution.

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