Some Limitations of Ancestral Character-State Reconstruction When Testing Evolutionary Hypotheses

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The recent explosion of phylogenetic information has had an impact far beyond the field of systematic biology. Workers in a variety of disciplines are now interested in generating and using phylogenies for their groups (or genes) of interest (e.g., Brooks and McLennan, 1991; Harvey et al., 1995; Martins, 1996). In large part, this new interest has been driven by the promise of using phylogenies to reconstruct ancestral character states, usually by parsimony. The simplicity of testing evolutionary hypotheses by mapping characters onto a phylogeny has an appeal that has not been lost on the biological community.

Despite its appeal, ancestral state reconstruction is an ambitious exercise. Although we are very familiar with the difficulty of accurately inferring phylogenies with thousands of characters (reviewed by Swofford et al., 1996), the challenges associated with reconstructing ancestral character states of individual characters are correspondingly more difficult (Swofford and Maddison, 1987, 1992; Maddison and Maddison, 1992; Collins et al., 1994a; Frumhoff and Reeve, 1994; Pagel, 1994; Maddison, 1995; Schluter, 1995; Schultz et al., 1996; Omland, 1997; Schluter et al., 1997; Cunningham et al., 1998).

Whereas we generally assume that data used for phylogenetic reconstruction are selectively neutral, many—if not most—of the characters we reconstruct to test evolutionary hypotheses are thought to be under selection (Brooks and McLennan, 1991; Harvey and Pagel, 1991). In fact, their presumed selective importance is often why we are interested in them in the first place. Therefore, the problems of convergence and parallel evolution that plague phylogenetic inference should be that much more serious when we test evolutionary hypotheses with ancestral state reconstructions.

In this paper, I discuss the limitations of using reconstructed ancestral character states to test evolutionary hypotheses. In particular, I argue that hypotheses of irreversible evolution (e.g., Dollo's law; Dollo, 1893) are particularly difficult to test by using ancestral character state reconstruction. I illustrate this point with two case studies of life history evolution in echinoderms.

DOLLO'S LAW AND THE BIASED LOSS OF LARVAL FEEDING IN ECHINODERMS

For two decades, Strathmann has argued persuasively that obligate larval feeding has been lost repeatedly throughout the evolutionary history of the echinoderms (Strathmann, 1978, 1985, 1993). Larval feeding appears to be homologous among the echinoderm classes and dates back at least to the Ordovician. Strathmann attributes the lack of larval feeding in many echinoderm species—including the entire Class Crinoidea—to this unidirectional bias of repeated losses (Strathmann, 1978, 1985, 1993).

In his reformulation of Dollo's law, Simpson (1953) argued that, once lost, complex characters should be difficult to regain. This idea is still widely held and can be expressed as follows: In many cases, the expression of a complex character might easily be interrupted by the action of a few genes. As time passes, the unused developmental pathways are thought to be progressively more difficult to restore (Raff et al., 1990; Maddison, 1994; Omland, 1997). Because echinoderm larval feeding requires a ciliary system and a full digestive tract, the loss of larval feeding certainly qualifies as the loss of a complex character (Strathmann, 1978).

In addition, Strathmann has proposed functional reasons for why regaining obligate feeding should be especially difficult in

echinoderm larvae. One proposed route to regaining larval feeding—coopting the post-metamorphic juvenile feeding apparatus—is unlikely because echinoderm benthic juvenile feeding mechanisms are poorly suited for planktonic feeding (Strathmann, 1978, 1985, 1993). Even if successful, any secondarily acquired larval structures should be clearly recognizable as analogous (Strathmann, 1978).

Here I consider two studies that have used ancestral reconstructions to test Strathmann's hypothesis of irreversible transitions to nonfeeding in echinoderms: the first in echinoids (sea urchins and their relatives), the second in asteroids (starfish).

Evolution of Larval Feeding in Sea Urchins

Wray (1996) carried out a phylogenetic analysis of larval feeding in sea urchins and their relatives. He identified an ordered series for three classes of larvae: obligate feeding → facultative feeding → nonfeeding. Using arguments similar to Strathmann's, Wray argued that obligate feeding is the primitive condition and has been replaced in many lineages by facultative feeding and nonfeeding (see also Emlet, 1986; Hart, 1996). Facultative feeders have very large egg yolks, and although they are able to feed, can undergo metamorphosis without feeding (Emlet, 1986; Wray and Raff, 1991; Hart, 1996). Because facultative feeders still have larval feeding structures, reversals to the state of obligate feeding are certainly possible. On the other hand, because nonfeeding larvae lack most structures associated with feeding, transitions to the nonfeeding condition may be irreversible (Strathmann, 1978; Wray, 1996). In the discussion below, I will consider only transitions to the presumably irreversible nonfeeding state.

An equally weighted parsimony analysis of larval feeding shows five transitions from obligate feeding to the presumably irreversible state of nonfeeding (Fig. 1). These changes appear to be consistent with Strathmann's hypothesis that there is a unidirectional bias in these transitions. But can they be used to falsify a null hypothesis of equal probability of gains and losses?

Recent workers have shown that equally weighted, unordered parsimony can be appropriately used to falsify hypotheses of equal probability of gains and losses (Sanderson, 1993; Jacobs et al., 1995). Because equally weighted parsimony allows reversibility, significant deviations from equal reversibility are illuminating. For example, Sanderson's method tests the null hypothesis of equal probability of gains and losses by using parsimony character-state reconstructions in a maximum-likelihood framework. In practice, only large phylogenies with many asymmetric character-state changes have the power to falsify this hypothesis (Sanderson, 1993).

When I applied Sanderson's method to Wray's phylogeny, the sample size was not sufficient to reject the null hypothesis of equal rates of gains and losses ($P > 0.4$). Because Wray's phylogeny includes only a few representative taxa, it is possible that broader taxonomic sampling may increase the power of Sanderson's method. However, the next study to be considered shows that even a higher degree of taxonomic sampling does not guarantee unambiguous interpretation of parsimony reconstructions.

Evolution of Larval Feeding in Starfish

Hart et al. (1997) carried out a molecular phylogenetic analysis of life-history evolution in two genera of starfish in the family Asterinidae. Whereas the sea urchin reconstruction showed only unidirectional transitions to nonfeeding (Fig. 1), in the starfish phylogeny equally weighted parsimony reconstructed one transition to nonfeeding followed by two independent gains of obligate larval feeding (Fig. 2). This result directly contradicts Strathmann's prediction of irreversible losses. Assuming the phylogeny is correct, this surprising reconstruction can be explained in two ways. First, the reconstruction may be right, and the traditional hypothesis of irreversible transitions to nonfeeding is wrong. Second, the irreversible losses of larval feeding may have happened so often that parsimony incorrectly reconstructs

a loss followed by gains (Schultz et al., 1996).

Which of these possibilities is more likely? On the one hand, it is known that the genus *Patiriella* includes "the greatest diversity of larval types and life histories known among extant starfish" (Hart et al., 1997). This suggests that evolution of larval feeding is indeed very rapid in this genus, and as mentioned above, rapid evolution can mislead parsimony reconstructions. On the other hand, rapid evolution does not preclude the possibility that larval feeding was lost and then regained. Indeed, the remarkable lability of this group may allow larval feeding to be regained especially easily. In the next section, I will explore the limitations of phylogenetic information as a means of resolving these issues.
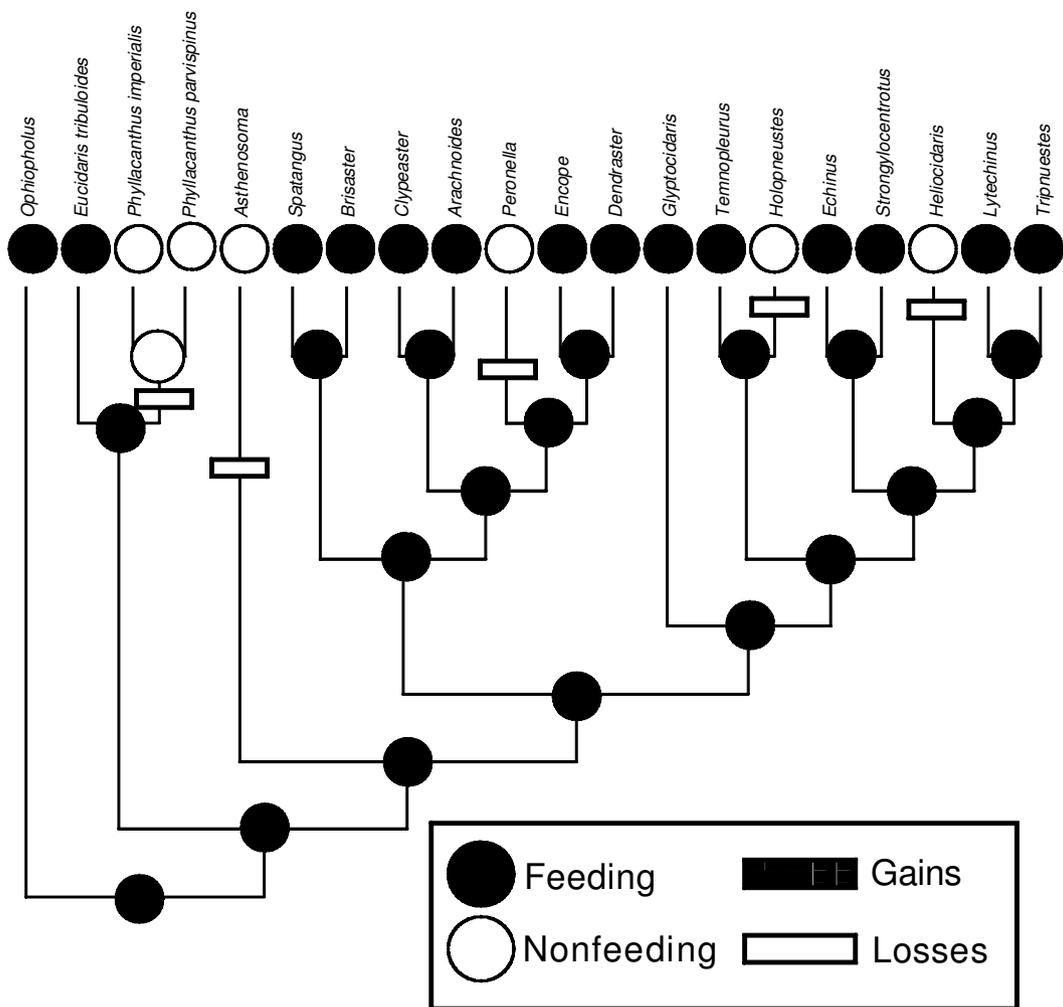

FIGURE 1. Equally weighted, unordered parsimony reconstructions of larval types of sea urchins and their relatives (echinoids). Species labeled as "feeding" include obligately feeding and facultatively feeding larvae. Based on Figure 2 in Wray (1996). Reconstructions performed by MacClade 3.0 (Maddison and Maddison, 1992).

THE LIMITATIONS OF PARSIMONY RECONSTRUCTIONS FOR TESTING EVOLUTIONARY HYPOTHESES

The parsimony reconstructions discussed so far are a valuable guide to evolutionary inference. In the sea urchin phylogeny, parsimony reconstructions are consistent with a hypothesis of unidirectional transitions from obligate feeding to nonfeeding (Fig. 1). In the starfish phylogeny, parsimony reconstructions suggest an alternative hypothesis that should be seriously considered—that larval feeding may have been lost and then regained (Fig. 2).

But these case studies also illustrate the limitations of equally weighted parsimony reconstructions as a means of testing evolutionary hypotheses. Although the sea urchin reconstructions are consistent with a hypothesis of irreversible transitions to nonfeeding (Fig. 1), there are not enough data to adequately test the null hypothesis of equal probability of gains and losses (discussed above).

In contrast, the starfish reconstructions actually appear to contradict Strathmann's hypothesis of irreversible evolution (Fig. 2; Strathmann, 1978; Wray, 1996). How can
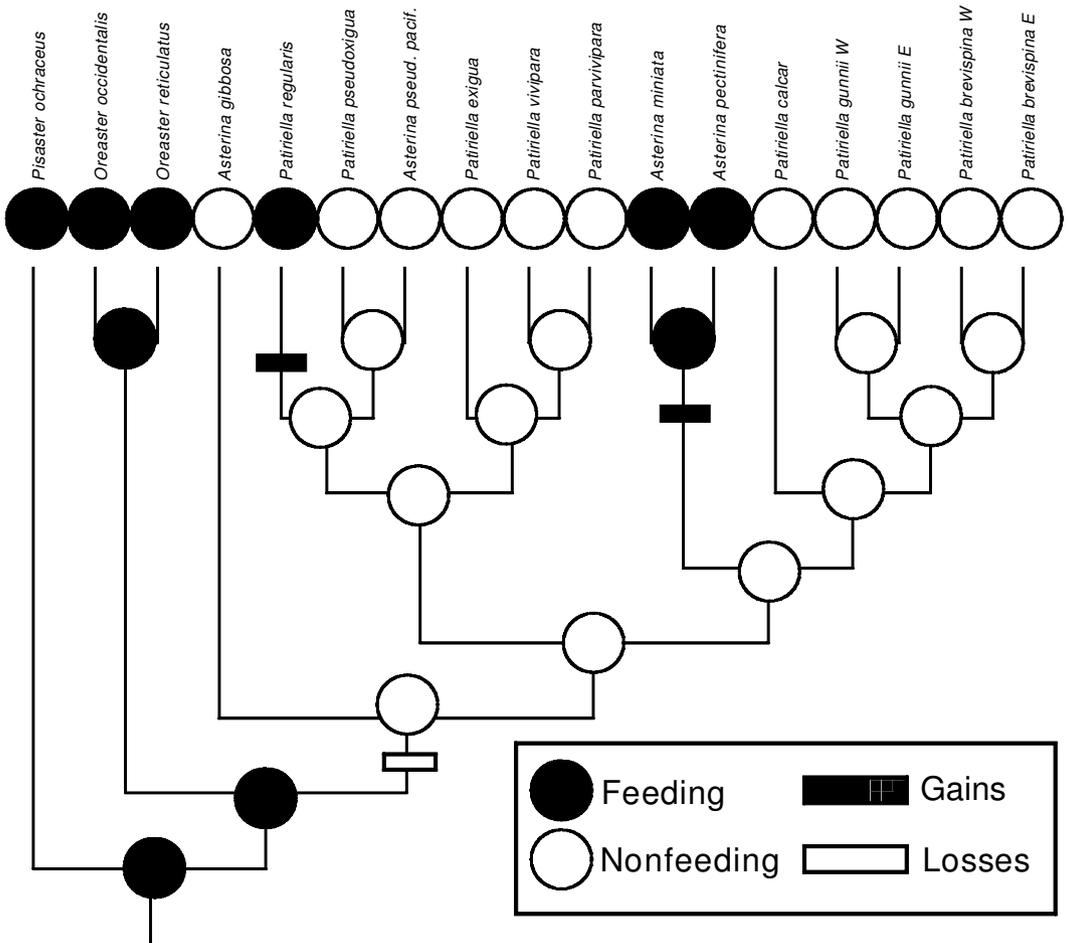

FIGURE 2. Equally weighted, unordered parsimony reconstructions of larval types of asteriniid starfish. Topology taken from Figure 1 in Hart et al. (1997), except that all three outgroup species are included.

we interpret parsimony reconstructions that contradict an a priori hypothesis of irreversible evolution? More to the point, do the starfish reconstructions falsify the traditional hypothesis of irreversibility (Fig. 2)?

In the starfish case, the answer is no. If irreversible changes (or any asymmetric changes) happen often enough, equally weighted parsimony is expected to fail in exactly the way shown in Figure 2—with apparent changes being followed by reversals (Frumhoff and Reeve, 1994; Schultz et al., 1996). In other words, the hypothesized process of frequent losses is predicted to generate the very errors in parsimony reconstruction that will appear to falsify it.

Furthermore, because the hypothesis to be tested is one of irreversibility, it may not be possible to falisify this hypothesis with a form of parsimony that allows perfect reversibility. Unfortunately, applying irreversible parsimony is not illuminating, because reversibility is prohibited. It may be useful to explore various degrees of asymmetric character-state weighting (Omland, 1997; Ree and Donoghue, 1999). But even these sensitivity analyses do not represent definitive tests of hypotheses of irreversibility.

A further drawback of parsimony is that reconstructions are usually represented as either unambiguous or ambiguous, often obscuring the actual degree of support for alternative character states. In the following section I explore estimating relative support for alternative character states in a maximum likelihood framework.

Do Maximum Likelihood Estimates of Support Change our Interpretation of Parsimony Reconstructions?

Schluter et al. (1997) proposed a maximum likelihood method for estimating the degree of confidence in ancestral reconstructions. Whereas most other maximum likelihood methods for ancestral reconstruction have been developed for molecular data (e.g., Yang et al., 1995; Koshi and Goldstein, 1996), the method of Schluter et al. can be applied to phenotypic characters. Their method is based on Pagel's (1994b)

continuous-time Markov model for character evolution and can be implemented for two-state characters by using the computer program Discrete 2.0 (Pagel, 1998). Aside from providing estimates of support, maximum likelihood reconstructions differ in several other important ways from parsimony reconstructions (reviewed in Schluter et al., 1997; Cunningham et al., 1998).

When maximum likelihood reconstructions are applied to the sea urchin phylogeny (forcing rates of gains and losses to be equal, as recommended by Schluter et al., 1997), the estimates of support for most internal nodes are ambiguous and only approach significance at 3 shallow nodes (Fig. 3). This is largely a consequence of the very high rate of evolution in this character, meaning that changes are likely along any branch (M. Pagel, pers. comm.). One might reasonably conclude, based on the maximum likelihood reconstructions, that this phylogeny is not very useful for making evolutionary inferences.

Aside from estimating ancestral states, Discrete 2.0 allows an asymmetric model to be applied that directly estimates the relative rates of gains and losses on the sea urchin phylogeny. Whereas parsimony only identified losses of obligate larval feeding (Fig. 1), maximum likelihood found the rate of gains to be twice as high as the rate of losses (0.98 vs. 0.42). This counterintuitive result should be treated with caution, however, because the asymmetric model does not fit the data significantly better than does a symmetric model that forces the rates of gains and losses to be equal (likelihood ratio test, $P > 0.40$).

When maximum likelihood reconstructions are applied to the starfish phylogeny, character support for the identity of the root node differs markedly depending on how branch lengths are treated. When branch lengths are considered equal (as in a parsimony analysis), maximum likelihood significantly favors an obligate feeding ancestor for the entire phylogeny (Fig. 4a). When branch lengths are estimated by using maximum likelihood and assuming a molecular clock, maximum likelihood finds almost
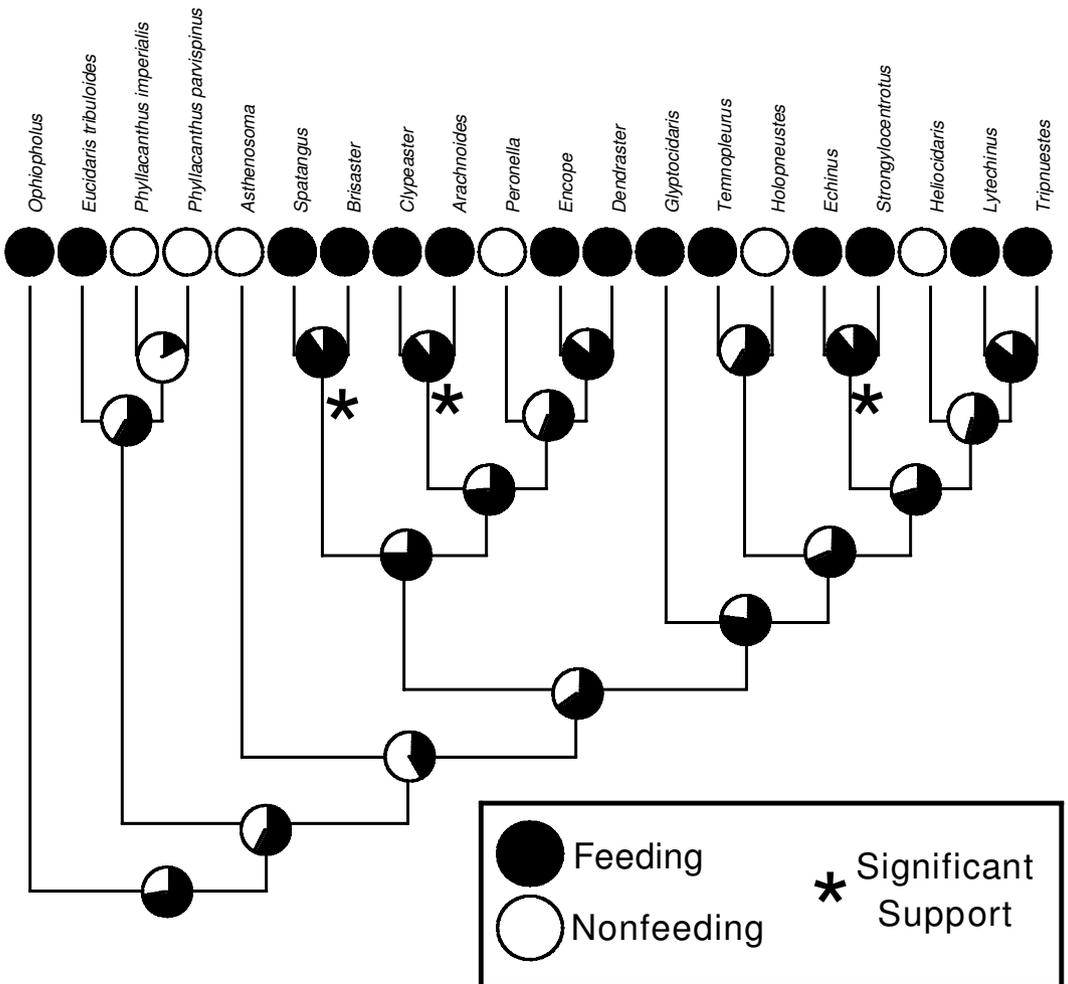
FIGURE 3. Larval evolution of sea urchins and their relatives, reconstructed by maximum likelihood with Discrete 2.0 (Pagel, 1998), as described by Schluter et al. (1997). Pie diagrams indicate the relative degree of support for alternative character states, with a ratio of 7:1 or greater considered significant (as in Schluter et al., 1997). All branch lengths are equal.

equal support for obligate feeding and nonfeeding in the root node (Fig. 4b).

The latter reconstruction may appear surprising to those familiar with parsimony reconstructions. Because all three outgroup taxa share the same character state, why is there so much uncertainty about the identity of the root? The difference between these reconstructions raises questions about the justification for using branch length information in ancestral reconstructions.

WHEN SHOULD INFERRED BRANCH LENGTHS BE USED IN MAXIMUM LIKELIHOOD RECONSTRUCTIONS?

Advocates of using maximum likelihood reconstructions for discrete phenotypic characters have cited in their favor the potential for using branch lengths inferred from molecular data as a source of temporal information (Schluter et al., 1997; Pagel, 1999). There are at least two reasons why relying entirely on a model incorpo-
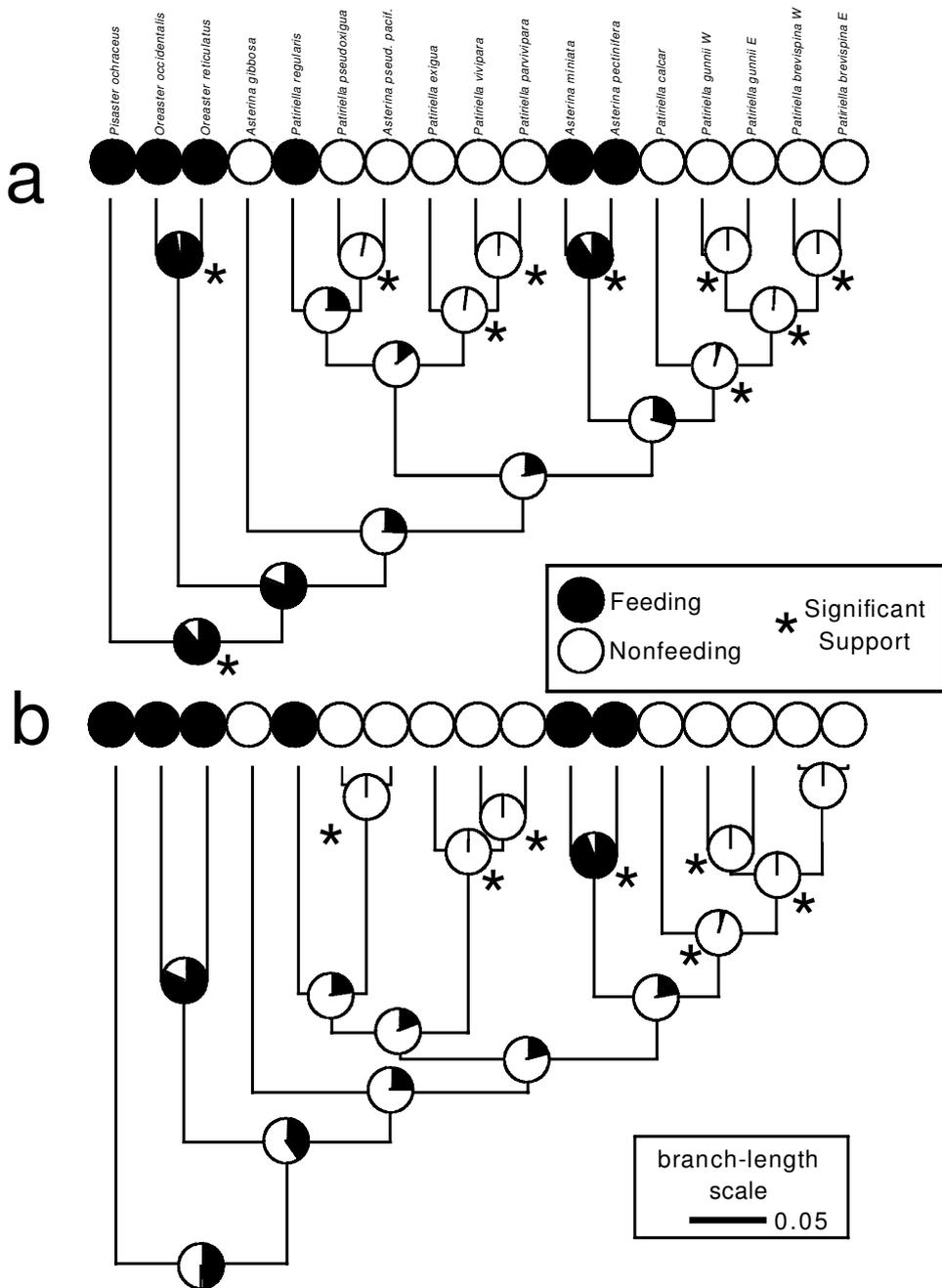
FIGURE 4. Larval evolution of asteriniid starfish, reconstructed by maximum likelihood as described in Figure 3. (a) Topology as in Figure 2, with all branch lengths equal, as in Figure 3. (b) Topology as in Figure 2, but with branch lengths estimated from all of the mitochondrial sequences (Hart et al., 1997). Branch-length estimates were calculated by using maximum likelihood under the assumption of a molecular clock with the general time-reversible model and an estimate of the proportion of invariant sites (Lanave et al., 1984; Yang, 1996). All estimates were made by using PAUP* 4.0d64.

rating inferred branch lengths may lead to misleading results. First, there are important limitations in the ability of DNA sequence data to accurately infer the length of internal branches. When DNA sequences are at all saturated, the length of internal branches may be systematically underestimated, even when an appropriate model of sequence is used (Fitch and Beintema, 1990; Graybeal, 1994; Cunningham et al., 1998). When this happens, terminal branches appear relatively long, and internal branches look relatively short. Because the maximum likelihood approach implemented here assumes a constant rate of character evolution across the entire phylogeny, character transitions are much more likely on long branches. In the starfish phylogeny, the longest branches lead to the outgroup, which explains why there is so much uncertainty about the identity of the root node (Fig. 4b).

The second reason why estimated branch lengths should be used with caution is that adaptive radiations are predicted to produce a great deal of evolutionary change along short internal branches. Even if the branch lengths are estimated perfectly, maximum likelihood may reconstruct the character changes as having occurred considerably after the actual radiation took place. In these cases, a model with equal branch lengths should be used as a point of comparison.

On the whole, though, maximum likelihood reconstructions are a promising method for studying phenotypic evolution. Not only do they provide estimates of support, they also allow the researcher to compare reconstructions based on different models of evolution.

CONCLUSIONS AND FUTURE PROSPECTS

I have argued, on the basis of two case studies of the evolution of larval feeding in echinoderms, that parsimony reconstructions have the potential to add further support to hypotheses of irreversible evolution. Unfortunately, using parsimony reconstructions to falsify these hypotheses is problematic. This is because frequent, unidirectional character-state transitions are predicted to cause parsimony to

incorrectly conclude that character reversals have taken place. The observation that parsimony reconstructions alone may not be sufficient to falsify hypotheses of irreversible evolution does not mean that these hypotheses are not interesting (e.g., Bull and Charnov, 1985)—or true, for that matter.

For the studies considered here, maximum likelihood revealed considerable uncertainty regarding the character states in several internal nodes, begging for caution when testing evolutionary hypotheses based on these reconstructions. Although maximum likelihood allows reconstructions to be based on branch lengths estimated from DNA sequences, exclusively relying on inferred branch lengths may in some cases lead to systematic errors in ancestral reconstruction. It may be advisable to consider other models of evolution as well, including specifying that all branch lengths are equal.

Some of the strongest evidence for reversibility will continue to be based on careful analysis of the homology of character states. For example, Lande (1978) reviews a striking example of character reversals in whales, where tiny vestigial hind-limbs appear in occasional individuals. Whales, of course, belong to a lineage where a fossil record strongly suggests that hind limbs have been absent for millions of years. Their rarity in extant populations—and their obviously reduced appearance—are both consistent with the hypothesis that these limbs represent true reversals.

Another case of proposed character reversal comes from the work of Boero et al. (1996), who argued that the medusae of the hydroid genus *Obelia* are so aberrant that they may be secondarily derived from ancestors that had previously lost the medusoid stage. Finally, in an example that is directly relevant to the sea urchin and starfish examples that form the central focus of this paper, Reid (1989) has noted that some gastropods with larval feeding have larval shells that are diagnostic of a clade of nonfeeding gastropods. He argues that these species with feeding larvae may be secondarily derived from ancestors that lacked feeding larvae.

Caution is also in order in cases where character transitions are not necessarily irreversible but tend to be asymmetric. For example, Collins et al. (1994) concluded that base compositional biases led to systematic errors in parsimony reconstructions of DNA sequences. In their review of phylogenies and larval evolution, Strathmann and Eernisse (1994) concluded that nonphylogenetic information about the probability of asymmetrical character transitions may be essential to fully understand the evolution of larvae in marine invertebrates (see also Omland, 1997; Ree and Donoghue, 1999). In their review, Strathmann and Eernisse (1994) came to the sobering conclusion that "[m]any controversies about the evolution of larvae would not be resolved even with perfect knowledge of relationships." As important as it is, phylogenetic information taken by itself does not represent the last word on understanding the course of character evolution. As always, homology assessment must include other sources of information in addition to careful phylogenetic analysis.

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