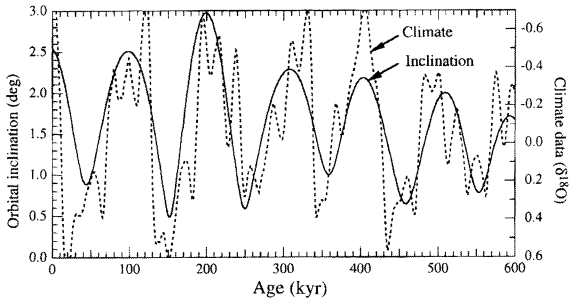

Comparison of orbital inclination (solid line, lagged by 33 kyr) and $\delta^{18}O$ climate data (dotted line) from SPECMAP[5].

reflect the percentage of the Earth's water frozen in ice. The figure shows $\delta^{18}O$ (dotted line) for the past 600,000 years from the SPECMAP compilation of data from five sea-floor sediment cores[5]. The figure also shows the orbital inclination (solid line), calculated by direct integration of planetary perturbations[6], transformed to the invariable plane (the plane of symmetry of the Solar System), and shifted to give the best least-squares fit to the $\delta^{18}O$ data. (Only three parameters were adjusted, one for the delay and two for the overall scale.) For the best fit, i preceded $\delta^{18}O$ by 33±3 kyr; as this is positive, there is no causality problem. Similarly, the presence of a strong variation in i near 400 kyr solves the Stage-11 problem.

The existence of the 100-kyr cycle of orbital inclination does not seem to have been noticed previously by climatologists or astronomers. It may have been missed for two reasons. Ever since Milankovitch, the implicit assumption has been that insolation is the driving force for climate cycles, and insolation is not directly affected by orbital inclination. Second, the 100-kyr cycle is not evident when i is calculated in the usual reference frame based on the present orbit of the Earth. Only when transformed to the invariable plane (or a plane near it) does the 100-kyr cycle unmix from the obscuring effect of a strong 70-kyr orbital precession cycle. We note that a 70-kyr cycle has been reported in $\delta^{18}O$ data from other sedimentary samples[7], and we suggest that this cycle may be related to orbital precession.

The only mechanism we have found that could link orbital inclination to climate is extraterrestrial accretion of meteoroids or dust. Such material can be detected in ice and sedimentary rock by analysis of iridium; Walter Alvarez has pointed out that extraterrestrial dust cycles could be detected using ^{3}He. If this mechanism is correct, a 100-kyr cycle should be seen in ice and sediment records of extraterrestrial accretion.

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Uncertainty in ancient phylogenies

SIR — Use of phylogenetic methods to estimate ancestral phenotypes is becoming widespread in evolutionary biology[1]. For example, Jermann *et al.*[2] estimated and then synthesized ribonucleases of early artiodactyl ancestors, beautifully demonstrating the power of these methods for elucidating molecular evolution. Essential to and frequently missing from such studies is a measure of the statistical uncertainty of estimated ancestral states needed to gauge their reliability. I have used a maximum-likelihood (ML) method to estimate the amino acid at position 38 of artiodactyl ribonuclease, the residue found[2] to be most crucial to enzyme catalytic activity*.

The method applies the Markov model of trait evolution[3], assuming that the rates of change between states are constant through time and over all branches of the phylogenetic tree. A given trait may have two states, i and j. The transition rate from state i to state j over an infinitesimally short time period is q_{ij}. This parameter and its converse, q_{ji}, can be estimated directly from data on modern species and their phylogenetic relationships. The ML estimates for the rates[3] are obtained by maximizing:

$$L(q_{ij},q_{ji})=\sum_{X_1=i}^{j}\sum_{X_2=i}^{j}\cdots\sum_{X_n=i}^{j}(P[S_1,S_2,\ldots,S_m,X_1,X_2,\ldots,X_n])$$

The term in parentheses on the right is the probability of arriving at the given trait values S of the m modern species when trait values at the n interior nodes (ancestors) are $X_1, X_2, \ldots, X_n$ (ref. 3). This equation can also be used to compute likelihoods of alternative states for any single ancestor as the portion of the sum contributed by each state at the given node. The state having highest likelihood is the ML ancestor state, conditional on the estimated values of q_{ij} and q_{ji}. The ratio of the two likelihoods measures the level of support for the ML estimate[4]. The procedure is similar for traits having three or more states[3]. The method evaluates each ancestor in turn, and while doing so assumes that possible states at remaining nodes have equal prior probability.

To simplify the analysis, I modelled only two states at position 38 of artiodactyl ribonuclease, Gly and Asp (denoted G and D in Fig. 1 of ref. 2), and I deleted the two species (nilgai and impala) having rarer amino-acid residues (Asn and Ser). This simplification does not affect ML ancestral states because the rates of transition between Gly or Asp and the rarer residues were estimated to be small, such that the likelihood of the rarer states in the ancestors was also small. It also does not significantly alter conclusions concerning the levels of support for Gly and Asp in the ancestors .

Computations were carried out using DISCRETE (ref. 3) and a reduced program developed independently. Maximum likelihood yielded $q_{\mathrm{Gly,Asp}} = 0.0164$ and $q_{\mathrm{Asp,Gly}} = 0.0102$. Thus, the half-life of Gly (the time interval over which a lineage in state Gly has a 50% chance of changing to state Asp) was 42 Myr, and the half-life of Asp was 68 Myr. Total tree length was 450 Myr (ref. 2); change at residue 38 is therefore not expected to be rare on this tree. These estimates are derived solely from the data on position 38 in artiodactyls rather than from data on all sites or for proteins in general[5]. This avoids the unnecessary assumption that transition rates and states at position 38 of artiodactyl ribonuclease are typical of sites on this or other proteins.

Asparagine was estimated to be the most likely state in all ancestral artiodactyls with one exception (the immediate ancestor to the two camel molecules, which was estimated to be Gly). This contrasts with the results from parsimony[2] which estimated that Gly was the residue at position 38 of the three most ancient species. More significantly, the uncertainty of the ML estimates for these three ancient nodes was high; likelihood ratios for all three were less than 1.4. By comparison, support limits for a ML estimate (analogous to 95% confidence intervals) generally encompass all values whose ln(likelihoods) are within 2 units of the maximum[4], corresponding to a likelihood ratio of e^2=7.4. Similarly, a likelihood ratio of 6.82 (corresponding to a χ_1^2=2 ln(6.82)=3.841) is required to reject a statistical null hypothesis[6]. Consequently, both states for early artiodactyls, Gly and Asp, are highly compatible with the data from contemporary species. In particular, a transition from Gly to Asp between the ancestors **g** and **h** is not supported by the likelihood analysis.

Variations on the above method are possible that differ in the degree to which likelihoods for a given state are conditional on the values of additional parameters in the likelihood model. For example, the states of all ancestors may be estimated

*J. Felsenstein (University of Washington) has independently developed a similar method for estimating ancestral nucleotide sequences at interior nodes of a ML phylogeny and Z. Yang (Pennsylvania State University) has recently developed an alternative bayesian approach.

simultaneously by choosing the single combination of ancestral states making up the largest portion of the sum, L. Support for the ML estimate at a given node is then evaluated by comparing its likelihood with that of the alternative state at the same node computed using the most likely arrangement of states at remaining nodes. The results were similar: Asp was the most likely state in early artiodactyls; likelihood ratios for the three ancients **h**, **i** and **j** were <2.6. I also tried another procedure in which the transition rates $q_{Gly,Asp}$ and $q_{Asp,Gly}$ were no longer fixed, but could vary depending on the state of the ancestor of interest. The residue at a given node was set to Gly, and the ML estimates for transition rates were then recomputed to best accommodate that ancestral value before obtaining the corresponding likelihood. These steps were repeated with the same ancestor set to Asp. Asparagine was again the most likely state in all three oldest ancestors, but the likelihoods were less than 1.4 times better than those for Gly.

These results used data from the artiodactyls[2] alone, whereas the parsimony reconstruction included information from older branches[7]. For example, whales are the sister taxon to the artiodactyls[7,8] and the one species surveyed has Gly at position 38 (ref. 7). Adding this species did not change the likelihood results for the earliest artiodactyls, presumably because a single branch ~55 Myr provides little information. Asparagine was again the most likely state in the three early antiodactyls and support remained low (<1.7). The results were also little changed when horse and rodents were added as lower branches. Addition of these taxa confirm that rates of change between Asp and Gly are relatively frequent: the horse has Gly whereas rodents include some species with Gly and others with Asp[7] (the casiragua with Glu was deleted from this analysis). Uncertainty of states in early artiodactyls is thus little diminished by the addition of these other taxa. In another analysis, pancreatic ribonuclease alone was used, the protein sequence from bovine seminal plasma being deleted. ML estimates and level of support for remaining ancestors **i** and **j** were little affected.

The above calculations assume that the phylogenetic tree of relationships among artiodactyls[7] is correct, including branch lengths, but the phylogenetic tree is itself merely an estimate. It should be possible to incorporate likelihoods of alternative trees into the calculation of ancestral states. Such a procedure might produce ML estimates different from those presented above, but it is unlikely that levels of support will be much improved. Uncertainties over ancient residues at position 38 of ribonuclease are high in large part because of a relatively high rate of transition between alternative states coupled with long spans of time. These are undoubtedly features of the correct phylogenetic tree, since variation at position 38 is seen at all levels of evolutionary relationships among taxa[7].

Perhaps the greatest weakness of the above method is the assumption that rates of evolution are constant throughout the tree. In truth, these may differ from lineage to lineage and through time. Its advantage is that methods can be devised to test the assumption of constant rates, and also to fit more complex models in which rates are allowed to vary, although many attempts to do this here did not reduce the uncertainty of ancestor estimates.

These results show that estimates of ancient ribonuclease sequences are highly uncertain, at least at the most critical position 38. More generally, they show that ancient sequences can be estimated in a probabilistic framework, and that uncertainty of estimates can be quantified. Such information will be valuable when designing studies to reconstruct ancient molecules or other characteristics of early ancestors.

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BENNER *ET AL.* REPLY — We agree that maximum likelihood (ML) methods are valuable for reconstructing ancient forms of life. We do not agree, however, that such methods are "frequently missing from such studies". In 1992, the Computational Biochemistry Research Group at the ETH in Zürich produced a tool (DARWIN) that makes automatic ML reconstructions[9]. DARWIN yields probabilistic ancestral sequences where each residue is represented as a vector of unit length in 20 dimensions[10]. The components of the vector in each dimension reflect the probability that each of the 20 natural amino acids was present at this position in the ancestor. We have used DARWIN to reconstruct some 50,000 amino acids encoded by the protogenome[11] (the most recent common ancestor of archaebacteria, eubacteria and eukaryotes[12]), and to propose a model for the metabolism of this ancestor[13].

Nor do we agree that ML "estimates of ancient ribonuclease sequences are highly uncertain." In the ancestral ribonuclease near the divergence of the brain, seminal and pancreatic ribonucleases (corresponding in the fossil record approximately to the origin of ruminant digestion), DARWIN reconstructs a Gly at position 38 with 99.5% certainty if all available ribonuclease sequences are considered[7]. Over the entire ancestral sequence, DARWIN assigns 118 of the 124 residues with >95% probability. Only one residue (at position 102) is assigned with a probability below 50% (ref. 2).

Why are Schluter's conclusions different from those of DARWIN? It is difficult to say from the information available. Differences in reconstructions are most often traced to different connectivities in the underlying evolutionary tree, which need not be clearly defined. DARWIN allows maximum likelihood factors to influence the positions and lengths of branches of that tree. The ML analysis discussed by Schluter is only concerned with the sequence variation, and assumed a tree generated by parsimony methods. We are not sure of the implications of such a hybrid approach. All we can say is that DARWIN, using a ML approach, consistently yields an ancestral reconstruction at position 38 not remarkably different from those yielded by consistent application of parsimony, even though the preferred connectivity of the evolutionary trees differs. If one moves away from the point of transition (Gly 38→Asp 38), DARWIN places a Gly at position 38 in more ancient sequences with only low statistical uncertainty and Asp in more recent sequences, again with low uncertainty. If the remaining uncertainty is unacceptable, other ancient sequences could be prepared and studied (as in ref. 2), or additional data collected to define the tree more precisely.

The most important point to be made by recent work in palaeomolecular bio-

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Scientific Correspondence

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