CHAPTER 13

RECONSTRUCTING ANCESTRAL TRAIT VALUES USING SQUARED-CHANGE PARSIMONY: PLASMA OSMOLARITY AT THE ORIGIN OF AMNIOTES

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INTRODUCTION

Biologists often wonder about the behavioral or physiological characteristics of extinct organisms. Were dinosaurs endothermic? How well could *Archaeopteryx* fly? Unfortunately, fossil information pertaining to behavior or physiology is generally unavailable (but see, for example, Lambert, 1992; Hillenius, 1994; Ruben, 1995; Ruben *et al.*, 1996). In some cases, extant forms exist that are morphologically very similar to ancient forms (Eldredge and Stanley, 1984). If one is willing to assume that the behavior and physiology of these "living fossils," such as the coelacanth, has also experienced relatively little evolutionary change, then a more or less direct window into the past is available (Thomson, 1991; but see Burggren and Bemis, 1990, p. 199).

Amniote Origins

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Usually, however, one is limited to a consideration of "ordinary" extant organisms as "models" for extinct forms (e.g., Ruben and Bennett, 1980; Burggren and Bemis, 1990; Ruben, 1991; Ruben and Parrish, 1991; Janis and Wilhelm, 1993; Garland and Carter, 1994; Hackstein and van Alen, 1996; Ruben *et al.*, 1996). The purpose of this chapter is to illustrate some ways in which data on the characteristics of living organisms can be combined with phylogenetic information and recently developed analytical methods to make inferences about the characteristics of hypothetical ancestral organisms.

For illustrative purposes, the phenotypic trait we consider is plasma osmolarity, which previous (nonphylogenetic) studies have shown to vary among phylogenetic lineages (clades) and in relation to ecology or habitat (e.g., freshwater versus saltwater). We have compiled from the literature osmolarity data for a total of 172 vertebrate taxa, including representatives of all major extant lineages (Figs. 1, 2, and Appendix 1). Some living forms are known to be highly derived as compared with their ancestors, and hence constitute "red herrings" with respect to making inferences about extinct forms (Gans, 1970). Given data for a wide variety of extant organisms, therefore, which ones should be used as models for extinct forms–all or a subset? Moreover, how, exactly, should one go about making an inference regarding extinct organisms? Most simply, one could estimate the value of a trait in a hypothetical ancestor as the simple mean of the values observed in its living descendants. Alternatively, one can use additional information on phylogenetic relationships to obtain a better estimate of the ancestral value.

The most common way to use phylogenetic information when making inferences about ancestral trait values is with the general analytical procedure termed "parsimony." Parsimony can be used to "map" or "optimize" a character onto a hypothetical phylogenetic tree, reconstruct values for hypothetical ancestral organisms (internal nodes of the tree), and hence "trace" character evolution (Brooks and McLennan, 1991; Stewart, 1993; Maddison, 1994; Maddison, 1995; Schultz *et al.*, 1996). Appendix 2 discusses the origin and present roles of parsimony analyses in systematic and comparative biology

(for some references pertaining to physiological traits, see Burggren and Bemis, 1990; Garland and Carter, 1994, p. 601).

The first and simplest outcome of a parsimony analysis is estimates of where and how many times a particular character state originated in a phylogenetic tree (e.g., Rosenberg, 1996). This allows one to address such questions as: How many times did endothermy arise during vertebrate evolution (Block *et al.*, 1993; Ruben, 1995)? How many times did flight evolve? Estimation of where in a phylogenetic tree a character state first evolved can also allow one to predict which descendant lineages should have it [i.e., before some living taxa have been actually measured (e.g., see Burggren and Bemis, 1990, p. 208 and 220), or for extinct taxa whose physiology or behavior cannot be measured (e.g., Bennett and Ruben, 1986)]. Moreover, parsimony analyses can elucidate the historical pathways of character evolution (e.g., whether a character increased or decreased in size over evolutionary time within a particular lineage), and hence whether the character state in a particular species is derived (sometimes termed "advanced") or similar to the ancestral value (sometimes termed "primitive"). Further, estimates of where, when, and how frequently particular character states evolved can be combined with information on biogeography or environmental changes (e.g., from the geological record) to get at possible *causes* of phenotypic evolution, such as adaptation in response to an altered selective regime [Eastman, 1993; Arnold, 1994: e.g., did changes in habitat aridity precede changes in evaporative water-loss rate of *Coleonyx* lizards? (Dial and Grismer, 1992)]. Attempts to identify "key innovations" also routinely involve parsimony analyses (Nitecki, 1990; Brooks and McLennan, 1991; Eastman, 1993, p. 200). Sometimes inferences about the traits of extinct forms can be combined with mathematical or physical models to "synthesize" extinct forms (e.g., Kingsolver and Koehl, 1985; Tracy *et al.*, 1986; Abler, 1992; Ryan and Rand, 1995).

Although systematists usually deal with discretely valued characters, comparative biologists often study characters that show continuous variation, both within and among species (e.g., body size, metabolic rate, maximal running speed, and home range area). For continuous-valued characters, the procedure termed "squared-change

parsimony" is often used to reconstruct values for hypothetical ancestors. In Appendix 3, we explain this procedure with worked examples and present computer simulations that compare its performance with an alternative, nonphylogenetic analysis.

The procedures we discuss are not ways of reconstructing phylogenetic trees. Rather, we use existing hypotheses about phylogenetic relationships (taken from the literature) to construct a composite estimate of phylogeny (as shown in Fig. 1) and then map onto this tree (with 172 tips) the plasma osmolarity data. These physiological data are *not* used to alter the phylogenetic topology that is employed in the analyses. Thus, our procedures are in the tradition of most recent work in "the comparative method," in which the "tip" data being analyzed (information on the phenotypes of a series of terminal taxa, typically species but sometimes populations or averages for genera or higher taxa) are independent of the phylogenetic framework used for analyses (reviews in Brooks and McLennan, 1991; Harvey and Pagel, 1991; Maddison and Maddison, 1992; Eggleton and Vane-Wright, 1994; Garland *et al.*, 1993; Garland and Adolph, 1994; Maddison, 1996; Martins, 1996b,c). Alternatively, one could use the physiological data to influence the estimate of phylogeny used for analysis. Whether the use of phylogenies that are "independent" of the characters under study is the best thing to do is unsettled (review in de Queiroz, 1996); nevertheless, it is often a practical necessity. Formal ways to combine fundamentally different types of data [e.g., DNA sequences, genetic distances (as from DNA hybridization studies), qualitative morphological characters, continuous-valued physiological traits that show broad overlap among the species being analyzed] into a single algorithm for estimation of phylogeny (e.g., Eernisse and Kluge, 1993) are the subject of much current controversy and no simple solution is readily available (de Queiroz *et al.*, 1995; Huelsenbeck *et al.*, 1996a).

PLASMA OSMOLARITY OF VERTEBRATES

For the illustrative purposes of this chapter, we have chosen to analyze plasma osmolarity as a representative physiological trait. No fossil indicator of this trait has ever been proposed, so inferences about

its value in extinct organisms must be based on consideration of living forms.

Plasma is the fluid component of blood; it can vary widely in solute concentration. The ability of solutes to cause osmotic pressure and osmosis is measured in terms of osmoles, the osmole being a measure of the total number of particles in a solution. Specifically, one osmole is defined as one gram of nonionizable and nondiffusable substance. *Osmolarity* is the osmolar concentration of a solution, and is expressed as osmoles per liter of solution. As shown in Figures 1 and 2, osmolarity varies widely among species of vertebrates.

Plasma osmolarity is a colligative property, measured as the total number of "osmotically active" particles in solution. If a molecule dissociates into two ions, both may contribute to the osmolarity. Additionally, particle size is unimportant: a small ion such as sodium contributes as equally to osmolarity as a larger macromolecule such as a protein. Thus, two solutions may have the same total osmolarity but differ greatly in chemical composition, as is seen when comparing intracellular and extracellular fluids (Withers, 1992). Physiologically, this means that total osmolarity can be regulated at a particular value concomitantly with some degree of freedom in the actual contents of the body fluids. As discussed below, different vertebrate taxa do indeed exhibit different ways of adjusting total plasma osmolarity.

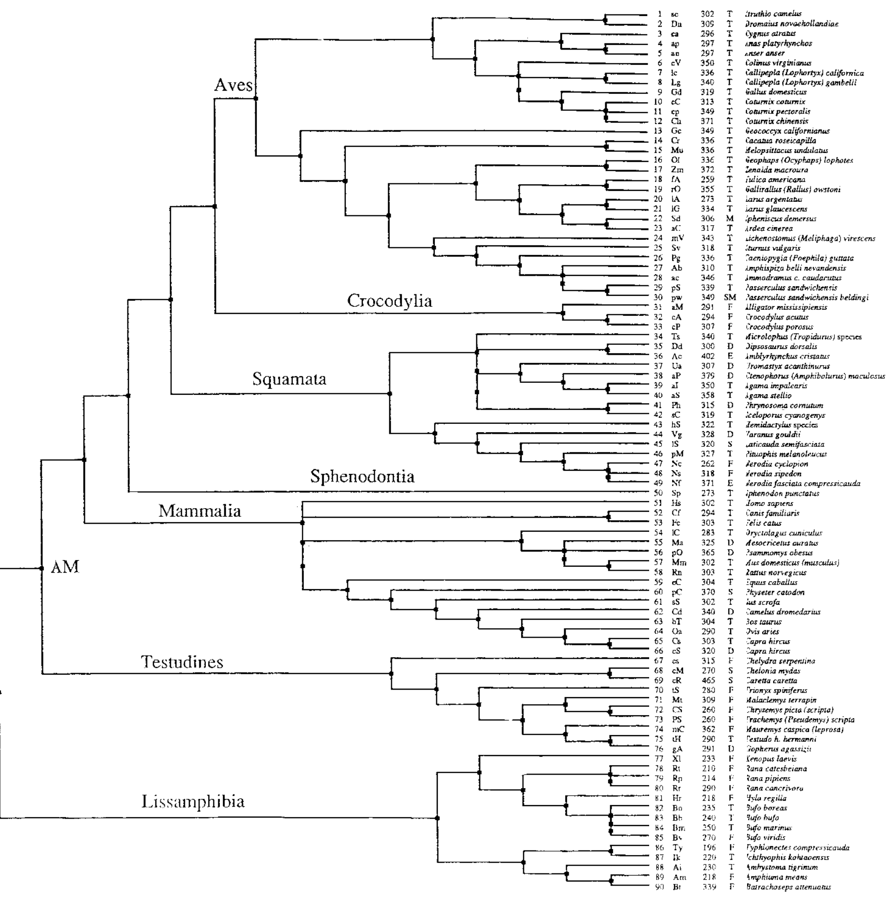
Although many invertebrates conform to the osmolarity of their surroundings, vertebrates typically regulate the osmolarity of internal fluids at levels different from those found in their environment (Bentley, 1971; Kirschner, 1991; Withers, 1992; Martin and Nagy, this volume). All freshwater vertebrates maintain plasma osmolarities higher than that of freshwater. Most marine vertebrates, on the other hand, maintain values lower than that of saltwater, which is typically about 1000 mOsm. Terrestrial vertebrates ingest freshwater, yet maintain plasma osmolarity at values higher than that of freshwater (freshwater fish do not usually drink their medium). Osmoregulation is an energetically costly process that is maintained presumably because of the biochemical and metabolic benefits of osmotic stability, much as thermoregulation and consequent relative thermal stability confers some benefits (Hochachka and Somero, 1984; Withers, 1992).

Even those marine fishes whose total osmolarity is isosmotic with seawater expend energy to regulate internal ion composition at levels that differ from their environment (Evans, 1979).

Although it is but one component of an animal's overall osmoregulatory scheme, plasma osmolarity offers some practical advantages for a broad-scale comparative study such as we discuss. First, it is a relatively simple trait to measure, so the data set available on vertebrate plasma osmolarity is sufficiently large to be interesting, but not so enormous as to be overwhelming. Second, plasma osmolarity is not dependent on body size or temperature, unlike many other physiological properties, such as metabolic rate. This independence simplifies comparisons among species. We stress that many other physiological properties deserve analysis in the context of amniote origins, including locomotor abilities, preferred body temperature, metabolic rate, blood oxygen carrying capacity, diet, and reproductive or life history traits.

Osmoregulatory requirements or abilities appear to play an important role in restricting most organisms to a particular habitat. The great majority of fish, for instance, are either freshwater or marine, and will quickly die if placed in the "wrong" osmotic environment (Holmes and Donaldson, 1969; Evans, 1979). Some animals, however, show osmoregulatory plasticity. "Anadromous" fish (those that move between fresh and sea water), for example, the true eels *Anguilla* and the salmonids *Onchorhynchus* and *Salmo*, undergo a predictable developmental physiological shift that allows

Figure 1; Panel 1. Hypothesis of phylogenetic relationships for 172 extant taxa (mostly different species, but also some subspecies or populations) for which data on plasma osmolarities are available (listed in Appendix 1). The first panel shows tetrapods, and the second shows all remaining vertebrates in our data set. The first column indicates the numerical order from top to bottom. The second column is a two-character identifying code (as required in the computer programs we used). The third column is plasma osmolarity in milliosmoles. The fourth column indicates habitat. Branch lengths in this figure are arbitrary (of the type suggested by Pagel, 1992); for all analyses, however, we assumed that each branch length was equal to one. **AM** indicates the node of interest in this chapter, the ancestor of all amniotes. Note that this tree contains several "soft" polytomies (multifurcations), which reflect uncertainty about relationships; for all of our analyses, however, we have assumed that they are actually hard polytomies (see Discussion). ➔

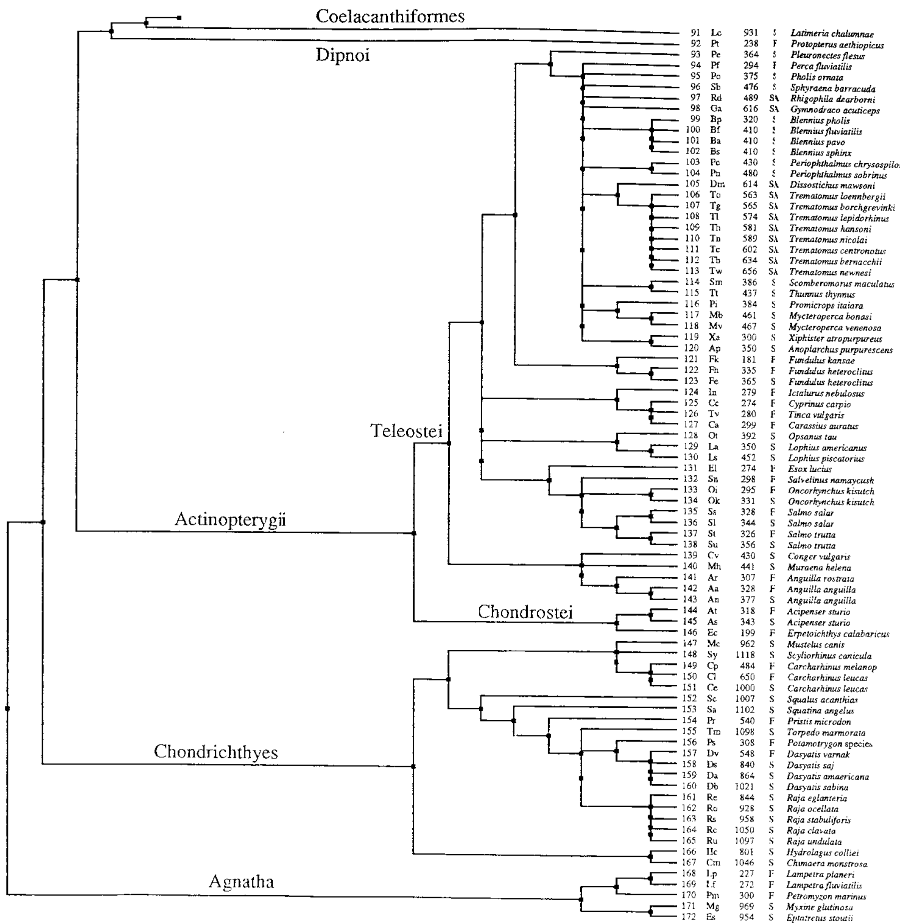


this transition at a particular life cycle stage (Holmes and Donaldson, 1969; Evans, 1979). Usually, external morphology reflects this shift. Some other fish, for example the salt marsh *Fundulus heteroclitus*, are euryhaline and can move readily between marine and freshwater habitats (Bentley, 1971).

A third type of osmoregulatory plasticity is seen in another estuarine animal, the crab-eating frog *Rana cancrivora* (Gordon *et al.*, 1961; tip 80 in Fig. 1). This frog can live in freshwater or marine habitats as an adult and typically consumes marine invertebrates that are isosmotic with seawater. Plasma osmolarity varies in relation to environmental salinity; for example, at 250 mOsm. in the environment, the plasma osmolarity is 340 mOsm, but at 800 mOsm, the plasma osmolarity is 830 mOsm (Gordon *et al.*, 1961; we use a value of 290 mOsm for this species, which represents animals acclimated to freshwater). The frog does this by increasing or decreasing internal concentrations of one molecule, urea, while regulating the other ions and proteins at constant levels, independent of habitat. A similar mechanism is at work in freshwater elasmobranchs, such as *Carcharhinus leucas* (Thorson *et al.*, 1973; tips 150-151 in Fig. 1) and some skates and rays (Holmes and Donaldson, 1969; Withers, 1992). Note that this isosmolarity with seawater is achieved differently from the method of the hagfishes (tips 171-172 in Fig. 1) and marine invertebrates, which retain an ionic composition much more similar to seawater (Withers, 1992).

Finally, a type of osmotic plasticity is seen among some Lissamphibia (Shoemaker *et al.*, 1992) and the desert tortoise *Gopherus agassizii* (Minnich, 1982). During desiccation, these animals may "relax" osmoregulation, allowing plasma osmolarity to increase. The physiological consequences of this are discussed in detail in the chapter by Martin and Nagy (this volume). Presumably, the ability to tolerate wide variations in plasma osmolarity or body

Figure 1; Panel 2. Hypothesis of phylogenetic relationships for 172 extant taxa (mostly different species, but also some subspecies or populations) for which data on plasma osmolarities are available (listed in Appendix 1). This second panel shows all remaining vertebrates in our data set. ➔



hydration has a genetic basis, which is expressed under certain environmental influences.

In summary, plasma osmolarity is a biologically meaningful physiological trait that is regulated by all vertebrates, but in many different ways. As with all aspects of the phenotype, plasma osmolarity of a particular individual (and hence the average value for a set of individuals from a particular species) will depend on both genetic and environmental factors, as well as genotype X environment interaction; it is also affected by short-term acclimation.

Attempts to analyze the evolutionary history of phenotypic traits on a phylogenetic tree presume that the traits are inherited phylogenetically; that is, passed on from ancestors to descendants (Brooks and McLennan, 1991; Harvey and Pagel, 1991; Maddison and Maddison, 1992; Eggleton and Vane-Wright, 1994; Martins, 1996b). Usually we think of genetically-based inheritance (Garland *et al.*, 1992, pp. 29-30), so we implicitly assume that the observed differences among species (e.g., the differences in plasma osmolarities shown in Fig. 1) reflect genetic differences, as opposed to environmental effects. That assumption can only be verified by raising all species under the same environmental conditions prior to measurement of the phenotype. However, a true "common garden" experiment (*sensu* Garland and Adolph, 1991) would be impossible (e.g., raising Antarctic ice fish in a desert environment) for more than a very few, exceptional animals in different clades. Moreover, even short-term acclimation can have strong effects on plasma osmolarities. Thus, in the absence of data from actual common garden experiments, it is unclear whether one should prefer to use plasma osmolarity values taken directly from animals in natural environments, or whether individuals of all species should be acclimated to some set of common conditions before taking measurements. But even acclimation to common conditions would be impossible for the range of species in Figure 1, thus the question is largely academic.

With data that are not derived from animals subject to common rearing and/or acclimation conditions, attempts to reconstruct ancestral plasma osmolarity values may be somewhat misleading. Also, relatively few vertebrates can move between osmotically different habitats (see above), therefore osmoregulatory ability must interact

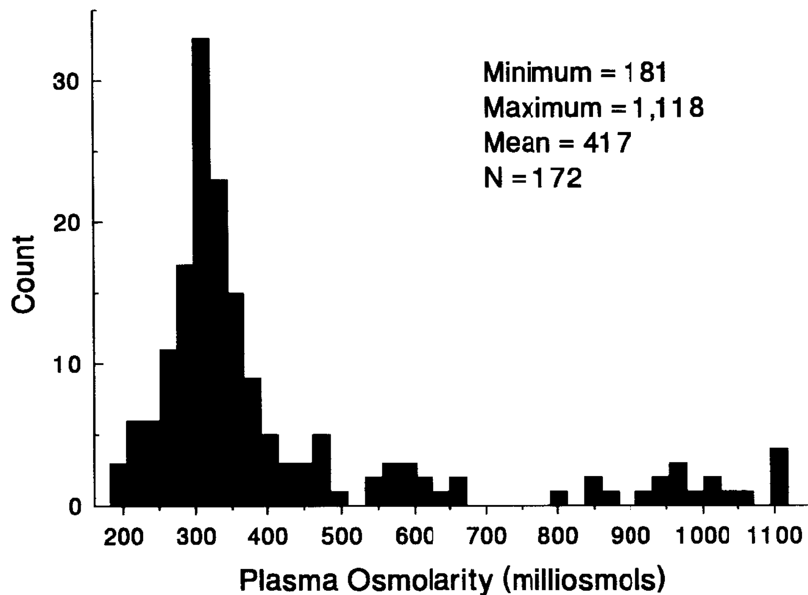


Figure 2. Frequency histogram of plasma osmolarities of 172 vertebrate taxa (data from Fig. 1 and Appendix 1).

with habitat occupancy, which is to some extent a behavioral trait. Also other physiological and biochemical properties can be expected to have coadapted (*sensu* Huey and Bennett, 1987) with plasma osmolarity. That is, natural selection should have favored the correlated evolution of "appropriate" combinations of (1) osmoregulatory "strategies" (e.g., regulating versus conforming), (2) the various physiological properties that are sensitive to plasma osmolarity, and (3) habitat occupancy (including the behavioral aspects of habitat "preference" or "selection"). Indeed, within various clades, plasma osmolarity seems to correlate with habitat type (for some formal statistical tests, see section below on "Choosing Appropriate Models ..."). This is understandable, particularly for aquatic organisms. Body fluids hypertonic to the environment can

lead to osmotic water gain, with the need to eliminate large amounts of water while retaining internal solute concentrations; animals with relatively hypotonic body fluids face the opposite problem of water loss and solute gain (Withers, 1992). Thus, one might intuitively expect higher plasma osmolarities in marine organisms than in freshwater organisms, because such a pattern would reduce the energetic cost of osmoregulation.

To reiterate, plasma osmolarity is perhaps best considered as one part of a complex, multivariate phenotype, involving aspects of biochemistry, physiology, morphology, and behavior. If so, then the analyses presented herein may be seriously inadequate; nonetheless, we view them as a useful first step in understanding the evolutionary physiology of osmoregulation. [Another issue that needs further study is the definition and identification of homology with respect to quantitative characters, such as plasma osmolarity (see Burggren and Bemis, 1990; Eggleton and Vane-Wright, 1994; Zelditch *et al.*, 1995; Martins, 1996b; Schultz *et al.*, 1996)].

With the foregoing caveats in mind, we searched the literature for data on plasma osmolarity. When more than one measurement was available for a particular species, usually the most recent measurement was used. Review papers and book chapters were consulted for consensus if multiple values were available. Although we have reported all data as osmolarity, some, particularly the older numbers, were actually measured as freezing point depression, from which we calculated osmolarity. For experiments that altered the internal fluid composition or osmolarity, the control values were used. Euryhaline animals may have more than one value, depending on the habitat; the "normal" or more common habitat for each of these is presented, or, where two habitats are reported, values reflect plasma osmolarities of animals taken directly from, or acclimated to, each habitat.

A caution applies to the higher osmolarity values (greater than 900 mOsm) reported for some of the marine fishes. The standard osmolarity of seawater is 1000 mOsm., but for fishes taken from the field, osmolarity may have been different at that location. Seawater osmolarity was reported as low as 935 and as high as 1108 mOsm in some of these studies. With the exception of hagfish, those marine vertebrates with osmolarity very close to seawater have plasma ion

concentrations of most substances that are very similar to those of freshwater animals, along with a larger amount of some osmolyte such as urea or trimethylamine oxide (Withers, 1992); together, these ions and osmolytes allow a match to the environmental osmolarity. Therefore, small differences between the highest values reported in Figure 1 may indicate a conforming response to the immediate marine conditions, rather than an inherent difference in osmoregulation. We have chosen to present the data in the osmolarity units for consistency, although a ratio of the measured plasma osmolarity to the measured seawater from which the fish was taken would in all of these cases be very close to unity.

The "habitat" listed in the fourth column of Figure 1 represents information in the original source, general references, or our knowledge of the animals. In some cases habitat is ambiguous, for example for birds that often occur in deserts but migrate and/or also occur in more mesic situations. Similar caveats apply to some of the lissamphibians, which can be difficult to categorize as terrestrial or freshwater. We should also note that the same habitat, such as freshwater, probably presents very different biological challenges for animals such as snakes (e.g., *Nerodia*) with impermeable integuments as compared with toads (e.g., *Bufo*), with much more permeable integuments or fishes of various types. We would encourage future workers to better define "habitat," including the possibility of describing aspects of habitat or ecology on a quantitative or semi-quantitative scale (see also Garland *et al.*, 1993, pp. 283-284).

PHYLOGENETIC RELATIONSHIPS OF VERTEBRATES

The method for reconstructing the plasma osmolarity of the ancestral amniote that we discuss, squared-change parsimony, requires an estimate of the phylogenetic relationships of all taxa included in the analysis. This same requirement applies to all of the "comparative methods" in current usage (Brooks and McLennan, 1991; Harvey and Pagel, 1991; Maddison and Maddison, 1992; Garland *et al.*, 1993; Eggleton and Vane-Wright, 1994; Garland and Adolph, 1994; Martins, 1996b).

Agreement as to the relationships of all 172 taxa shown in Figure 1 does not exist. We have, therefore, synthesized the available literature in an informal way to provide a composite estimate of the phylogeny (as in the Appendix of Garland *et al.*, 1993). Readers should note that we use the term "composite" specifically because it does not have a formal meaning in systematic biology, unlike such words as "consensus." Composite is used, in its simple dictionary sense, to mean "made up of distinct parts or elements; compounded; not simple in structure" (Oxford English Dictionary, 1971). The term has been used in this way several times in recent papers (e.g., Garland and Janis, 1993; Garland *et al.*, 1993; Purvis, 1995; Pyron, 1996). Our main point in this chapter is to illustrate existing methodology; thus, we invite future researchers to reanalyze the data presented herein as new and better phylogenetic information, data on plasma osmolarities, and analytical methodologies become available. We took phylogenetic relationships of the main vertebrate clades from Ridley (1993), Pough *et al.* (1996), and references therein. Most of these relationships are widely accepted, although the order of the splits within the groups lungfish-coelacanths-tetrapods, and turtles-mammals-squamates, are controversial (e.g., on the position of turtles, see Lee, 1993, 1995 versus Laurin and Reisz, 1995). Under Discussion, we give an example of the effects on our results of changing some of these topological relationships.

Within fishes (Agnatha, Chondrichthyes, Actinopterygii), we followed Nelson's (1994) classification, with additions from de Carvalho (1996) for elasmobranchs. For lissamphibians, we followed Hay *et al.* (1995), Hedges and Maxson (1993), and Larson and Dimmick (1993). For Testudines, we followed Gaffney and Meylan (1988). For squamates, we followed Garland (1994), with additions from Frost and Etheridge (1989) and Greer (1989) for "lizards" and Heise *et al.* (1995) for snakes. For mammals, we followed Garland *et al.* (1993), with additional information from Miyamoto and Goodman (1986), Novacek (1992), and Milinkovitch (1995). For Aves, we followed Sibley and Ahlquist (1990), with additions from Leeton *et al.* (1994) and Bleiweiss *et al.* (1995). For all the tree–in the absence of contrary information–we assumed families and genera (e.g., *Blennius* and *Trematomus*) to be monophyletic, which allowed us to "resolve"

some relationships within higher-order groups (see Purvis, 1995). In some cases, we could have resolved particular polytomies with a phenetic criterion, under the assumption that species that are more similarly phenetically (e.g., with respect to plasma osmolarity) are also more closely related (Harvey and Pagel, 1991, p. 157; Pagel, 1992, p. 441). We have not done so because of concern that it might bias our final conclusions (see de Queiroz, 1996).

We used the above references and Barbadillo (1989), Corbet and Hill (1991), Conant and Collins (1991), Frost (1992), Christidis and Boles (1993), Monroe and Sibley (1993), Wilson and Reeder (1993), and Zug (1993) to check species names for accuracy and consistency with the latest taxonomies.

CHOOSING APPROPRIATE MODELS FROM EXTANT FORMS

> "Parsimony and other assumptions are helpful guiding principles; they should not be accepted uncritically, however, but should be combined with our knowledge of the general biology of the animals we study." (Ryan, 1996, p. 7)

In general, one should use all available evidence when tackling a given problem. This notion motivates the "total evidence" approach to phylogenetic inference (e.g., Eernissee and Kluge, 1993). Thus, one could use all available data on plasma osmolarities of vertebrates (as shown in Fig. 1) to infer the value of the ancestral amniote. But doing so would not actually use "all available evidence." For example, Table 1 indicates that plasma osmolarity seems to vary in relation to phylogeny (and habitat–see below). In other words, variation in plasma osmolarity does not seem to be distributed randomly across the tips of the phylogenetic tree. Instead, species within certain clades (lineages) seem to resemble each other more closely than species from some other part of the tree. Members of the Nototheniidae [Antarctic ice fishes (tips 105-113 in Fig. 1)], for instance, have relatively high plasma osmolarities (mean = 597.6, min = 563, max = 656) as compared with most other Actinopterygii.

Resemblance of closely related species is expected (Harvey and Pagel, 1991), even under a simple Brownian motion model of

Table 1. Mean (+/- SE), Range, and Sample Size, of Plasma Osmolarity values (mOsm) of the Vertebrate Clades, Classified by Habitat (Data from Appendix 1; total $N = 172$).

	Saltwater	Saltwater, Antarctic	Freshwater	Estuarine	Terrestrial	Desert
Aves					326 ± 26.6 259-372 30	
Crocodylia			297 ± 8.5 291-307 3			
Squamata	320 1		290 ± 39.6 262-318 2	386 ± 21.9 371-402 2	336 ± 15.9 319-358 6	326 ± 31.5 300-379 5
Sphenodontia (tuatara)					273 1	
Mammalia	370 1				299 ± 7.0 283-304 11	338 ± 20.2 320-365 4
Testudines	368 ± 137.9 270-465 2		298 ± 39.3 260-362 6		290 1	291 1
Lissamphibia			243 ± 47.0 196-339 9		235 ± 11.2 220-250 5	
Coelacanth-iformes	931 1					
Dipnoi (lungfish)			238 1			
Actinopterygii	394 ± 50.4 300-480 27	589 ± 44.3 489-656 11	288 ± 43.4 181-335 16			
Chondrichthyes	983 ± 103.4 801-1118 16		506 ± 125.8 308-650 5			
Cephalaspido-morphi			266 ± 36.8 227-300 3			
Myxini	961 ± 10.6 954-969 2					

Here, the penguin, *Spheniscus demersus*, and the sparrow *Passerculus sandwichensis beldingi*, have been classified as terrestrial. The two animals classified as estuarine are the Galapagos marine iguana (*Amblyrhynchus cristatus*) and the water snake (*Nerodia fasciata compressicauda*). Both of these reptiles may spend time in seawater and fresh water, so may have osmotic loads unlike those of typical terrestrial or freshwater animals. For the squared-change parsimony analyses described in the text, we emphasize the 63 taxa classified here as desert ($N = 10$) or terrestrial ($N = 53$ excluding the penguin).

character evolution (see Appendix 3 section on "Computer Simulations to Test ..."). Thus, statistical tests comparing average values of different clades must incorporate phylogenetic information (Garland *et al.*, 1993). To illustrate that plasma osmolarity is not distributed randomly across the phylogeny for our 172 taxa, we performed a phylogenetic analysis of variance by use of computer-simulated null distributions, as described in Garland *et al.* (1993; see also Martin and Clobert, 1996; Reynolds and Lee, 1996; Harris and Steudel, 1997). For simplicity, we just compared the four clades with a reasonably large number of "Terrestrial" members, as indicated in Table 1 (Aves, N = 30; Squamata, N = 6; Mammalia, N = 11; Lissamphibia, N = 5). Inspection of the summary statistics shown in Table 1 suggests that these four clades differ in average plasma osmolarity. Indeed, a conventional one-way ANOVA yields an F statistic of 28.90, which is highly significant with 3 and 48 degrees of freedom (P < 0.0001). This P value, however, cannot be trusted, because it is based on comparison with a conventional F distribution, which effectively assumes that species' plasma osmolarities constitute independent and identically distributed values. In other words, the conventional F distribution is constructed under the assumption of a "star" phylogeny (Garland *et al.*, 1993: see Discussion below). As can be seen from Figure 1, the terrestrial birds (tips 1-30), squamates (subset of the tips 34 - 49), mammals (subset of tips 51-66), and amphibians (subset of tips 77-90) are instead related in a strongly hierarchical fashion.

To account for phylogenetic relationships, we can use Monte Carlo computer simulations to construct a phylogenetically correct distribution of F statistics (Garland *et al.*, 1993). We did so by pruning the tree shown in Figure 1 to include only the 52 taxa of interest and set all branch lengths equal to unity (except for those collapsed in polytomies). We then used the PDSIMUL program to simulate the evolution of plasma osmolarity. We used starting and ending values of 365 mOsm, expected variances at the tips equal to the variance of the real data (1253 mOsm), limits of 181 and 1118 mOsm, and a Brownian motion model. For each of 1000 simulated data sets, we performed a one-way ANOVA with the program PDSINGLE. We then ordered the F values from lowest to highest and determined how

they compared with the corresponding F value for the real data set of 52 species. Only 33 of the 1000 F values for simulated data were greater than the real F of 28.90, thus we conclude that the four clades actually do differ significantly (i.e., P < 0.05) in average plasma osmolarity.

In addition to variation in relation to clade, Table 1 suggests that plasma osmolarity may vary in relation to ecology or habitat. Within the Actinopterygii, for example, the 27 saltwater forms (mean = 394) tend to have higher values than do the 16 freshwater forms (mean = 288). Similarly, within the Chondrichthyes, saltwater forms (mean = 983, N = 16) also tend to have higher values than do freshwater forms (mean = 506, N = 5). Within the Mammalia, desert forms (mean = 338, N = 4) tend to have higher values than do non-desert ("Terrestrial") forms (mean = 299, N = 11).

To illustrate a phylogenetically based statistical test for habitat differences, we can again use computer simulation. Consider the 54 Actinopterygii. One-way ANOVA of the plasma osmolarities of saltwater, saltwater Antarctic, and freshwater forms yields F = 133.05, which is nominally highly significant with 2 and 51 df (P < 0.0001). We performed simulations using the same parameters as listed above. For the 1000 simulated data sets, F statistics ranged from 0.004 to 34.27. Thus, not one was greater that the F for the real data, and we conclude that habitat differences are statistically significant at P < 0.001 (this P value could be even smaller, but we only analyzed 1,000 simulated data sets). We could also perform a two-way analysis of variance, with factors of clade and habitat, but we have not done this for simplicity.

The foregoing differences among cladistically or ecologically defined groups make biological sense. Saltwater forms, for example, must maintain relatively high osmolarities because their aqueous environment contains much higher concentrations of ions than does freshwater. Otherwise, they would tend to lose water to the environment (unless they had impermeable integuments) and hence dehydrate (Withers, 1992). The high plasma osmolarities of the Antarctic ice fishes (tips 105-113 in Fig. 1) prevent freezing in sea water that falls below zero degrees Celsius and contains ice (Dobbs and DeVries, 1975; Withers, 1992; Eastman, 1993). This and some

other associations between plasma osmolarity and ecology almost certainly represent evolutionary adaptations, that is, genetically based phenotypes that are the result of past and/or current natural selection.

If the characteristic of interest tends to vary in relation to either phylogeny or ecology, then reconstructing the value of an ancestral form should be facilitated by use of only those forms that are "similar" to the ancestral form. With respect to phylogenetic similarity, this would mean an emphasis on closely related taxa. With respect to ecology, we should focus on forms that live in habitats similar to that of the ancestral form we are trying to reconstruct.

The foregoing two principles seem intuitively obvious. In practice, however, applying them may be complicated. First, consider phylogenetic relatedness. To reconstruct the value of a particular node on a phylogenetic tree, we obviously should begin by considering all available data for descendant taxa. Thus, to reconstruct the plasma osmolarity of the hypothetical organism at the node labeled **AM** in Figure 1 (i.e., the "mother" of all amniotes), we should begin by considering all available data for Testudines, Mammalia, Sphenodontida, Squamata, Crocodylia, and Aves (these being the only amniotes for which data on plasma osmolarity are available). But we should also consider "outgroups" to the amniote clade (see also Huey, 1987). The closest outgroup clade in our data set is the Lissamphibia (see Laurin and Reisz, this volume), for which we have data on 14 species (including some Anura, Gymnophiona, and Urodela). But we can also consider additional outgroups; the next closest cladistically would be the coelacanth and then the lungfish. Should we stop here, or should we include further outgroups? If the latter is the case then we can go to the rest of the bony fishes, then the Chondrichthyes, and finally the jawless fishes (lamprey and hagfish). Should we include all of these outgroups or only a subset?

Now consider habitat type (Table 1). If we knew the habitat of the ancestral form we are trying to reconstruct, then an argument could be made to include in our analyses only those forms that occur in similar habitats. As discussed elsewhere (Berman *et al.*, this volume; Martin and Nagy, this volume), it is likely that the first amniote was fairly terrestrial but had access to freshwater. The adults, we presume, were terrestrial before the eggs were. Fossil evidence for these

transitional forms seems to come mostly from terrestrial rather than aquatic sediments (Carroll, 1988; Berman *et al.*, 1992; Lombard and Sumida, 1992; Berman *et al.*, this volume; Hotton *et al.*, this volume). Although the habitat of the ancestral amniote is controversial (see Sumida, this volume), no one has suggested that it lived in Antarctic seawater or was freeze-tolerant. Thus, we might reasonably exclude the nine Nototheniidae (tips 105-113 in Fig. 1) before trying to reconstruct the ancestral amniote's plasma osmolarity.

If a fair amount of data are available, as in the present example of plasma osmolarities (N = 172), then the practitioner probably will be faced with tough decisions about which taxa should be included in attempts to reconstruct an ancestral phenotype. Some simple guidelines are possible (see also Huey, 1987; Burggren and Bemis, 1990). First, one should emphasize close relatives (including some outgroups) and forms thought to be ecologically similar to the extinct form of interest (e.g., based on paleontological information). Second, one should perform several analyses, using the entire data set as well as different subsets, and examine results for consistency: check for taxa that have a large influence on the reconstructed value. An important area for future research on how best to reconstruct ancestral values will be the development of more complicated parsimony-like procedures that allow incorporation of additional information, such as apparent evolutionary shifts in relation to habitat or phylogeny. In their absence, we will, for illustrative purposes, analyze our data set as several different subsets, but concentrate on a subset of 63 terrestrial and desert taxa (see Table 1 and below).

Finally, we emphasize that the original investigators did not collect the present 172 data points for the purpose of reconstructing the plasma osmolarity of the ancestral amniote. Sampling of the vertebrate lineages is obviously very incomplete and uneven, and in no sense should the available data set be considered optimal for our purposes. The bony fishes alone comprise perhaps 20,000 extant species, of which our sample represents but 0.3%. We hope, therefore, that our analyses will prompt other workers to measure additional taxa that would help to refine our estimates of ancestral values and to more thoroughly study covariation between plasma osmolarity and phylogeny or ecology.

Table 2. Effects of Deleting Certain Terminal Taxa on Squared-change Parsimony Reconstructions of Plasma Osmolarity (milliosmols) at Interior Nodes on the Phylogenetic Tree Shown in Figure 1.

Taxa Included	*N*	Simple nonphylogenetic mean	Root node	Ancestor of all amniotes
All vertebrates	172	417	644	365
No "ordinary" saltwater forms	122	358	429	293
Only terrestrial and desert	63	314	263	280#
All amniotes (tips 1-76 in Fig. 1)	76	321	310	310*
Only terrestrial amniotes	58	321	294	294*

For comparison, simple means (i.e., ordinary, nonphylogenetic averages) are also shown. The topology used for analyses was as in Figure 1, but all branch lengths were set equal to unity.
As discussed in the text, we emphasize this subset of 63 taxa as the preferred models for estimation of the plasma osmolarity of the ancestral amniote (node AM in Fig. 1).
* These values are the same as those under root node column because no "outgroups" are present in the reduced phylogeny; thus, this node *is* the root node.

RECONSTRUCTING PLASMA OSMOLARITY AT THE ORIGIN OF AMNIOTES

We now turn to analysis of the data at hand. We use squared-change parsimony to reconstruct the plasma osmolarity of the ancestor of all amniotes, represented by node **AM** in Figure 1. (For comparative purposes, in the next section we also present results for the ancestor of all vertebrates, the basal node in Fig. 1.) For all analyses, we assume that each branch segment is of equal length. These branch lengths are arbitrary, but a justification of their use is presented under Discussion.

Considering all 172 terminal taxa, the squared-change parsimony estimate for the ancestor of all amniotes (node **AM** in Fig. 1) is 365 mOsm. This value is lower than the simple mean (i.e., the ordinary, nonphylogenetic mean, computed in the usual way) of 417

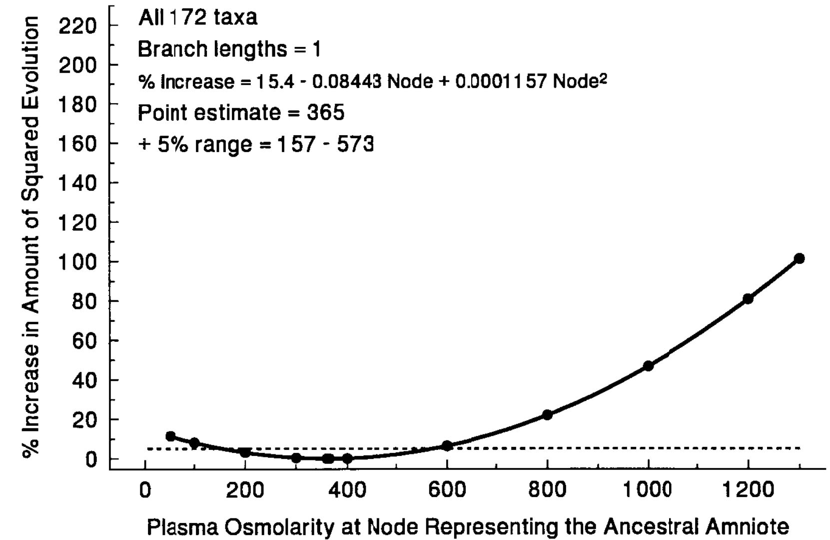


Figure 3. Consequences of fixing nodal value for ancestral amniote at values other than that indicated by unconstrained squared-change parsimony; all 172 taxa. Horizontal dashed line indicates 5% increase in the sum of squared evolutionary changes, compared with the amount required for the unconstrained squared-change parsimony reconstructions.

(Table 2). Figure 3 shows the effect of changing the nodal value for the ancestral amniote to something other than the unconstrained squared-change parsimony reconstruction of 365 mOsm. Any other nodal value requires there to have been more squared change summed over the entire phylogeny. As a heuristic gauge of the uncertainty in this reconstruction (see Appendix 3), we point out the range of nodal values over which this increase is 5%: 157-573 (note that 5% is an arbitrary value).

Effects of deleting various taxa are shown in Table 2. For example, excluding "ordinary" saltwater forms but retaining the Antarctic saltwater forms (leaving N = 122), the point estimate for the

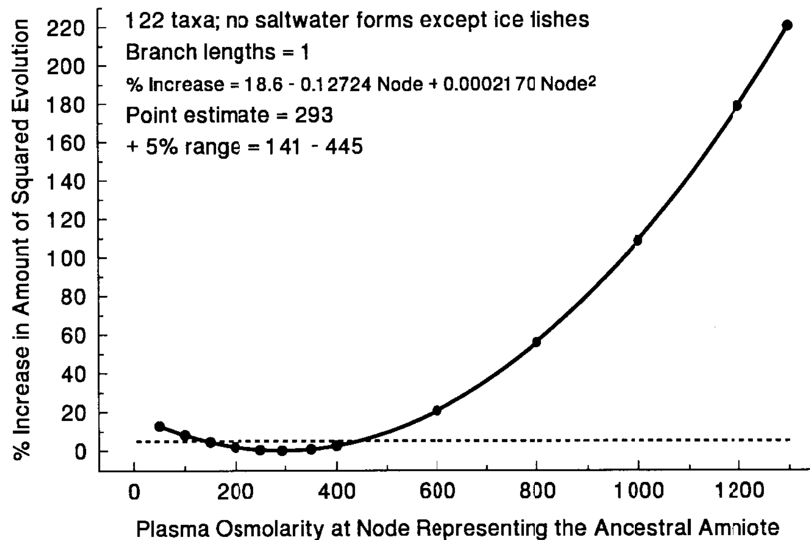


Figure 4. Consequences of fixing nodal value for ancestral amniote at values other than that indicated by unconstrained squared-change parsimony; subset of 122 taxa.

ancestral amniote decreases to 293. The +5% range also decreases to 141-445 (Fig. 4). Considering only the 76 amniotes in our data set, the squared-change parsimony estimate for ancestral amniote is reduced to 310 mOsm, which is only slightly lower than the nonphylogenetic mean of 321 (Table 2).

As discussed in the previous section and in the chapter by Martin and Nagy (this volume), the best "models" for the ancestral amniote may be terrestrial forms. In the remainder of our analyses, therefore, we concentrate on a subset of 63 taxa including only the "Terrestrial" and "Desert" forms of Table 1 (but also excluding the penguin, *Spheniscus demersus*). For this favored subset of 63 terrestrial plus desert taxa, Figure 5 indicates a point estimate of 280 with a +5% range of only 254-306. Thus, as we have restricted the

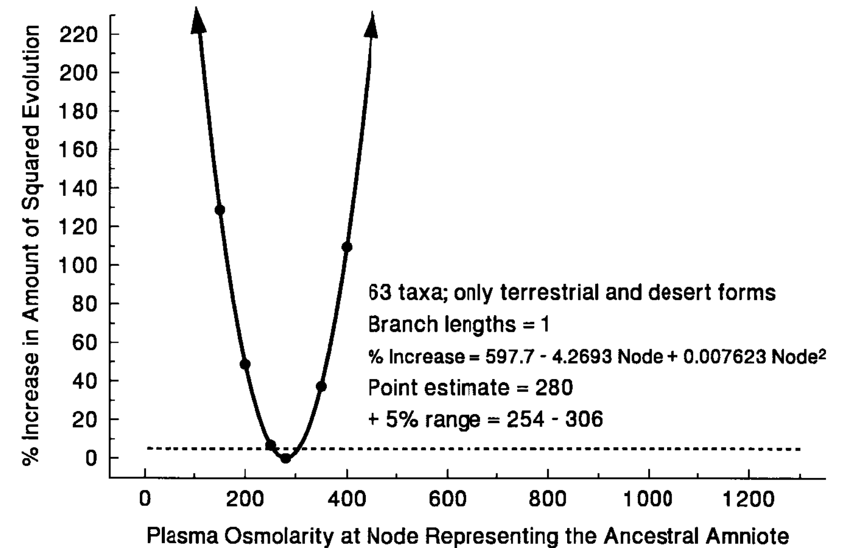


Figure 5. Consequences of fixing nodal value for ancestral amniote at values other than that indicated by unconstrained squared-change parsimony; preferred subset of 63 terrestrial taxa (see text).

taxa under consideration, eliminating those that (as argued above) are not thought to be good models for the ancestral amniote, the point estimate has changed somewhat (decrease of 23.3%), but the main effect has been to decrease dramatically the +5% range (by 87.5%).

Still considering only the 63 taxa, a delete-one jackknife yields an approximate standard error of 6 for the value reconstructed at the ancestral amniote. If the appropriate degrees of freedom were about 60, then we would use a t-statistic of 2.0 for $\alpha = 0.05$. This would yield a nominal 95% confidence interval of 268-292. However, because the taxa that we are jackknifing are related in a strongly hierarchical fashion, they cannot be considered as independent data points. Thus, we can only offer this range as a heuristic (see Appendix

3). Still, it is interesting to note that both of these ranges exclude the simple nonphylogenetic mean of 314 mOsm for the 63 terrestrial plus desert taxa.

RECONSTRUCTING PLASMA OSMOLARITY AT THE ORIGIN OF VERTEBRATES

Although estimation of the plasma osmolarity of the ancestor of all vertebrates (i.e., the root node of Fig. 1) is not the focus of this chapter, we will comment briefly on the value reconstructed by squared-change parsimony. As noted in Appendix 3, for the root node and only the root node, this value is identical to the value computed by Felsenstein's (1985) independent contrasts procedure. This is convenient, because a formal analytical procedure for putting a 95% confidence interval on the root node constructed by independent contrasts has recently been developed (T. Garland, Jr., and A. R. Ives, unpublished). Considering all 172 terminal taxa, the squared-change parsimony estimate for the root node of the entire phylogeny is 644 mOsm with a 95% confidence interval of 467 - 821 mOsm [under the assumption, as elsewhere in our computations, that all of the polytomies in Figure 1 are actually hard (see Discussion)]. These values can be compared with the simple (nonphylogenetic) mean of 417 mOsm and a (nonphylogenetic) confidence interval of 383-450 mOsm. Note the lack of overlap of the 95% confidence intervals!

Both squared-change parsimony and independent contrasts (Felsenstein, 1985; see Appendix 3) effectively assume Brownian motion character evolution. This assumption also holds when either method is applied to a star phylogeny, in which case the estimate at the root node would be the same as the nonphylogenetic mean. One component of the Brownian motion assumption is that no directional trends in character evolution have occurred (see Appendix 3). These assumptions, and the others inherent to comparative analyses (see Discussion and Appendix 3), should be kept in mind as we consider reconstructed values and gauges of their uncertainty, including formal confidence intervals.

Inspection of Table 1 or Figure 1 indicates that the only living vertebrates with values of approximately 644 milliosmols are some

freshwater Chondrichthyes and the Antarctic ice fishes. To our knowledge, no one has proposed that the ancestor of all vertebrates lived in subzero seawater. Thus, we would reject the inference that the ancestral vertebrate was like extant ice fishes. Eliminating that possibility leaves us with the possible interpretation that the ancestral vertebrate osmoregulated like extant freshwater Chondrichthyes, a clade that is predominately marine. Thorough consideration of this hypothesis is beyond our scope, but is does constitute an example of how the analytical approach we discuss can provide new perspectives on old problems. Existing fossil and morphological/physiological evidence is contradictory as to whether the earliest vertebrates were freshwater or marine (Wake, 1979). If this issue could be settled, then we could instead focus on estimates derived from analyses using only freshwater or marine forms. For example, including only the 45 freshwater taxa, the simple mean is 303 mOsm and the squared-change parsimony reconstruction is 335 mOsm (with all branch lengths set equal to unity). One might also argue that the freshwater Chondrichthyes are specialized (derived) and hence “red herrings” (Gans, 1970). If the five freshwater Chondrichthyes are pruned from the tree (so N = 40), the values are reduced to 277 and 272 mOsm, respectively. All of these are much lower than the corresponding values for the entire 172 taxa (first line of Table 2), and well below the lower bounds of the 95% confidence intervals given above.

In closing, note that we have analyzed the raw plasma osmolarity values. For all 172 taxa, these values show a highly skewed distribution (Fig. 2), although the skew is much less for the subset of 63 taxa that we emphasized in the previous section. When skewness is present, performance of squared-change parsimony reconstructions might be improved by transformation of the data prior to analysis (followed by back-transformation to the original scale of measurement). We see this as an interesting area for future study (see also Díaz-Uriarte and Garland, 1996).

DISCUSSION

Phylogenetic versus Nonphylogenetic Estimates of Ancestral Values

For the subset of 63 terrestrial and desert taxa that we have emphasized as preferred "models" for the ancestral amniote, the squared-change parsimony algorithm reconstructs a value of 280 for the plasma osmolarity of the node corresponding to the ancestor of all amniotes. Two heuristic indicators of uncertainty in this reconstructed value are 254-306 (the range that causes a +5% increase in the sum of squared changes: see Fig. 5) and 268-292 (the nominal 95% confidence interval obtained by jackknifing). The simple (nonphylogenetic) mean for the 63 taxa is 314, which falls outside of either of the foregoing uncertainty ranges. Our limited simulation results (see above) and numerous previous studies of phylogenetically based comparative methods demonstrate that phylogenetic approaches are almost always superior to their nonphylogenetic alternatives (Grafen, 1989; Brooks and McLennan, 1991; Harvey and Pagel, 1991; Martins and Garland, 1991; Maddison and Maddison, 1992; Garland *et al.*, 1993; Eggleton and Vane-Wright, 1994; Garland and Adolph, 1994; Purvis *et al.*, 1994; Díaz-Uriarte and Garland, 1996; Martins, 1996a,b). Thus, although the foregoing ranges are only heuristic, they do suggest that we have gained some degree of accuracy and precision by employing the more complicated phylogenetic estimation procedures.

Let us make clear the relationship between squared-change parsimony reconstructions on a phylogenetic tree and the alternative of ignoring phylogenetic relationships. Imagine that we wished, for whatever perverse reason, to ignore the available phylogenetic information. This is generally equivalent to assuming that the phylogenetic relationships are adequately represented as a single hard polytomy, or "star" phylogeny, with all terminal taxa at the end of equal-length branches that radiate from a single basal node (e.g., Fig. 2 in Garland and Carter, 1994). If we were to apply squared-change parsimony to such a phylogeny, then only a single node (the root) would exist to be estimated, and its value would be estimated as the mean of all adjacent nodes, which would be all of the tips. Thus, the value would be "reconstructed" as the simple mean of the tip values.

Therefore, a conventional analysis, ignoring phylogeny, might estimate the ancestral value as a simple mean of the values for all extant taxa, and this would be exactly equivalent to a squared-change parsimony reconstruction on a star phylogeny. This point has also been made with respect to other phylogenetically based statistical methods (e.g., see Garland *et al.*, 1993; Purvis and Garland, 1993; Garland and Adolph, 1994, p. 822). Analysis of the plasma osmolarity data presented herein (see Table 2), the made-up example in Figure A1, and many other examples in the literature (e.g., Huey and Bennett, 1987; Chevalier, 1991) demonstrate that ancestral values reconstructed with reference to phylogenetic relationships can be quite different from the simple mean of the tip values. Differences are also apparent in the computer simulations described in Appendix 3; for instance, the correlation between the squared-change parsimony value for the root node and the simple mean was only 0.471 under simple Brownian motion and 0.456 under the model simulating an evolutionary trend with limits. The squared-change parsimony reconstructions make use of more information (the estimated phylogenetic relationships) and are arguably "better" in every sense of the word. Thus, they should be used whenever any phylogenetic information is available.

Assumptions of the Squared-Change Parsimony Method

All methods of analyzing comparative data make various assumptions that may be invalid. According to Martins and Hansen (1996, p. 40), "the sum of squared changes algorithm assumes that the distribution of evolutionary changes which gives the smallest sum of squared changes is the most likely to be true"; a second assumption is that "within-species variation does not exist or is negligible in comparison to the level of among-species variability." A third assumption is that the measured differences among the species' mean values (the plasma osmolarities being analyzed) are assumed to reflect genetic differences among the species. This would only be the case if all species had been bred and raised under the same environmental conditions–a common garden experiment (Garland and Adolph, 1991; Garland *et al.*, 1992). This is rarely done for broad-scale comparative studies, let alone for the 172 taxa included in Figure 1. In any case, this third assumption applies to *all* analyses of comparative data, phylogenetic or not.

A fourth assumption is that the phylogeny is known without error. This is unlikely ever to be the case for a real data set involving more than a few taxa. "Ignoring" phylogeny and using conventional statistical estimators does not avoid this problem, however, because it is simply the special case of assuming a star phylogeny with equal-length branches. A star phylogeny is absolutely known to be wrong for most sets of organisms that might be studied; hence, ignoring phylogeny is indefensible. Whatever phylogenetic information is available is likely to be closer to reflecting the truth than is a star, therefore it can and should be used to advantage (e.g., Grafen, 1989; Purvis and Garland, 1993; Losos, 1994; Purvis *et al.*, 1994; Martins, 1996c).

Uncertainty about Phylogenetic Topology

Some ways of dealing with topological uncertainty have been discussed for some other phylogenetic comparative methods (e.g., Garland *et al.*, 1993; Purvis and Garland, 1993; Losos, 1994; Martins, 1996c). Here, we have simply represented obvious topological uncertainty as unresolved nodes (see Fig. 1). For performing the iterative squared-change parsimony algorithm, we have treated these as "hard" polytomies (reflecting true, simultaneous multiway speciation events) rather than "soft" polytomies (reflecting lack of knowledge about what is actually a fully bifurcating topology). In fact, most of the polytomies in Figure 1 actually are soft. However, ways of performing squared-change parsimony computations to account for this uncertainty have not yet been developed. If and when they are developed, the result will almost certainly be to decrease the "confidence" we have in the reconstruction for any particular node.

Some of the phylogenetic relationships depicted in Figure 1 are controversial. An informal way of dealing with this uncertainty would be to redo the analysis with all, or a large number of, the possible alternatives that have been proposed (cf. Losos, 1994). For example, considering the major vertebrate lineages, one could repeat the analysis with alternative arrangements of lungfish-coelacanths-tetrapods and/or testudines-mammals-squamates. Doing so can become quite laborious if many alternatives are considered. For illustrative purposes, we have simply redone our analysis for one such case, switching the position of the lungfish (*Protopterus*) and the

coelacanth (*Latimeria*). This particular switch would seem to have the potential for a major effect, because these lineages are only two or three nodes removed from the node we wish to estimate. For all 172 taxa, the topology of Figure 1, and all branch lengths set equal to one, the squared-change parsimony estimate for the ancestral amniote is 365 mOsm, whereas the rearrangement yields a value of 322 mOsm. This effect occurs because the lungfish, which inhabits freshwater, has a much lower plasma osmolarity (238 mOsm) than does the coelacanth (931 mOsm), which inhabits saltwater (Thomson, 1991). Nevertheless, the lower nodal estimate is still well within the +5% range shown in Figure 3 (157-573 mOsm). Moreover, neither of these organisms is retained in our preferred subset of 63 taxa (as used to produce Fig. 5).

Uncertainty About Phylogenetic Branch Lengths

Another assumption of the squared-change parsimony procedure is that the branch lengths are known without error in units of (or proportional to) expected variance of evolutionary change for the character of interest. For expediency, we have simply assumed that all of the branch segments are of equal length, which corresponds to a "speciational" model of character evolution (as in Martins and Garland, 1991; Díaz-Uriarte and Garland, 1996).

As a partial test of the adequacy of our branch lengths, we performed the diagnostic check described by Garland et al. (1992; see also Garland et al., 1991; Díaz-Uriarte and Garland, 1996), which was originally intended for Felsenstein's (1985) method of phylogenetically independent contrasts. Both independent contrasts and squared-change parsimony effectively assume Brownian motion character evolution, thus it seems appropriate to use this diagnostic for the latter as well. For all 172 taxa, the correlation of the absolute values of the standardized contrasts with their standard deviations was -0.056 for all branch lengths set equal. This weak and statistically nonsignificant correlation suggests that the branch lengths are adequate for analyses

Another set of arbitrary branch lengths, corresponding to those depicted in Figure 1, was suggested by Pagel (1992). These branch lengths are constructed by setting all internode branch segments equal to a length of one and then making all branches leading to terminal

taxa line up evenly across the top of the tree. These branch lengths are often employed by systematists when presenting line drawings of phylogenetic hypotheses (e.g., as in Fig. 1). For these branch lengths, the correlation of the absolute values of the standardized contrasts with their standard deviations was -0.137. The 2-tailed critical value (α = 0.05) for a correlation coefficient with 170 df is 0.150, therefore these branch lengths seem marginally adequate.

The second set of arbitrary branch lengths that we used corresponds to those suggested by Grafen (1989, his Fig. 2). For these, the correlation of the diagnostic was -0.161 ($P < 0.05$), thus they seem marginally inappropriate for analyses of plasma osmolarity. As discussed elsewhere (Grafen, 1989; Garland et al., 1992; Díaz-Uriarte and Garland, 1996), these arbitrary branch lengths could also be transformed to improve the diagnostic statistic, but in the sake of brevity we have not done so. In any case, a number of examples have now emerged in which branch lengths set equal to unity perform as well as, or better than, others that have been tried (T. Garland, unpublished).

Although the foregoing diagnostic test suggests that branch lengths set equal to unity may be adequate for our purposes, it is still of interest to ask how much our results would change if we used other branch lengths. Table 3 shows the results of using the two other sets of arbitrary branch lengths suggested by Pagel (1992) and by Grafen (1989). For the data set containing only 63 terrestrial plus desert taxa, the value reconstructed for the ancestral amniote increased from 280 to 297 and 294, respectively. These changes are still well within the +5% range shown in Figure 5. For the analysis of all 172 taxa, the three values were 365, 376, and 371, which constitutes a very minor change in relation to the +5% range of 157-573 (Fig. 3). Thus, reconstruction of the plasma osmolarity of the ancestral amniote seems to be relatively insensitive to alterations of phylogenetic branch lengths.

Use of Fossil Information

Our analysis of a physiological trait, plasma osmolarity, has necessarily involved data only for extant forms. For traits such as physiology or behavior, fossil information will rarely be available (Ruben, 1995; Martins, 1996b). If one were studying a morphometric

trait, however, such as limb length, then it might be possible to obtain data from both extant and extinct forms. These data could easily be combined in analyses such as we have presented. The only requirement would be that the extinct forms could be placed on the phylogenetic tree; that is, their relationship to extant taxa would need to be known. If branch lengths in units of estimated divergence times were being employed, then extant taxa would line up contemporaneously along the tips of the phylogeny, with extinct forms at the ends of branches terminating below the tips. If one had data for some fossil forms that were closely related to the node of interest (i.e., were topologically close) and occurred not too far away in chronological time (i.e., were at the ends of relatively short branches, assuming that the branch lengths being used for analysis were in units proportional to divergence times), then the extinct forms would have a relatively large effect on the node being reconstructed (because averaging involves adjacent nodes and weighting by the reciprocal of branch length). Thus, inclusion of data for fossil forms could help to narrow the "confidence interval" about the reconstructed nodal value.

Other Uses of Squared-Change Parsimony

In the example we have presented, and in most other published applications of squared-change parsimony procedures (e.g., Huey and Bennett, 1987), a primary goal is to estimate the value of a hypothetical ancestral organism, as represented by an internal node of the phylogenetic tree. Minimization of the sum of the squared changes over the entire phylogenetic tree is taken as the optimality criterion by which to choose a set of internal nodes, including the one(s) of primary interest.

Similar logic could be used to estimate the value of an extant or extinct species for which data were unavailable but which could be placed on the phylogenetic tree; that is, the value for a terminal node rather than an internal node. For example, one might wish to estimate the limb proportions, locomotor abilities, and/or hunting behavior of an extinct mammal that was know only from fragmentary fossils (cf. Garland and Janis, 1993; Janis and Wilhelm, 1993; Harris and Steudel, 1997). In conservation biology, one might wish to estimate the home range area (e.g., Garland *et al.*, 1993) of some (endangered) species that had yet to be studied. Various approaches would be possible,

Table 3. Effects of Different Branch Lengths (All of Which Are Arbitrary) on Squared-change Parsimony Reconstructions of Plasma Osmolarity (mOsm).

Taxa Included (N)	All = 1		Pagel, 1992		Grafen, 1989	
	Root node	Ancestor of all amniotes	Root node	Ancestor of all amniotes	Root node	Ancestor of all amniotes
All vertebrates (172)	644	365	511	376	544	371
All amniotes (76)	310	310*	314	314*	313	313*
No saltwater forms (122)	429	293	398	328	407	314
Only terrestrial (63)	263	280	292	297	289	294
Only terrestrial amniotes (58)	294	294*	309	309*	306	306

* These values are the same as those under Root Node column because no "outgroups" are present in the reduced phylogeny; thus, this node is the root node.

including squared-change parsimony. A range of values for the focal species could be analyzed, and then one would take as the "best" estimate that value which minimized the sum of squared changes over the entire phylogeny. Many of the indices for indexing uncertainty discussed elsewhere in this chapter could also be applied in this context.

Returning to interior nodes, once values have been reconstructed by a parsimony procedure, then simple subtraction can be used to make inferences can be made about the character changes that occurred along particular branch segments. Huey and Bennett (1987), for example, drew inferences about whether particular lineages of lizards had increased or decreased their thermal preferences and tolerances (and whether these changes were associated with biological shifts, such as diurnality versus nocturnality, or nondesert versus desert). In the example presented in this paper, we can also make such inferences. Bear in mind, however, that the usefulness of such inferences depends very directly on the accuracy of the nodal reconstructions. We have presented two ways of indexing uncertainty about nodal reconstructions (see above and Appendix 3); others are discussed below. When one singles out a particular species or lineage to determine if it shows change from the ancestral value, it would make sense first to consider whether its value is outside of such uncertainty intervals (e.g., see Chevalier, 1991, who also incorporated information on within-species variation). (The method of phylogenetically independent contrasts can also be employed to compare single species with a set of others; see Fig. 4 of Garland and Adolph, 1994; McPeek, 1995; Martinez del Rio *et al.*, 1995.)

One example of an evolutionary change in plasma osmolarity is the apparent decrease seen in the lineage leading to the lampreys (Class Cephalaspidomorphi, *Lampetra* and *Petromyzon*: tips 168-170 in Fig. 1). Extant lampreys are anadromous; they have a freshwater larval stage and may enter seawater as adults (Nelson, 1994). One of the species in our data set, *Petromyzon marinus*, is anadromous (Mathers and Beamish, 1974); however, the data for all three species were taken from landlocked freshwater lampreys found in the Great Lakes of North America. Available data for *Petromyzon* caught at sea (cited in Morris, 1971) and for lampreys in 1037 mOsm sea water

(Logan *et al.*, 1980) indicate plasma osmolarities in the range of about 309-361 mOsm. All of these values are much lower than the value of 644 mOsm that is estimated by squared-change parsimony for the ancestor of all 172 vertebrates in our data set (see first row of Table 2). The closest living relatives of the lampreys, the hagfishes (Nelson, 1994: Class Myxini, *Myxine* and *Eptatretus*: tips 171-172) lack a larval stage, are marine, and have much higher plasma osmolarities (954-969 mOsm), values similar to those of saltwater Chondrichthyes. Although the arbitrary branch lengths of Figure 1 do not reflect the fact, it is important to note that lampreys and hagfishes diverged phylogenetically at least 400-500 million years ago (Nelson, 1994); this distant relationship is reflected by their placement in different taxonomic Classes (despite some superficial similarities).

Given that changes along branch segments can be inferred for one trait, then the same can be done for two or more traits. These inferred changes along branch segments can then be correlated. This provides a way to study correlated character evolution (e.g., see Huey, 1987; Huey and Bennett, 1987; Garland *et al.*, 1991; Walton, 1993; Westneat, 1995). Simulation studies indicate that squared-change parsimony estimates of correlated character evolution may be better than some alternatives [e.g., Felsenstein's (1985) phylogenetically independent contrasts] in some cases (Martins and Garland, 1991; Martins, 1996a; see also Pagel, 1993; Bjorklund, 1994). The CMSINGLE program of Martins and Garland (1991) performs the necessary computations. Note, however, that the number of branch segments along which changes can be inferred is greater than the number of original data points (i.e., the data for the tip species). Moreover, the inferred changes are not independent in the statistical sense, because the nodes are computed as averages of surrounding values (Martins and Garland, 1991; Pagel, 1993). Thus, conventional critical values cannot be used for hypothesis testing. Instead, Monte Carlo computer simulations can be used to create empirical null distributions for hypothesis testing (Garland *et al.*, 1991; Martins and Garland, 1991), and computer programs to do so are available from the senior author (CMSINGLE and CMMEANAL of Martins and Garland, 1991; PDSIMUL of Garland *et al.*, 1993). Phylogenetic randomization procedures, as mentioned in the next section, could also be used for this purpose.

With respect to plasma osmolarity, one might test for correlated evolution with habitat shifts. However, the crude categorizations of "habitat" that we have compiled do not constitute a continuous-valued character that ranges simply along a single dimension. Rather, "habitat" encompasses at least one qualitative difference, between aquatic and terrestrial. Phylogenetically based statistical methods for correlating changes in a continuous variable with multiple changes in a complicated, qualitative variables are not well worked out (see Grafen, 1989; Garland *et al.*, 1992, 1993; Garland and Adolph, 1994; McPeek, 1995; Martins, 1996b; Pyron, 1996), so we leave such analyses for the future. Nonetheless, we have shown (see above section on "Choosing Appropriate Models ...") that plasma osmolarity differs significantly among habitat types for the 54 Actinopterygii. This covariation implies that osmolarity has indeed evolved in concert with habitat occupancy.

Future Possibilities for Indexing Uncertainty in Nodal Reconstructions

In closing, we mention some other possibilities for indexing uncertainty in squared-change parsimony reconstructions. Most of these are computer-intensive "resampling" methods of the type that have only recently become popular in ecology and evolutionary biology, although some have a long history (Sokal and Rohlf, 1981; Noreen, 1989; Manly, 1991; Crowley, 1992; Efron and Tibshirani, 1993; Lapointe and Legendre, 1992, 1995; Lapointe *et al.*, 1994). Jackknifing, as we have used above (and see Appendix 3), is one of these methods. Bootstrapping is similar to jackknifing, except that the resampling is done with replacement. The possibility of sampling species with replacement (i.e., a given species' value could appear multiple times in a single resampled data set) does not seem to make much sense. How, for instance, would that species be placed multiple times on a hierarchical phylogeny (as a polytomy?)? Thus, we will not consider bootstrapping. The two other resampling methods that we will consider are Monte Carlo simulations and randomization procedures; the latter can be considered a special case of the former.

Monte Carlo computer simulations have been used to test hypotheses about comparative data (e.g., Garland *et al.*, 1991; Garland *et al.*, 1993; Westneat, 1995; Martin and Clobert, 1996; Reynolds and Lee, 1996; Harris and Steudel, 1997). These procedures involve simulating the evolution of one or more characters along a specified evolutionary tree (topology and branch lengths) under a specified model of evolutionary change (see also Bjorklund, 1994; Díaz-Uriarte and Garland, 1996). For each simulated data set, the statistic of interest is then computed in exactly the same fashion as for the one real data set. The model of evolutionary change is assumed to be representative of the actual processes that resulted in the data about which one wishes to draw inferences (e.g., the average plasma osmolarities of various species of vertebrates). [Simulations can also be used to test the performance of different analytical methods (e.g., Grafen, 1989; Martins and Garland, 1991; Purvis *et al.*, 1994; Martins, 1996a); in Appendix 3, we compare the performance of squared-change parsimony and a conventional nonphylogenetic analysis for estimating the root node of a phylogeny.]

At first thought, Monte Carlo simulations might seem ill-suited to testing hypotheses about values reconstructed for internal nodes. Among other parameters, one must specify the starting value for a simulation, i.e., the value of the character at the root of the phylogenetic tree. If the node of interest *is* the root node, or even one adjacent or almost adjacent to the root, then the results would depend very strongly on a user-specified value, i.e., an assumption of the procedure. This could lead to "inappropriately biasing the analysis" (see de Queiroz, 1996). Nevertheless, further thought suggests the possible utility of computer simulations.

First, if independent fossil (or other) information allowed specification of the starting value (e.g., Garland *et al.*, 1993), then the potential for *inappropriate* bias could be greatly reduced if not eliminated. Second, one could use the simulated data only to gauge variability about the point estimate, but not the point estimate itself. In other words, for the best estimate of the value at the node of interest, one could use the estimate from the real data set (alternatively, see Reynolds and Lee, 1996, p. 740). For the estimate of uncertainty, one could simulate many data sets (e.g., 1000), determine the range of

values from the 2.5th to the 97.5th percentiles, divide by two, and use this as an index of a +/- 95% confidence interval about the point estimate from the real data set (cf. Reynolds and Lee, 1996). This "confidence interval" would, of course, be completely predicated on the chosen simulation model (as well as the specified topology and branch lengths). This sort of procedure would be analogous to the "parametric bootstrap" that has recently gained favor in studies of uncertainty in the reconstruction of phylogenetic trees (Huelsenbeck *et al.*, 1996; see also Crowley, 1992, p. 429).

Randomization tests are another example of a computer-intensive resampling method, but these procedures completely avoid the specification of starting values. Randomization tests reshuffle the real data (e.g., the species' mean plasma osmolarities), rather than sampling a subset of it (jackknifing) or creating new data sets by Monte Carlo simulation. Each time the real data are reshuffled, the statistic of interest is recomputed (e.g., the squared-change parsimony value reconstructed for a particular node).

Conventional randomization tests reshuffle the data equiprobably, which, in the context of comparative data, is equivalent to assuming that the character(s) evolved along a star phylogeny with equal branch lengths and by Brownian motion (i.e., it is assumed that nothing like character displacement has occurred). F.-J. Lapointe and the senior author are currently developing a phylogenetic randomization procedure and associated computer programs. This will allow the tip data to be reshuffled in a way that preserves the phylogenetic structure in the data (i.e., closely related species tend to have similar phenotypes). In general, this would mean that values permuted from a given tip are most likely to go back to that tip itself or its sister, next most likely to go to their next closest relative, and so on. As with Monte Carlo simulations, each of the permuted data sets could then be submitted to a squared-change parsimony analysis, a histogram constructed, and the spread of this histogram used to indicate the uncertainty with which we should view the point estimate of the node, i.e., the value reconstructed for the real data set.

Finally, as noted in Appendix 3 (section on "Squared-Change Parsimony and Independent Contrasts"), the root node value reconstructed by squared-change parsimony is the same as that

computed by independent contrasts. In principle, independent contrasts solve the problem of the tip data being nonindependent. Thus, for the root node, we believe that jackknifing (applied above and discussed in Appendix 3) may actually have more than heuristic value. This suggestion has not yet been studied. Also for the root node, an analytical procedure based on independent contrasts has been developed (T. Garland, Jr., and A. R. Ives, unpublished results). With the assumption that character evolution can be modeled as Brownian motion, this allows the computation of standard parametric confidence intervals for root nodes (see above section on "Reconstructing Plasma Osmolarity at the Origin of Vertebrates"). Extensions to incorporate within-species variation are also possible (J. Felsenstein, pers. comm.).

The suitability of the resampling methods discussed in this section depends, in part, on how one views interspecific comparative studies in relation to issues of sampling, populations, and the desired nature of the inference. Some of the procedures assume that the available data represent a random sample from some population and/or that the parameters of that population can be fully defined. Exactly what assumptions are made by each method is a complicated subject and beyond the scope of this chapter (Sokal and Rohlf, 1981; Noreen, 1989; Manly, 1991; Crowley, 1992; Efron and Tibshirani, 1993; see also Díaz-Uriarte and Garland, 1996; Martins and Hansen, 1996) But we can mention how some of these issues pertain to our example. The "population" might be considered as all (extant) vertebrates. We cannot fully define that "population," however, because we obviously do not have data for all vertebrates. Moreover, the 172 species in our data set probably should not be considered as a random sample. In general, comparative physiologists do not choose study organisms by reference to a table of random numbers and a list of all extant species (which themselves are probably not a random sample of all species that have ever lived!). Instead (see Garland and Carter, 1994), species are often studied because they are of particular interest [e.g., because they live in extreme environments, such as deserts or the Antarctic (e.g., Eastman, 1993)], because they are particularly suitable for certain physiological measurements, or sometimes just because they happen to be available. And finally, note that we emphasized a still less random subset of 63 taxa for most of our analyses. Future studies

will need to consider carefully the optimal application of different computer-intensive resampling methods for making various sorts of inferences from comparative data.

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APPENDIX 1

Complete data set employed in the analyses ($N = 172$). Species are ordered as in the phylogeny shown in Figure 1, and Tip# indicates position of species from top to bottom. Osm. is osmolarity in mOsm. Species names show the most recent taxonomic conventions (see text). Genus or species names in parentheses are the ones used in original sources. Ha, habitat; D, desert; E, estuarine; F, freshwater; M, marine; S, saltwater; SA, saltwater Antarctic; SM, salt marsh; T, terrestrial.

Clade	Species	Tip#	Osm	Ha	Source
Aves	*Struthio camelus*	1	302	T	Altman and Dittmer, 1971
Aves	*Dromaius novaehollandiae*	2	309	T	Skadhauge, 1974
Aves	*Cygnus atratus*	3	296	T	Hughes, 1976
Aves	*Anas platyrhynchos*	4	297	T	Deustch *et al.*, 1979
Aves	*Anser anser*	5	297	T	Zucker *et al.*, 1977
Aves	*Colinus virginianus*	6	350	T	McNabb, 1969
Aves	*Callipepla (Lophortyx) californica*	7	336	T	McNabb, 1969
Aves	*Callipepla (Lophortyx) gambelii*	8	340	T	McNabb, 1969
Aves	*Gallus domesticus*	9	319	T	Skadhauge, 1967
Aves	*Coturnix coturnix*	10	313	T	Osono and Nishimura, 1994
Aves	*Coturnix pectoralis*	11	349	T	Roberts and Baudinette, 1984
Aves	*Coturnix chinensis*	12	371	T	Roberts and Baudinette, 1984
Aves	*Geococcyx californianus*	13	349	T	Ohmart, 1972

Clade	Species	Tip#	Osm	Ha	Source
Aves	*Cacatua roseicapilla*	14	336	T	Skadhauge, 1974
Aves	*Melopsittacus undulatus*	15	336	T	Krag and Skadhauge, 1972
Aves	*Geophaps (Ocyphaps) lophotes*	16	336	T	Skadhauge, 1974
Aves	*Zenaida macroura*	17	372	T	Smyth and Bartholomew, 1966
Aves	*Fulica americana*	18	259	T	Carpenter and Stafford, 1970
Aves	*Gallirallus (Rallus) owstoni*	19	355	T	Carpenter and Stafford, 1970
Aves	*Larus argentatus*	20	273	T	Ensor and Phillips, 1972
Aves	*Larus glaucescens*	21	334	T	Hughes, 1977
Aves	*Spheniscus demersus*	22	306	M	Erasmus, 1978
Aves	*Ardea cinerea*	23	317	T	Lange and Staaland, 1966
Aves	*Lichenostomus (Meliphaga) virescens*	24	343	T	Skadhauge, 1974
Aves	*Sturnus vulgaris*	25	318	T	Braun, 1978
Aves	*Taeniopygia (Poephila) guttata*	26	336	T	Skadhauge and Bradshaw, 1974
Aves	*Amphispiza belli nevandensis*	27	310	T	Moldenhauer and Wiens, 1970
Aves	*Ammodramus (Ammospiza) c. caudacutus*	28	346	T	Poulson, 1969
Aves	*Passerculus sandwichensis*	29	339	T	Goldstein *et al.*, 1990
Aves	*Passerculus sandwichensis beldingi*	30	349	SM	Goldstein *et al.*, 1990
Crocodylia	*Alligator mississipiensis*	31	291	F	Lauren, 1985
Crocodylia	*Crocodylus acutus*	32	294	F	Minnich, 1982; Dill and Edwards, 1931
Crocodylia	*Crocodylus porosus*	33	307	F	Minnich, 1982; Grigg, 1981
Squamata	*Microlophus (Tropidurus)* species	34	340	T	Minnich, 1979; Roberts and Schmidt-Nielsen, 1966
Squamata	*Dipsosaurus dorsalis*	35	300	D	Minnich, 1979; House, 1974
Squamata	*Amblyrhynchus cristatus*	36	402	E	Nagy and Shoemaker, 1984
Squamata	*Uromastyx acanthinurus*	37	307	D	Minnich, 1979; Tercafs and Vassas, 1967
Squamata	*Ctenophorus (Amphibolurus) maculosus*	38	379	D	Minnich, 1982; Braysher, 1976
Squamata	*Agama impalearis*	39	350	T	Minnich, 1979; Tercafs and Vassas, 1967
Squamata	*Agama stellio*	40	358	T	Minnich, 1979; Frenkel and Kraicer, 1971

Clade	Species	Tip#	Osm	Ha	Source
Squamata	*Phrynosoma cornutum*	41	315	D	Minnich, 1979; Roberts and Schmidt-Nielsen, 1966
Squamata	*Sceloporus cyanogenys*	42	319	T	Minnich, 1979; Stolte *et al.*, 1977
Squamata	*Hemidactylus* species	43	322	T	Minnich, 1979; Roberts and Schmidt-Nielsen, 1966
Squamata	*Varanus gouldii*	44	328	D	Minnich, 1979; Green, 1972
Squamata	*Laticauda semifasciata*	45	320	S	Minnich, 1982; Dunson and Taub, 1967
Squamata	*Pituophis melanoleucus*	46	327	T	Minnich, 1979; Komadina and Solomon, 1970
Squamata	*Nerodia cyclopion*	47	262	F	Minnich, 1979; LeBrie and Elizondo, 1969
Squamata	*Nerodia sipedon*	48	318	F	Minnich, 1982; Dessaur, 1970
Squamata	*Nerodia fasciata compressicauda*	49	371	E	Minnich, 1982; Dunson, 1980
Sphenodontia	*Sphenodon punctatus*	50	273	T	Minnich, 1982; Schmidt-Nielsen and Schmidt, 1973
Mammalia	*Homo sapiens*	51	302	T	Guyton, 1991
Mammalia	*Canis familiaris*	52	294	T	Papanek and Raff, 1994
Mammalia	*Felis catus*	53	303	T	Altman and Dittmer, 1971
Mammalia	*Oryctolagus cuniculus*	54	283	T	Keil *et al.*, 1994
Mammalia	*Mesocricetus auratus*	55	325	D	Gottschalk *et al.*, 1963
Mammalia	*Psammomys obesus*	56	365	D	Jamison *et al.*, 1979
Mammalia	*Mus musculus*	57	302	T	Altman and Dittmer, 1971
Mammalia	*Rattus norvegicus*	58	303	T	Ullrich *et al.*, 1963
Mammalia	*Equus caballus*	59	304	T	Altman and Dittmer, 1971
Mammalia	*Physeter catodon*	60	370	S	Gordon, 1982
Mammalia	*Sus scrofa*	61	302	T	Altman and Dittmer, 1971
Mammalia	*Camelus dromedarius*	62	340	D	Schmidt-Nielsen, 1964
Mammalia	*Bos taurus*	63	304	T	Spector, 1956
Mammalia	*Ovis aries*	64	290	T	Dunham *et al.*, 1993
Mammalia	*Capra hircus*	65	303	T	Altman and Dittmer, 1971
Mammalia	*Capra hircus*	66	320	D	Chosniak *et al.*, 1984
Testudines	*Chelydra serpentina*	67	315	F	Minnich, 1982; Dessaur, 1970
Testudines	*Chelonia mydas*	68	270	S	Minnich, 1982; Dessauer, 1970
Testudines	*Caretta caretta*	69	465	S	Minnich, 1982; Schoffeniels and Tercafs, 1965
Testudines	*Trionyx spiniferus*	70	280	F	Minnich, 1982; Dunson and Weymouth, 1965
Testudines	*Malaclemys terrapin*	71	309	F	Minnich, 1979; Gilles-Baillien, 1970
Testudines	*Chrysemys picta (scripta)*	72	260	F	Minnich, 1979; Dantzler and Schmidt-Nielsen, 1966
Testudines	*Trachemys (Pseudemys) scripta*	73	260	F	Minnich, 1982; Platner, 1950
Testudines	*Mauremys caspica (leprosa)*	74	362	F	Minnich, 1982; Schoffeniels and Tercafs, 1965

Clade	Species	Tip#	Osm	Ha	Source
Testudines	*Testudo h. hermanni*	75	290	T	Minnich, 1982; Gilles-Baillien and Schoffeniels, 1965
Testudines	*Gopherus agassizii*	76	291	D	Minnich, 1982; Minnich, 1977
Lissamphibia	*Xenopus laevis*	77	233	F	Shoemaker *et al.*, 1992; McBean and Goldstein, 1970
Lissamphibia	*Rana catesbeiana*	78	210	F	Alvarado, 1979; Yoshimura *et al.*, 1961
Lissamphibia	*Rana pipiens*	79	214	F	Alvarado, 1979; Campbell *et al.*, 1967
Lissamphibia	*Rana cancrivora*	80	290	F	Shoemaker *et al.*, 1992; Gordon *et al.*, 1961
Lissamphibia	*Hyla regilla*	81	218	F	Alvarado, 1979; Mullen, 1974
Lissamphibia	*Bufo boreas*	82	235	T	Alvarado, 1979; Mullen, 1974
Lissamphibia	*Bufo bufo*	83	240	T	Alvarado, 1979; Ferreira and Jesus, 1973
Lissamphibia	*Bufo marinus*	84	250	T	Alvarado, 1979; Middler *et al.*, 1969
Lissamphibia	*Bufo viridis*	85	270	F	Bentley, 1971; Balinsky, 1981
Lissamphibia	*Typhlonectes compressicauda*	86	196	F	Stiffler *et al.*, 1990
Lissamphibia	*Ichthyophis kohtaoensis*	87	220	T	Stiffler *et al.*, 1990
Lissamphibia	*Ambystoma tigrinum*	88	230	T	Alvarado, 1979; Alvarado, 1972
Lissamphibia	*Amphiuma means*	89	218	F	Stanton, 1988
Lissamphibia	*Batrachoseps attenuatus*	90	339	F	Shoemaker *et al.*, 1992; Balinsky, 1981
Coelacanthiformes	*Latimeria chalumnae*	91	931	S	Griffith *et al.*, 1974
Dipnoi	*Protopterus aethiopicus*	92	238	F	Evans, 1979; Smith, 1930
Teleostei	*Pleuronectes flesus*	93	364	S	Bentley, 1971; Lange and Fugelli, 1965
Teleostei	*Perca fluviatilis*	94	294	F	Gordon, 1982; Lutz, 1975
Teleostei	*Pholis ornata*	95	375	S	Bridges, 1993; Barton, 1979
Teleostei	*Sphyraena barracuda*	96	476	S	Evans, 1979; Becker *et al.*, 1958
Teleostei	*Rhigophila dearborni*	97	489	SA	Dobbs and DeVries, 1975
Teleostei	*Gymnodraco acuticeps*	98	616	SA	Dobbs and DeVries, 1975
Teleostei	*Blennius pholis*	99	320	S	Bridges, 1993; House, 1963
Teleostei	*Blennius fluviatilis*	100	410	S	Bridges, 1993; Muller *et al.*, 1973
Teleostei	*Blennius pavo*	101	410	S	Bridges, 1993; Muller *et al.*, 1973
Teleostei	*Blennius sphinx*	102	410	S	Bridges, 1993; Muller *et al.*, 1973
Teleostei	*Periophthalmus chrysospilos*	103	430	S	Bridges, 1993; Lee *et al.*, 1987
Teleostei	*Periophthalmus sobrinus*	104	480	S	Bridges, 1993; Gordon *et al.*, 1965

Clade	Species	Tip#	Osm	Ha	Source
Teleostei	*Dissostichus mawsoni*	105	614	SA	Dobbs and DeVries, 1975
Teleostei	*Trematomus loennbergii*	106	563	SA	Dobbs and DeVries, 1975
Teleostei	*Trematomus borchgrevinki*	107	565	SA	Dobbs and DeVries, 1975
Teleostei	*Trematomus lepidorhinus*	108	574	SA	Dobbs and DeVries, 1975
Teleostei	*Trematomus hansoni*	109	581	SA	Dobbs and DeVries, 1975
Teleostei	*Trematomus nicolai*	110	589	SA	Dobbs and DeVries, 1975
Teleostei	*Trematomus centronotus*	111	602	SA	Dobbs and DeVries, 1975
Teleostei	*Trematomus bernacchii*	112	634	SA	Dobbs and DeVries, 1975
Teleostei	*Trematomus newnesi*	113	656	SA	Dobbs and DeVries, 1975
Teleostei	*Scomberomorus maculatus*	114	386	S	Evans, 1979; Becker *et al.*, 1958
Teleostei	*Thunnus thynnus*	115	437	S	Holmes and Donaldson, 1969; Becker *et al.*, 1958
Teleostei	*Promicrops itaiara*	116	384	S	Holmes and Donaldson, 1969; Becker *et al.*, 1958
Teleostei	*Mycteroperca bonasi*	117	461	S	Holmes and Donaldson, 1969; Becker *et al.*, 1958
Teleostei	*Mycteroperca venenosa*	118	467	S	Holmes and Donaldson, 1969; Becker *et al.*, 1958
Teleostei	*Xiphister atropurpureus*	119	300	S	Bridges, 1993; Evans, 1967
Teleostei	*Anoplarchus purpurescens*	120	350	S	Bridges, 1993; Barton, 1979
Teleostei	*Fundulus kansae*	121	181	F	Evans, 1979; Stanley and Fleming, 1964
Teleostei	*Fundulus heteroclitus*	122	335	F	Bentley, 1971; Pickford *et al.*, 1966
Teleostei	*Fundulus heteroclitus*	123	365	S	Bentley, 1971; Pickford *et al.*, 1966
Teleostei	*Ictalurus nebulosus*	124	279	F	Gordon, 1982; Umminger, 1971
Teleostei	*Cyprinus carpio*	125	274	F	Evans, 1979; Houston and Madden, 1968
Teleostei	*Tinca vulgaris*	126	280	F	Holmes and Donaldson, 1969; Keys and Hill, 1934
Teleostei	*Carassius auratus*	127	299	F	Gordon, 1982; Umminger, 1971
Teleostei	*Opsanus tau*	128	392	S	Bentley, 1971; Lahlou *et al.*, 1969
Teleostei	*Lophius americanus*	129	350	S	Gordon, 1982; Forster and Berglund, 1956
Teleostei	*Lophius piscatorius*	130	452	S	Evans, 1979; Brull and Nizet, 1953
Teleostei	*Esox lucius*	131	274	F	Holmes and Donaldson, 1969

Clade	Species	Tip#	Osm	Ha	Source
Teleostei	*Salvelinus namaycush*	132	298	F	Holmes and Donaldson, 1969; Hoffert and Fromm, 1966
Teleostei	*Oncorhynchus kisutch*	133	295	F	Holmes and Donaldson, 1969; Conte, 1965
Teleostei	*Oncorhynchus kisutch*	134	331	S	Holmes and Donaldson, 1969; Conte, 1965
Teleostei	*Salmo salar*	135	328	F	Evans, 1979; Parry, 1961
Teleostei	*Salmo salar*	136	344	S	Evans, 1979; Parry, 1961
Teleostei	*Salmo trutta*	137	326	F	Evans, 1979; Gordon, 1959
Teleostei	*Salmo trutta*	138	356	S	Evans, 1979; Gordon, 1959
Teleostei	*Conger vulgaris*	139	430	S	Holmes and Donaldson, 1969; Boucher-Firley, 1934
Teleostei	*Muraena helena*	140	441	S	Holmes and Donaldson, 1969; Boucher-Firley, 1934
Teleostei	*Anguilla rostrata*	141	307	F	Holmes and Donaldson, 1969; Butler *et al.*, 1969
Teleostei	*Anguilla anguilla*	142	328	F	Evans, 1979; Sharatt *et al.*, 1964
Teleostei	*Anguilla anguilla*	143	377	S	Evans, 1979; Sharatt *et al.*, 1964
Chondrostei	*Acipenser sturio*	144	318	F	Holmes and Donaldson, 1969; Magnin, 1962
Chondrostei	*Acipenser sturio*	145	343	S	Holmes and Donaldson, 1969; Magnin, 1962
Chondrostei	*Erpetoichthys calabaricus*	146	199	F	Gordon, 1982; Lutz, 1975
Chondrichthyes	*Mustelus canis*	147	962	S	Holmes and Donaldson, 1969; Doolittle *et al.*, 1960
Chondrichthyes	*Scyliorhinus canicula*	148	1118	S	Evans, 1979; Payan and Maetz, 1971
Chondrichthyes	*Carcharhinus melanop*	149	484	F	Holmes and Donaldson, 1969; Smith, 1931
Chondrichthyes	*Carcharhinus leucas*	150	650	F	Gordon, 1982; Thorson *et al.*, 1973
Chondrichthyes	*Carcharhinus leucas*	151	1000	S	Gordon, 1982; Thorson *et al.*, 1973
Chondrichthyes	*Squalus acanthias*	152	1007	S	Holmes and Donaldson, 1969; Murdaugh and Robin, 1967
Chondrichthyes	*Squatina angelus*	153	1102	S	Holmes and Donaldson, 1969; Pora, 1936
Chondrichthyes	*Pristis microdon*	154	540	F	Holmes and Donaldson, 1969; Smith, 1931
Chondrichthyes	*Torpedo marmorata*	155	1098	S	Holmes and Donaldson, 1969; Pora, 1936
Chondrichthyes	*Potamotrygon* species	156	308	F	Withers, 1992; Thorson *et al.*, 1967
Chondrichthyes	*Dasyatis varnak*	157	548	F	Holmes and Donaldson, 1969; Smith, 1931
Chondrichthyes	*Dasyatis saj*	158	840	S	Holmes and Donaldson, 1969

Clade	Species	Tip#	Osm	Ha	Source
Chondrichthyes	*Dasyatis amaericana*	159	864	S	Holmes and Donaldson, 1969; Bernard *et al.*, 1966
Chondrichthyes	*Dasyatis sabina*	160	1021	S	Evans, 1979; deVlaming and Sage, 1973
Chondrichthyes	*Raja eglanteria*	161	844	S	Holmes and Donaldson, 1969; Price and Creaser, 1967
Chondrichthyes	*Raja ocellata*	162	928	S	Holmes and Donaldson, 1969; Maren *et al.*, 1963
Chondrichthyes	*Raja stabuliforis*	163	958	S	Holmes and Donaldson, 1969; Maren *et al.*, 1963
Chondrichthyes	*Raja clavata*	164	1050	S	Bentley, 1971; Murray and Potts, 1961
Chondrichthyes	*Raja undulata*	165	1097	S	Holmes and Donaldson, 1969; Pora, 1936
Chondrichthyes	*Hydrolagus colliei*	166	801	S	Holmes and Donaldson, 1969; Urist, 1966
Chondrichthyes	*Chimaera monstrosa*	167	1046	S	Kirschner, 1991; Robertson, 1976
Cephalaspidomorphi	*Lampetra planeri*	168	227	F	Holmes and Donaldson, 1969; Bull and Morris, 1967
Cephalaspidomorphi	*Lampetra fluviatilis*	169	272	F	Evans, 1979; Pickering and Morris, 1970
Cephalaspidomorphi	*Petromyzon marinus*	170	300	F	Gordon, 1982; Mathers and Beamish, 1974
Myxini	*Myxine glutinosa*	171	969	S	Evans, 1979; Robertson, 1976
Myxini	*Eptatretus stoutii*	172	954	S	Evans, 1979; McFarland and Munz, 1965

APPENDIX 2: PARSIMONY IN SYSTEMATIC AND COMPARATIVE BIOLOGY

The English word "parsimony" is derived from the Latin stem "pars-," which denotes "to spare, save" (Oxford English Dictionary, 1971). As a logical principle, parsimony dictates, in essence, that simple explanations are generally to be preferred. Parsimony is often used as a procedure to infer the phylogenetic relationships of organisms and, as we discuss in this chapter, the evolution of particular characters in the context of an (independent) hypothesis of phylogenetic relationships. Use of parsimony as a philosophical or as an operational principle in systematic biology seems to have at least two origins (reviews in Farris, 1983; Sober, 1988; Edwards, 1996). At present, the justification of parsimony as a procedure for phylogenetic inference remains quite controversial, in part because "parsimony" means different things to different people [cf. Kluge and Wolf (1993) and Sanderson (1995) on the meaning of "cladistics"].

What might be termed "cladistic parsimony" seems to derive from the "Law of parsimony: the logical principle that no more causes or forces should be assumed than are necessary to account for the facts" (Oxford English Dictionary,

1971). To quote Farris (1983, p. 7): "Most phylogeneticists recognize that inferring genealogy rests on the principle of parsimony, that is, choosing genealogical hypotheses so as to minimize requirements for ad hoc hypotheses of homoplasy." "Homoplasy" describes characters or states of characters that are shared by two or more taxa but are not homologous (derived from a common ancestor). "Put simply, homoplasy exists if...two taxa showing the character have a common ancestor that does not have the character" (Wiley, 1981, p. 12). Homoplasy can result from parallel or convergent evolution or from evolutionary reversals.

Cladistic parsimony argues that we should prefer phylogenetic hypotheses that minimize the amount of homoplasy in the characters used in the analysis (e.g., DNA sequences and morphology). For a given data set, this generally translates into a preference for the phylogenetic tree that requires the fewest total number of steps across all characters being analyzed. However, an important point to note here is that, with categorical variables, the actual amount of evolutionary (genetic) change (on some unspecified biological scale) is not necessarily implied to be the same from, say, a coded state of 0 to 1 as from a state of 1 to 2. Thus, minimizing the amount of homoplasy (misinterpreted homologies, representing parallel and/or convergent evolution) does not necessarily equate to finding the phylogenetic tree that implies the least amount of biological change.

The other origin of parsimony procedures in systematic biology hinges on their being, in some cases, an approximation to the maximum-likelihood solution for a model of random evolution (Edwards, 1996). This is a statistical justification of what might be called "distance parsimony," or a preference for topologies that invoke the minimum net amount of evolutionary change (Edwards, 1996). Related to this is the more colloquial idea that, as expressed in a recent textbook in evolutionary biology, "The parsimony principle is reasonable because evolutionary change is improbable. ... it is more likely that a character will be shared by common descent than by independent, convergent evolution. For any set of species, a phylogeny requiring less evolutionary change is more plausible than one requiring more" (Ridley, 1993, pp. 449-450).

In its purest form, "cladistic parsimony" applies only to discretely valued characters (e.g., Stewart, 1993), although an algorithm for continuous-valued characters is available (Kluge and Farris, 1969; Farris, 1970; Huey and Bennett, 1987; Losos, 1990; Maddison and Maddison, 1992; Miles and Dunham, 1996; Butler and Losos, in review). With respect to morphological characters, systematists generally code them into discrete states (e.g., 0 versus 1) and describe each taxon as being characterized by one state or the other, with some characters coded as multistate (e.g., see Brooks and McLennan, 1991). Sometimes this coding is easy to do, as when a particular feature is either present or absent from all individuals that have been examined for a given taxon. Sometimes the categorization is fuzzier, as when a morphological feature is described as "small, medium or large." Categorization is also difficult when taxa show polymorphism (more than one character state) among its members (Wiens, 1995). Finally, categorization can be performed even when the character under consideration is inherently continuous-valued and polygenic (Falconer and Mackay, 1996), such as body size or shape, or

metabolic rate (e.g., Dial and Grismer, 1992; Zelditch *et al.*, 1995; Strait *et al.*, 1996). If the phenotypes of the set of taxa under consideration fall cleanly into a small number of non-overlapping categories, then categorization may not be too disputable. When overlap is considerable, however, then it becomes debatable whether such features of organisms can even provide useful information for systematic purposes (see also comments in Garland and Adolph, 1994, pp. 817-821).

Irrespective of how characters are coded, for a given data set, several or even many different topologies may be equally parsimonious, leaving the investigator with ambiguity as to which particular tree should be preferred. This ambiguity can stem from the data (they may contain insufficient information to allow complete resolution of all relationships, or they may imply no unique resolution) and/or from the methods used to draw inferences from the data. Ways of dealing with these sorts of uncertainty are debatable and beyond the scope of this chapter. As well, in addition to parsimony, which itself has multiple variants (Maddison, 1994; Maddison, 1995; Edwards, 1996), many other ways of constructing and/or choosing phylogenetic trees are available, such as maximum likelihood. For all of these issues, we must refer the reader to other literature (e.g., see Wiley, 1981; Friday, 1987; Felsenstein, 1988a,b, 1992; Sober, 1988; Lynch, 1989; Sarich *et al.*, 1989; Springer and Krajewski, 1989a,b; Goldman, 1990; Mayr and Ashlock, 1991; Miyamoto and Cracraft, 1991; Wiley *et al.*, 1991; Lapointe and Legendre, 1992, 1995; Lapointe *et al.*, 1994; de Queiroz *et al.*, 1995; Hillis, 1995; Purvis, 1995; Zelditch *et al.*, 1995; Hillis *et al.*, 1996; Huelsenbeck *et al.*, 1996; Huelsenbeck, Hillis, and Jones, 1996; Lee and Spencer, this volume; Laurin and Reisz, this volume).

As noted in the Introduction, we are here concerned not with inferring phylogenetic relationships but, rather, with mapping characters onto independently-derived hypotheses of phylogenetic relationships (see de Queiroz, 1996). Our mapping of a character (in our case, plasma osmolarity) will not be used to alter the phylogeny itself. The phylogeny that we will use for analysis is taken from the literature, as an informal synthesis of available information from many different sources (as, for example, in Garland *et al.*, 1993; for a more formal type of synthesis, see Purvis, 1995).

Parsimony as a principle can be used to guide how we map characters onto a phylogenetic tree. It would suggest finding that reconstruction (i.e., set of values at interior nodes) that minimizes the amount of change of the character of interest across the entire phylogenetic tree (also termed "character optimization"). For discretely valued characters, this can be termed Manhattan, Wagner, absolute-change, or linear parsimony. For a given character and a given phylogenetic tree, it is often the case that several or many different parsimony reconstructions can be found which result in the same sum of absolute change (Losos, 1990; Dial and Grismer, 1992; Maddison, 1994; Maddison, 1995; Miles and Dunham, 1996; Butler and Losos, in review). Thus, the most parsimonious reconstruction for a given internal node may be a range of character values. Linear parsimony can be used only with dichotomous trees (Maddison and Maddison, 1992, p. 304). Moreover, different types of parsimony exist, including (1) variants that require a priori

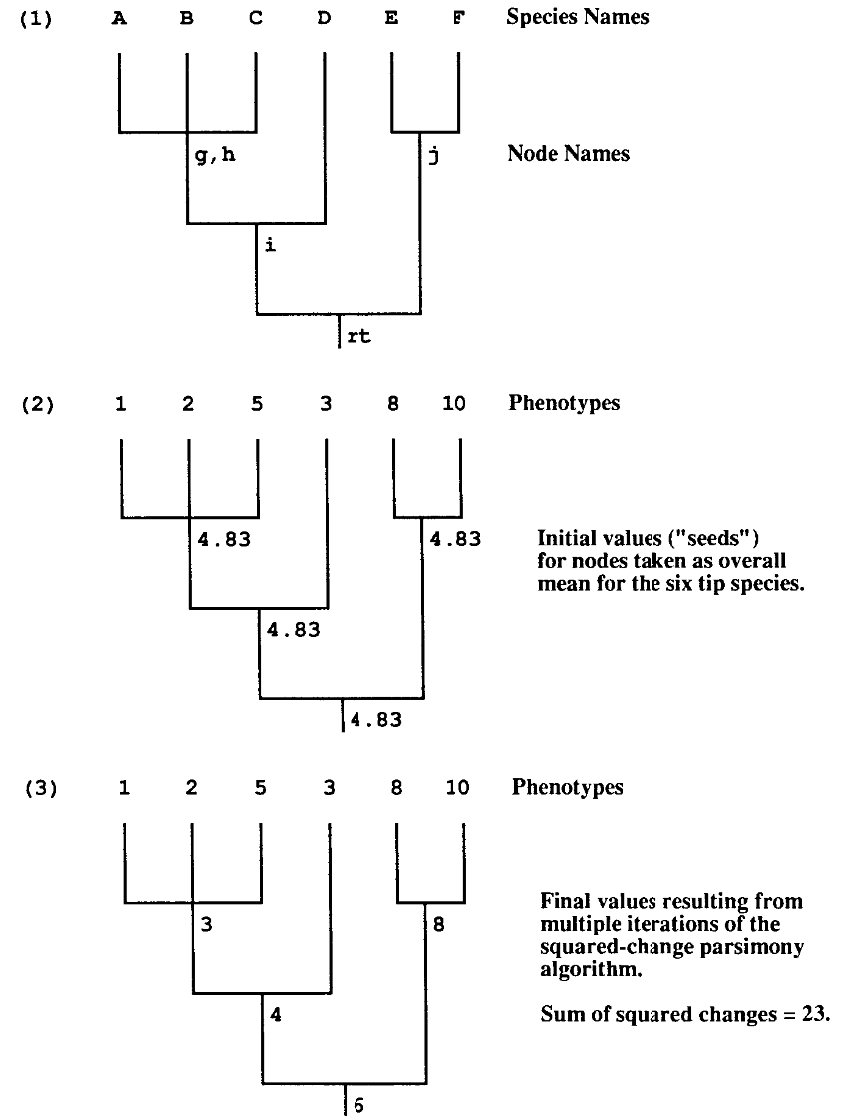
specification of the character state at the root of the tree or not and (2) variants that allow reversals of character state or not (Maddison and Maddison, 1992; Maddison, 1994).

APPENDIX 3: SQUARED-CHANGE PARSIMONY

For continuous-valued characters, another procedure exists for mapping characters onto specified phylogenetic trees. This algorithm obviates the need to code different taxa as having one of two or more discrete values. It is now most commonly termed "squared-change parsimony," although the terms "minimum evolution" and "minimum squared-change evolution" are also used (Huey and Bennett, 1987; Losos, 1990; Maddison, 1991; Martins and Garland, 1991; Maddison and Maddison, 1992; Walton, 1993; McArdle and Rodrigo, 1994; Westneat, 1995; Miles and Dunham, 1996). Unlike linear parsimony, squared-change parsimony can reconstruct values at internal nodes as having intermediate states not observed in the actual data (the terminal taxa or tips). An interesting area for future research with continuous-valued characters will be to compare squared-change parsimony with (1) linear parsimony and (2) linear parsimony applied after the character has been coded into a small number of discrete categories (cf. Huey and Bennett, 1987; Losos, 1990; Dial and Grismer, 1992; Miles and Dunham, 1996; Butler and Losos, in review).

Squared-change parsimony is relatively easy to implement through an iterative algorithm as used herein, through a recursive algorithm (Maddison, 1991), or by direct computation (McArdle and Rodrigo, 1994). The iterative algorithm works as follows. First, values for the phenotypes of a series of species are placed onto the tips (terminal nodes or taxa) of a phylogenetic tree. Second, arbitrary values are placed (seeded) at each of the internal nodes of the tree, including the root (or basal) node. Typically, the overall mean value of the tips is used to seed the nodes (so that convergence occurs more rapidly), but any arbitrary value can be used with no effect on the final results. Third, working from one side of the tree to the

Figure A1. Illustration of the squared-change parsimony algorithm. (1) An hypothesis of phylogenetic relationships for six extant species, named A-F. Internal nodes on the tree are given the names g, h, i, j, and rt (for root). Note that nodes g and h are effectively a single node in a polytomy, because one of the internode branches has been set to zero length; this "hard" polytomy indicates a multiway speciation event. (2) The hypothetical measured phenotypes for a continuous-valued character (e.g., body size, plasma osmolarity) for each of the six species (shown as integers only for simplicity). The algorithm begins by "seeding" each of the nodes with an arbitrary value for its phenotype; in (2) the simple mean of the six extant species is used. (3) Final results of the iterative algorithm (i.e., after values converge); these values at the nodes are those which minimize the sum of the squared changes (23 in this example) over the entire tree. The branch lengths shown here are arbitrary; for computations, all were set to equal length one. ➔



other, then back and forth many times, the nodal values are replaced with the average of the values of all adjacent nodes. [If branch segment lengths are not all set equal to one, then this average is computed by weighting by the reciprocal of the branch lengths (see Maddison, 1991; Martins and Garland, 1991).] In a fully bifurcating phylogenetic tree (no polytomies), each node is thus replaced with the average of the three adjacent nodes (which may be tip nodes or internal ones), except for the root node, which is replaced with the average of its two adjacent nodes. At each pass through the tree, the nodal values will change. Eventually, however, the magnitude of the changes becomes smaller and smaller, and the entire set of nodal values converges on the set of values which minimizes the (weighted) sum of the squared changes over the entire tree. These values are unique; no other values for any or all of the nodes would yield a smaller sum of squared changes over the whole tree. This procedure for finding a set of nodal values was hence termed "minimum evolution" by Huey and Bennett (1987; and also by Martins and Garland, 1991; Garland *et al.*, 1991), who first employed it in the present context, but is more accurately termed "squared-change parsimony" (or weighted squared-change parsimony: Maddison, 1991).

Squared-change parsimony also works with "hard" polytomies, reflecting true, simultaneous multiway speciation events. With hard polytomies, a node is again reconstructed as the (weighted) mean of all of its adjacent nodes, which may be more than three (or, for the root node, more than the usual two). A program that computes squared-change parsimony reconstructions with hard polytomies (PDSQCHP) is available from the senior author. As mentioned in the Discussion, formal ways of dealing with the uncertainty represented by "soft" polytomies (which reflect lack of knowledge about the true topology) have yet to be developed.

All parsimony procedures (including those for discrete characters) share the characteristic that they cannot possibly reconstruct values at interior nodes that are outside of the range of values observed at the tips of the tree. This is an unrealistic limitation in some cases, because evolutionary trends may actually result in terminal taxa (e.g., species alive today) that have phenotypes very different from those of their ancestors (e.g., hypothetical example in Fig. 10 of Grafen, 1989; real example in Fig. 2 of Garland *et al.*, 1993). In the context of a given data set, the only way to increase the possible range of ancestral values reconstructed by parsimony procedures is to add additional outgroup taxa to the data set.

A Simple Example of Squared-Change Parsimony

Figure A1 illustrates the squared-change parsimony algorithm with a simple example, including a polytomy (a node with more than two descendants). (For simplicity, we have set all branch segments equal to unity for computations.) Note that the value reconstructed for node **i**, 4, is somewhat different from the simple mean of the six tip species (4.83). The value reconstructed at the root of the tree, 6, differs even more from the simple mean of the tip values.

Justification of Squared-Change Parsimony

Although the procedure has been used many times, the general justification of squared-change parsimony has not been thoroughly considered (see Huey and Bennett, 1987; Losos, 1990; Harvey and Pagel, 1991; Maddison, 1991; Martins and Garland, 1991; Maddison and Maddison, 1992; Pagel, 1993; McArdle and Rodrigo, 1994; Miles and Dunham, 1996). Under a Brownian motion model of character evolution, squared-change parsimony reconstructions are "similar but not equivalent to a maximum-likelihood estimate" (Huey and Bennett, 1987, p. 1103; see also references in Edwards, 1996). This claim applies if the branch lengths used in computations are in units equal or proportional to expected variance of character evolution (Felsenstein, 1985; Martins and Garland, 1991). In the example shown in Figure A1, all branch lengths were effectively set to one for computations. With variable branch lengths, the algorithm also works, but nodal values are computed as weighted averages, with weighting based on the reciprocal of the branch lengths connecting each node to its adjacent nodes (see Maddison, 1991; Martins and Garland 1991). Brownian motion evolution with all branch segment lengths set equal to unity is equivalent to a "speciational" model of character evolution (termed "punctuational" in Huey and Bennett, 1987; Martins and Garland, 1991) (for more discussion of alternative models for simulating character evolution, see Garland *et al.*, 1993; Bjorklund, 1994; Díaz-Uriarte and Garland, 1996).

According to Maddison (1991, pp. 311 and 312), the squared-change parsimony reconstruction has maximum posterior probability under a Brownian motion model. In other words, minimizing the sum of (weighted) squared changes maximizes the Bayesian posterior probability (i.e., the probability of the reconstructed ancestral character states, given the tip data). (Maximizing the posterior probability is not the same as maximum likelihood estimation: the likelihood of the hypothesis is only one part of the posterior probability, the other two terms being the probability of the hypothesis and the probability of the data.) This, of course, is conditional on the correctness of the model of character evolution (Brownian motion), the topology of the tree, and the branch lengths (which should be in units proportional to expected variance of character evolution).

A closely related view as to the justification of squared-change parsimony stems from the more general context of fitting a model to some empirical data. We have a set of observed data, including the mean phenotypes of a series of species and the relevant phylogenetic topology and branch lengths. To these data we fit a model, i.e., the set of estimates for the phenotypes at all of the internal nodes. We should prefer that model which "best" fits the data (i.e., is most consistent with the data). If our operational definition of "best" is the smallest amount of squared change summed over the whole tree, then we can define "parsimony" in terms of consistency between model and data.

Finally, squared-change parsimony might be justified in any particular application by performing computer (Monte Carlo) simulations and comparing its performance with that of other comparative methods (e.g., Martins and Garland, 1991; Martins, 1996a). This type of justification would, of course, depend on the simulation parameters (including the model of evolution, topology, and branch lengths) being reasonable for the character(s) under study. We have performed one such set of simulations, and the results are described in the next section.

Although they should not be seen as justifications per se, squared-change parsimony also possesses some convenient properties. First, unlike the linear-parsimony alternatives (Butler and Losos, in review), it provides a unique point estimate, a single number, for the value of a continuous-valued character at each of the interior nodes (hypothetical ancestors) on a phylogenetic tree. Indeed, Losos (1990, p. 387) stated that "The major advantage of the squared-change parsimony approach is its analytical tractability." Second, squared-change parsimony is relatively easy to apply. Solutions can be found within minutes or hours, even for hundreds of taxa, with existing computer programs and personal computers. Like it or not, ease of application drives many decisions regarding analytical procedures.

Computer Simulations to Test Performance of Squared-Change Parsimony

We compared how well squared-change parsimony estimates the value at the root node of Figure 1 with the nonphylogenetic alternative of simply computing the mean value for all 172 taxa. We used the PDSIMUL program of Garland *et al.* (1993) to simulate simple Brownian motion character evolution (no limits on how far the phenotype could evolve), with all branch lengths set equal to one (this can be called "speciational" Brownian motion). We started each simulation at a value of 416.55, which is the simple mean of all 172 taxa. For each of 1,000 simulated data sets, we then used the PDERROR program of Díaz-Uriarte and Garland (1996) to compute (1) the simple mean of the simulated tip data and (2) the squared-change parsimony value at the root node. We focused on the root node for convenience: the estimate for the root node is the same for squared-change parsimony and for independent contrasts (see next section), and the latter is much faster to compute (and what PDERROR actually produces).

The simple means of the 1000 simulated data sets ranged from -105 to 955, with an overall mean of 416.10 (+/- standard error = 4.623, variance = 21,371). The squared-change parsimony reconstructions spanned a narrower range, from 167 to 636, with an overall mean of 416.67 (+/- standard error = 2.408, variance = 5,800). Obviously, the means of these distributions do not differ significantly from the true value at the root of the tree (416.55), so both yielded unbiased estimates of the value at the root of the tree. However, comparison of the standard errors (or variances) indicates that the spread of the distributions of the two estimators was very different. The squared-change parsimony reconstruction is much less variable, and hence performs much better.

Figure A2. Worked examples of fixing nodal values when using the squared-change parsimony algorithm. Node **i** (names shown in panel (1) of Fig. A1) is fixed at different values, and the algorithm is applied. When one node is thus fixed, reconstructed values at other nodes are different than when all nodes are free to change (see Fig. A1). Moreover, the sum of squared changes over the whole tree is always greater when any node is fixed at other than its freely-reconstructed value (in Fig. A1, node i was reconstructed as a value of 4, and the sum of squared changes was 23). See Fig. A3 for a graphical representation of this effect. ➔

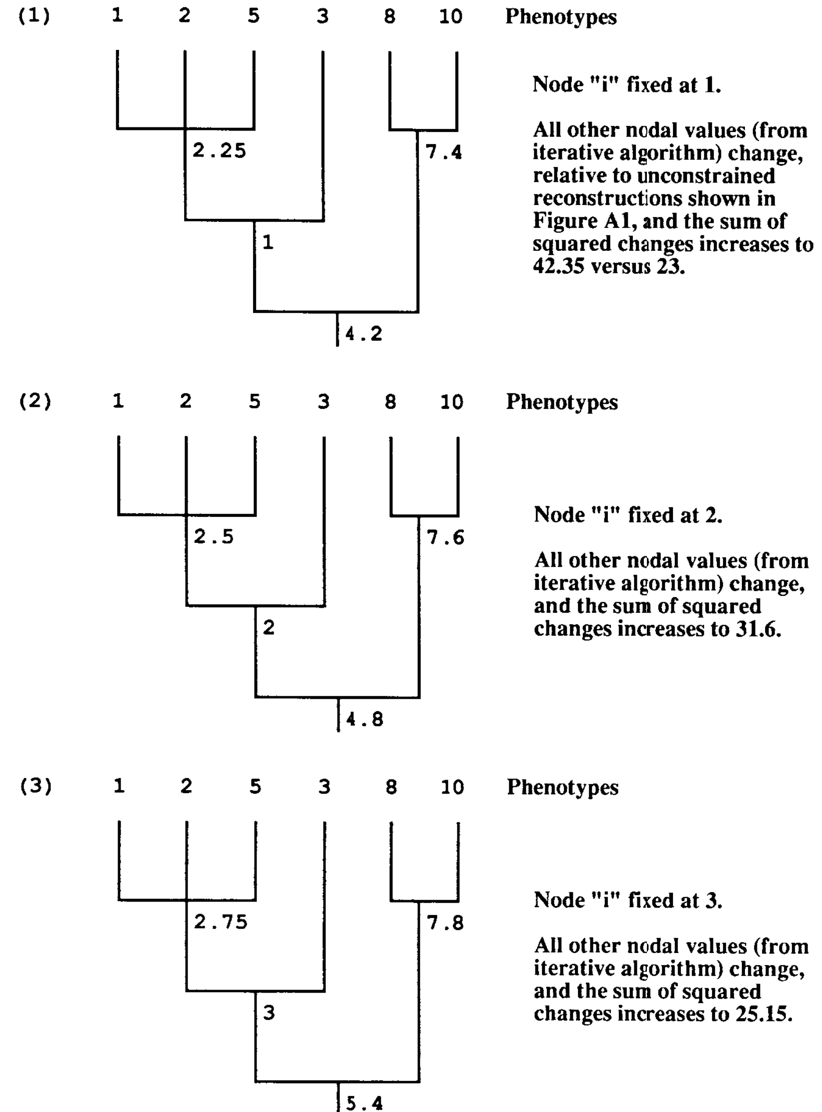


Figure A2; Panel 1.

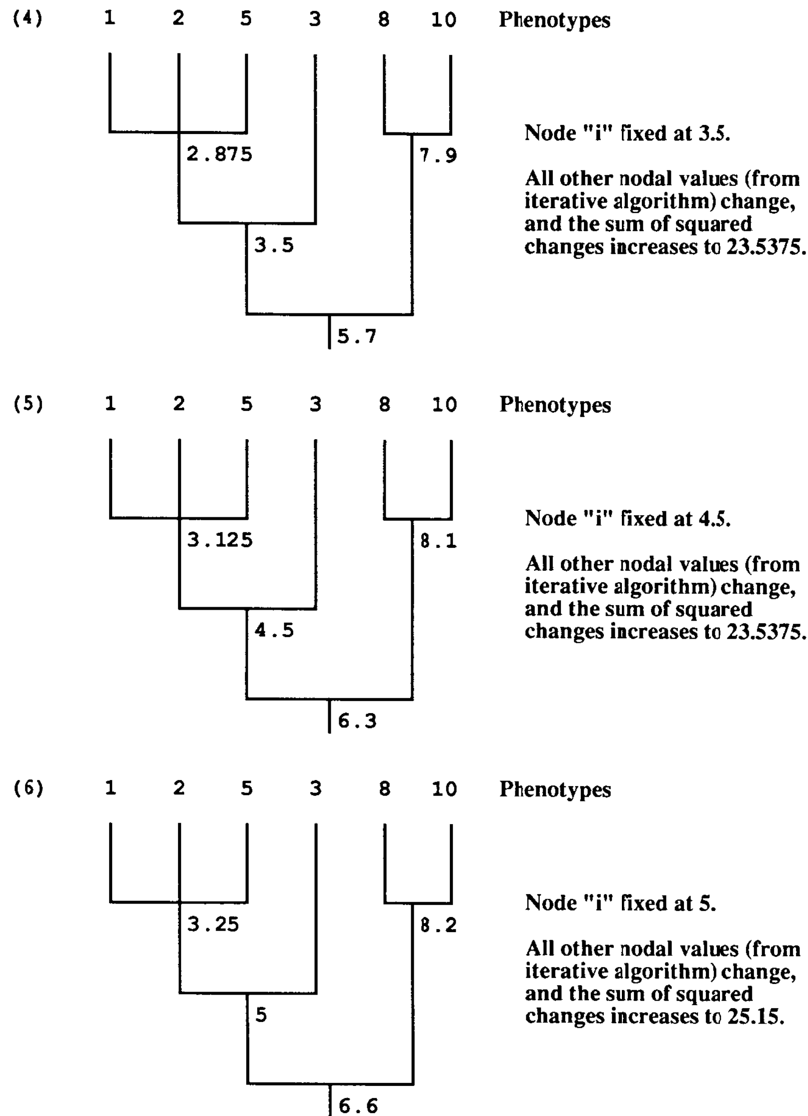


Figure A2; Panel 2.

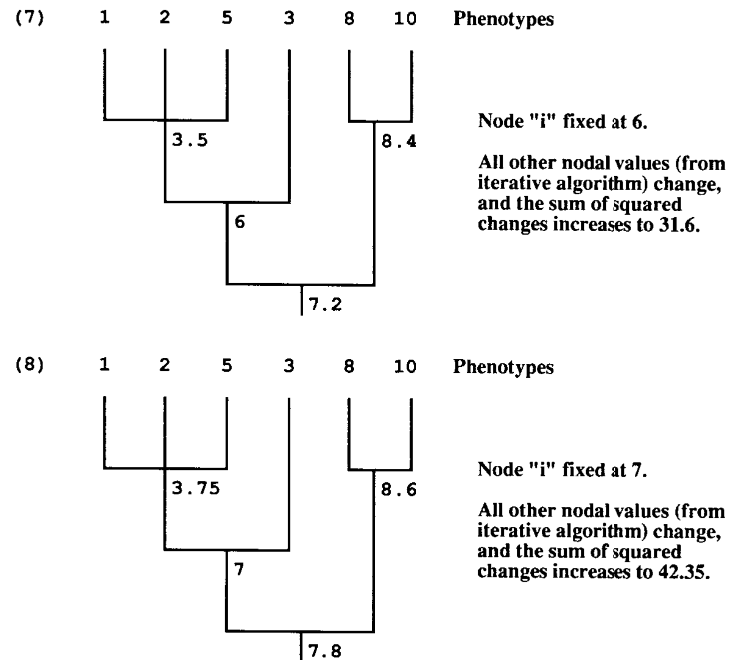


Figure A2; Panel 3.

In the foregoing simulations, simple Brownian motion models a character that is equally likely to increase or decrease in value at any point during its evolution; the magnitude of possible changes is set proportional to the length of the branches on the phylogeny (which can be set to equal length, as we have done, or can be of variable length); the magnitude of possible increases and decreases is also set to be equal, on average, so no overall trends in evolution will occur (except by chance); and no limits are placed on how far the character can evolve. This is the simplest possible model for the evolution of continuous-valued characters, and is the usual starting point in simulation studies. Brownian motion is considered to be a reasonable model for phenotypic evolution by random genetic drift and for evolution in response to certain forms of stochastically changing selection (see Felsenstein, 1985, 1988a).

Obviously, however, Brownian motion may not be a realistic model for the evolution of plasma osmolarities in vertebrates. For example, plasma osmolarity does not evolve without bounds (e.g., see Fig. 2; also note that negative values are physically impossible). Consequently, we also performed a second simulation, intended to be more biologically realistic. Many additional simulations are possible (e.g., see Garland *et al.*, 1993; Díaz-Uriarte and Garland, 1996), but are beyond the scope of this chapter. This second simulation was like the first, except that initial

values were set at 365 mOsm (this is the value reconstructed by squared-change parsimony for all 172 taxa; see Fig. 3) and desired final values at 416.55 (the actual simple mean for all 172 taxa), such that a trend for increasing plasma osmolarity was modeled. In addition, we used the "Replace" option of PDSIMUL to put limits on how far the trait could evolve, equal to the range of values in our real data set (181 and 1118; see Fig. 2). (The desired variances across the tips of the simulated data sets were left to be the same as the actual data, which is the default in PDSIMUL.)

With the more complicated model, simulating an evolutionary trend and limits, the squared-change parsimony estimates of the root value were again superior to the simple mean. The simple means ranged from 326 to 804, with an overall mean of 483.69 (+/- standard error = 2.913, variance = 8487). The squared-change parsimony reconstructions spanned a slightly narrower range, from 272 to 693, with an overall mean of 409.01 (+/- standard error = 2.000, variance = 3999). The means of both of these distributions differ significantly from the true value at the root of the tree (365); thus, both estimators were biased and tended to overestimate the value at the root of the tree. The squared-change parsimony estimator was, however, considerably less biased and also had a lower variance.

Squared-Change Parsimony and Independent Contrasts

Squared-change parsimony is not the same as another comparative method used commonly with continuous-valued characters, Felsenstein's (1985) phylogenetically independent contrasts (see Grafen, 1989; Losos, 1990; Martins and Garland, 1991; Garland *et al.*, 1992, 1993; Bjorklund, 1994; McPeek, 1995; Díaz-Uriarte and Garland, 1996; Martins, 1996a; Martins and Hansen, 1996). Squared-change parsimony is a "directional" comparative method (*sensu* Harvey and Pagel, 1991), whereas independent contrasts is a "nondirectional" or "cross-sectional" (Pagel, 1993) method. Directional methods reconstruct ancestral states at each node of a phylogeny and then compute changes between the inferred nodes and other nodes, including the measured tip values. Independent contrasts, on the other hand, uses phylogenetic information (topology and branch lengths) to transform the tip data into a set of N-1 contrasts (worked examples in Garland and Adolph, 1994) that are independent and identically distributed (at least in principle), and which can then be used in many conventional statistical procedures. Independent contrasts also involve calculation of values for interior nodes as an intermediate part of its computations. These nodal values constitute what can be termed "local parsimony" reconstructions, but they do not provide the globally most parsimonious solution because only the information from daughter nodes is used to estimate a given node (Maddison, 1991). Independent contrast nodal values are computed in this way because the goal is to provide independent data points that can then be used in subsequent statistical analyses. The nodal values from squared-change parsimony, on the other hand, are not statistically independent, because the value of each affects the value of all others (for example, Fig. A2). Independent contrast nodal values do not have any of the properties discussed above under the section titled "Justification of Squared-Change Parsimony," therefore they should not be used as estimators of ancestral values per se.

One special case exception exists. The value reconstructed at the root (basal) node is identical for independent contrasts and squared-change parsimony. This makes sense, because the local parsimony of independent contrasts is the same as global parsimony when the entire tree is considered (i.e., from the root node through all descendants). Computer programs for independent contrasts are much faster than those for squared-change parsimony, therefore if one is interested only in the root node, then the former can be used (e.g., PDTREE of Garland *et al.*, 1993; PDERROR program of Díaz-Uriarte and Garland, 1996; others cited in Martins and Hansen, 1996).

Recently, Ryan and Rand (1995; see also Ryan, 1996) have used nodal values from independent contrasts in preference to those from squared-change parsimony, but this practice is unjustified. Ryan and Rand (1995) stated that the two analyses gave similar results in their application, but this will not always be the case.

Indexing Uncertainty in Squared-Change Parsimony Reconstructions

As noted above, linear parsimony reconstructions of discretely valued characters often yield ambiguous values for nodes (Maddison, 1994, 1995). This may seem inconvenient, but it is also useful as an indicator of our incertitude in the reconstructed values (see Appendix 2 in Losos, 1990; Butler and Losos, in review). Recently, Maddison (1995) has discussed ways of calculating probability distributions of ancestral states reconstructed by linear parsimony, given the assumption of a stochastic model of character evolution (see also Schultz *et al.*, 1996). Comparable methods have not yet been fully developed for squared-change parsimony reconstructions, but several heuristic approaches are possible. We explain and then apply two of them, and consider some other possibilities under Discussion. Here we assume that the only data available to the comparative biologist are estimates of the mean phenotype for a series of taxa (the tip values); if estimates of the phenotypic variances for each species are available, then additional procedures are possible (Lynch, 1991; Maddison, 1991; Martins, 1994; McArdle and Rodrigo, 1994; J. Felsenstein, pers. comm.; see also Chevalier, 1991).

Fixing Interior Nodes.–One approach was developed by Huey and Bennett (1987; see also Chevalier, 1991) and is illustrated in Figures A2 and A3. This approach involves fixing a node at an arbitrary value, one other than that which the (unconstrained) squared-change parsimony algorithm delivered. The squared-change parsimony algorithm can then be rerun, but with the value at one (or more) node(s) constrained not to change. The result of this constrained character optimization will always be higher values for the (weighted) sum of the squared changes over the whole tree.

Figures A2 and A3 illustrate that, for node i, fixing it at any value other than 4–the one reconstructed by the unconstrained squared-change parsimony algorithm (Fig. A1)–results in different values at all other internal nodes and a greater amount of squared change over the entire phylogenetic tree. The same principle would hold for any other internal node. If we have reason to believe that

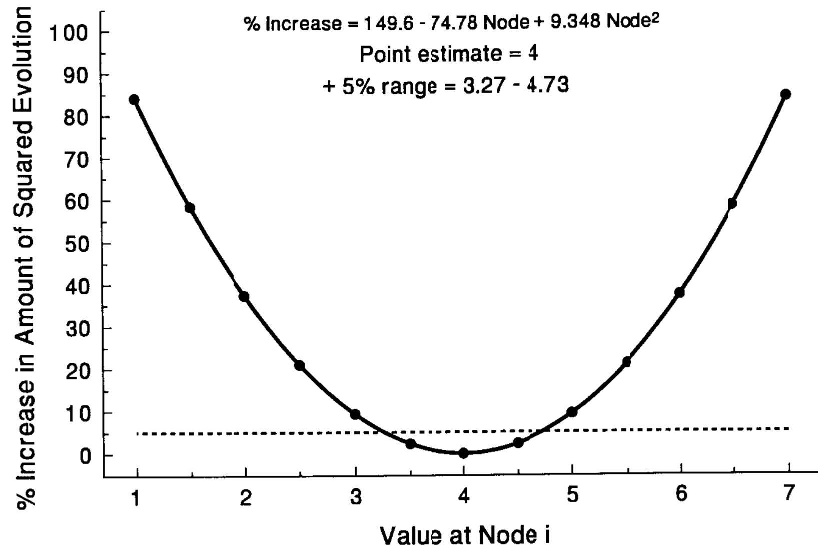


Figure A3. Graphical summary of consequences of fixing nodal values when using the squared-change parsimony algorithm (from Fig. A2).

greater amounts of squared change are less likely, then these other values for node i should themselves be considered less likely to accurately reflect the true (but unknown) value for that ancestral node.

Figure A3 (like Fig. 4 of Huey and Bennett, 1987) shows that over a range of values around the point estimate of 4 the sum of squared changes over the whole phylogeny does not increase very much. For example, in the range 3.27-4.73, the amount of squared evolution increases by only 5%. We caution readers that these intervals cannot be interpreted as "confidence intervals" in the conventional statistical sense.

It is also possible to consider the consequences of fixing a node at a value that is actually outside of the range of values observed for the tips. For example, if we fix node i at a value of 11, the sum of squared change increases to 128.35, or 458% greater than if the value of node i is 4. In general, we can consider any value as a possibility for any ancestral node, but some of these hypothetical values would require there to have been a relatively huge amount of evolution, as compared with the minimum possible, which occurs when all nodes are taken as the values reconstructed by unconstrained squared-change parsimony. In real examples, some values may not be physically (e.g., body mass or plasma osmolarity less than 0) or

biologically possible (e.g., vertebrate body temperature greater than about 50 Celsius), and so can be excluded a priori from consideration.

Jackknifing.–Another heuristic way to index uncertainty (or dispersion) in nodal values reconstructed by squared-change parsimony is to use the statistical procedure called jackknifing (Sokal and Rohlf, 1981, pp. 795-799; Manly, 1991; Crowley, 1992; Efron and Tibshirani, 1993). Jackknifing is a way of using the observed data points themselves to gauge the uncertainty in some statistic that can be computed from the data. The general procedure is to first compute the statistic of interest, such as the mean, based on all N data points. As in ordinary statistics, this estimate based on the entire sample is taken as the best estimate of the statistic of interest.

The second step in jackknifing is to recompute the statistic N times, based on deleting each data point from the sample in turn. This is termed resampling. [This "delete-one jackknife" is common and computationally the simplest, but greater numbers of data points can also be deleted and doing so provides better results in some cases (e.g., see Wu, 1986, and discussions following]. The deletion of data points is always done without replacement, unlike the more general method termed "bootstrapping" (see Efron and Tibshirani, 1993).] Thus, if we had a sample of 100 data points, we would recompute the mean 100 times after deleting each of the data points in turn. We would then compute "pseudovalues" for each of these as (N * mean for whole sample) - (N-1) (mean with one data point deleted). The approximate standard error of our original mean is then taken as the square root of (variance of the pseudovalues/N). This approximate standard error can then be used to set confidence intervals by reference to a t-distribution; degrees of freedom are usually assumed to be N-1. In the text, we present the jackknifing computations for the ancestral amniote's plasma osmolarity, as reconstructed by squared-change parsimony. Again, however, this is only a heuristic device, because the degrees of freedom associated with the computations are generally unknown in the face of phylogenetic relatedness and hence nonindependence of the tip values (e.g., see Harvey and Pagel, 1991; Martins and Garland, 1991; Garland et al., 1993; Pagel, 1992, 1993). In addition, the validity of jackknifing needs to be proved analytically or justified by computer simulation for each different type of application (Efron and Tibshirani, 1993).

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