

# RESURRECTING ANCIENT GENES: EXPERIMENTAL ANALYSIS OF EXTINCT MOLECULES

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There are few molecular fossils: with the rare exception of DNA fragments preserved in amber, ice or peat, no physical remnants preserve the intermediate forms that existed during the evolution of today's genes. But ancient genes can now be reconstructed, expressed and functionally characterized, thanks to improved techniques for inferring and synthesizing ancestral sequences. This approach, known as 'ancestral gene resurrection', offers a powerful new way to empirically test hypotheses about the function of genes from the deep evolutionary past.

## BILATERIAN

An animal that shows bilateral symmetry across a body axis. Bilaterians include chordates, arthropods, nematodes, annelids and molluscs, among other groups.

## ORTHOLOGUES

The 'same' gene in more than one species. Orthologues descend from a speciation event.

The evolution of gene function is a central issue in molecular evolution: by what mechanisms and dynamics have the diverse functions of modern-day genes emerged? This question is usually addressed by inferring past processes from extant patterns, using statistical methods to detect the traces of ancient evolutionary events in the sequences of modern-day genes (see, for example, REFS 1,2). Thanks to the recent surge in knowledge of structure–function relationships, evolutionists can better interpret indicative patterns — such as biases or changes in evolutionary rates — by focusing on specific parts of gene sequences that are most likely to change gene function (for some examples, see REFS 3,4). Despite the important insights that the statistical approach has made possible, however, this method remains inferential, without empirical tests to refute or corroborate the evolutionary hypotheses that sequence patterns suggest.

Recent advances in phylogenetics and DNA-synthesis techniques have made it possible to experimentally test molecular evolutionary hypotheses by resurrecting ancient genes in the laboratory. To resurrect a gene, the sequence of an ancient protein is inferred using phylogenetic methods, a DNA molecule coding for that protein is synthesized, the extinct protein is expressed *in vitro* or in cultured cells and its functions are assayed using molecular techniques. Half a dozen recent publications have reported the resurrection of ancestral genes from the distant evolutionary past, including

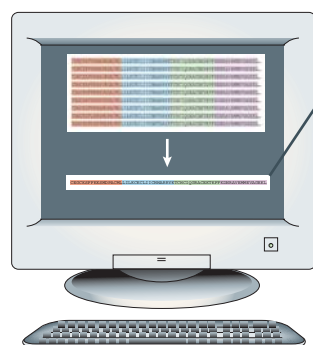
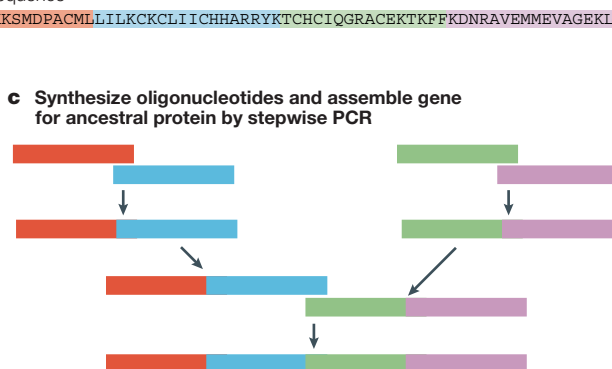
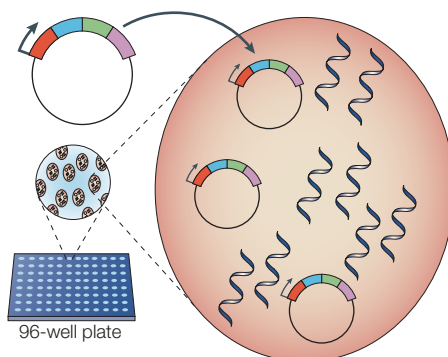
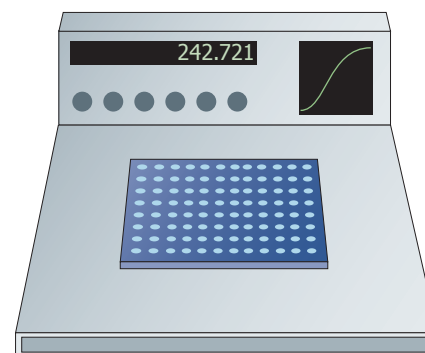
genes from the last common ancestors of bacteria, of BILATERIAN animals and of vertebrates. These studies have shed light on fascinating questions about primordial environmental adaptations and the evolution of crucial gene functions.

Here, I review ancestral gene resurrection, the technical advances that have made it feasible and the studies that have applied it. I discuss the historical development of the technique and its methodological basis, with an emphasis on the previously unattainable insights it has allowed. I also highlight the limitations and pitfalls of this strategy, which should be kept in mind when designing studies and interpreting results, and I conclude with suggestions for future extensions and applications of the technique.

## How to raise a gene from the dead

A gene resurrection study, as with all good science, begins with a question — in this case, one that could be answered if we knew the functions of the ancestral or intermediate forms that existed during the evolution of modern-day genes. For example, a question about the physiological or biochemical traits of an extinct species could be answered by resurrecting and studying the ORTHOLOGUE, in that species, of a gene that determines the trait in modern-day species. If the question concerns how a gene family and its functions diversified, then the ancestral gene that gave rise to the family through gene duplications can be resurrected and characterized, as

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**a Infer phylogenetic tree from aligned sequences and determine best-fitting evolutionary model****b Reconstruct protein sequence at ancestral node by maximum likelihood****c Synthesize oligonucleotides and assemble gene for ancestral protein by stepwise PCR****d Subclone assembled gene into vector, transform cultured cells and express ancestral protein****e Purify ancestral protein (if necessary) and characterize function using *trans*-activation, binding or other assay**

**Figure 1 | The ancestral gene resurrection strategy.** Flow chart of the stages required to resurrect and characterize an ancestral gene. A hypothetical protein from the ancestral vertebrate is shown as an example. See main text for details of each step. mya, million years ago.

**OUTGROUP SEQUENCES**

In phylogenetics, sequences that are known *a priori* to be more distantly related to the other sequences in the analysis (the ingroup sequences) than the ingroup sequences are to each other.

**CODON BIAS**

Preferential use of certain DNA codons over others that code for the same amino acid.

**BINDING ASSAYS**

A family of biochemical procedures used to determine the affinity and specificity with which a protein binds a specific ligand or substrate.

can ancestors from intermediate points during the proliferation of that family.

Resurrecting an ancestral gene involves five steps (FIG. 1). In the first step, sequences that are descended from the ancestral gene are obtained and aligned — along with OUTGROUP SEQUENCES — and the tree of their relationships is inferred (or imposed if it is known *a priori*). Amino-acid sequences are typically used because they contain less ‘noise’ than DNA sequences, which are more subject to convergence and reversal. Phylogenetic methods, such as maximum parsimony or maximum likelihood (ML; see later sections), are then used to infer the best estimate of the ancestral state for each sequence site given the present-day sequence data. In the second step, a DNA sequence that codes for the ancestral protein is inferred on the basis of the genetic

code; CODON BIAS for the system in which the gene will be expressed can be introduced to improve the translation rate. This coding sequence is produced *de novo* (the third step) by synthesizing overlapping oligonucleotides and assembling them by PCR or by restriction digest/ligation; site-directed mutagenesis can be used if the ancestral sequence can be made by introducing only a few changes into an extant gene. In the fourth step, the ancestral gene is cloned into a plasmid that allows high-level expression, and the plasmid is then transfected into bacterial or mammalian cells in culture. Finally, the ancestral protein can be purified if necessary and its functions characterized using experimental tests such as reporter-gene expression assays, ligand- or substrate-BINDING ASSAYS, or assays that measure enzyme specificity and turnover rate.

### Modernizing gene resurrection techniques

In 1963, **L. Pauling** and E. Zuckerkandl prophesied that it would be possible one day to infer the gene sequences of ancestral species, to “synthesize these presumed components of extinct organisms ... and study the physicochemical properties of these molecules”<sup>5</sup>. Not until 1990, however, were methods for ancestral sequence inference and DNA synthesis sufficiently mature to make the first such work possible (BOX 1). Five gene resurrection studies were published during the following seven years; all involved genes were from the relatively recent past, such as transposons in the genomes of mice and salmonids, and digestive RNases in ancestral artiodactyls (TABLE 1).

A hiatus of more than half a decade followed, in which no new gene resurrection studies were published. Recent advances in statistical techniques for ancestral sequence reconstruction, however, have allowed the gene resurrection strategy to be extended into much more ancient evolutionary time. Meanwhile, improvements in DNA synthesis, protein expression and functional characterization have made the approach more practical and accessible. As a result of these advances, half a dozen gene resurrections have been published in the past two years, addressing more ambitious evolutionary questions about evolutionary events that are far more ancient than could be studied using earlier methods.

**More accurate phylogenetic methods.** The advent of statistical techniques for inferring the sequences of ancestral genes is the most important development for modern gene resurrection studies. The first phylogenetic technique for inferring ancestral states was the

maximum parsimony method, which was developed in the early 1970s but not applied to or adapted for gene sequences until the first phylogenetic computer programs emerged in the mid 1980s (BOX 1). Maximum parsimony was an important advance over consensus methods, in which the most common state among extant sequences is assumed to be the ancestral state, irrespective of phylogenetic relations. The consensus approach is extremely sensitive to the sample of sequences chosen, however, and it will always err if a change from the ancestral state occurred on a deep branch that subtended a highly speciose group (BOX 2). To take a morphological example, the consensus method would lead to the inference that the vertebrate ancestor had jaws, because there are far more species of TELEOSTS and tetrapods than there are of (jawless) lampreys and hagfish. Despite its weaknesses, the consensus method was used in several early gene resurrection studies<sup>6,7</sup>.

Maximum parsimony, by contrast, takes account of the phylogenetic relationships among extant sequences. Simulation studies show that this feature makes maximum parsimony generally accurate and effective for sequences that are reasonably closely related to each other<sup>8</sup>. An empirical study of the accuracy of parsimony reconstruction is even more persuasive: in 1992, while seeking to develop a system to directly evaluate phylogenetic methods, D. Hillis and colleagues evolved an ‘experimental phylogeny’ of nine viral lineages in the laboratory by a repeated process of lineage splitting. They characterized the DNA of the terminal and ancestral viruses, used parsimony to predict the ancestral states and compared these inferences with the actual ancestral states. They found that parsimony accurately reconstructed 98.6% of all ancestral states<sup>9</sup>.

#### TELEOSTS

The class of bony vertebrate fish with ray-like fins and symmetrical tails. It includes the vast majority of marine and freshwater bony fishes.

#### PARSIMONY PRINCIPLE

The principle that the best-supported evolutionary inference is the one that requires the fewest number of character changes. This criterion rests on the assumption that identical character states among closely related species are more likely to have descended from the same state in the species’ common ancestor than to have evolved multiple times.

#### ARTIODACTYL

A member of the animal taxon that includes cows, sheep, pigs, giraffes, camels, oxes, whales, hippopotami and other two-toed hoofed mammals.

### Box 1 | Ancient history

The rise of ancestral gene resurrection has been long and slow. More than 40 years ago, Pauling and Zuckerkandl suggested the idea of resurrecting ancient genes<sup>5</sup>. The first important step towards realizing that vision was made in 1971 by W. Fitch, who developed the first phylogenetic algorithm to reconstruct ancestral character states on the basis of present-day states. Relying on the **PARSIMONY PRINCIPLE** and the theoretical work of **W. Hennig**, Fitch’s method determines the states at every internal node on a phylogenetic tree and places evolutionary changes on specific branches, so as to minimize the total number of changes that are required on the tree<sup>30</sup>.

In the 1980s — after computer programs such as **PAUP**<sup>31</sup> made Fitch’s algorithm tractable for gene sequences — several studies used the method to infer the sequences of ancestral genes (see, for example, REF 32). Not enough was known, however, about protein structure–function relationships to make these reconstructions particularly rewarding. It was not yet feasible to synthesize and characterize an ancestral gene, although at least two studies did analyse the functional effect of specific substitutions using site-directed mutagenesis of extant genes<sup>33,34</sup>. Meanwhile, many evolutionary biologists began to use ancestral state inference to understand the evolution of morphological and behavioural characters<sup>35</sup>, and a rich literature emerged on the power and pitfalls of Fitch’s method (see, for example, REFS 36,37).

In the same period, improved methods for DNA synthesis set the stage for the first realization of Zuckerkandl’s and Pauling’s vision. In 1990, **S. Benner** and colleagues<sup>38</sup> used the parsimony principle to infer the protein sequence of the highly conserved gene that encodes digestive ribonuclease from the last common ancestor of three organisms — swamp buffalo, river buffalo and ox — that lived 5–10 million years ago (mya). They produced DNA coding for this protein by sequentially ligating short (10–22 bases) overlapping DNA oligonucleotides, followed by site-directed mutagenesis<sup>39</sup>. The resurrected proteins degraded RNA at least as effectively as the extant proteins, demonstrating that it was possible to resurrect a fully functional ancestral gene. In subsequent work, Benner’s group resurrected a series of even more ancient ribonucleases from ancestral species dating up to 40 mya on the **ARTIODACTYL** phylogeny. They found that the emergence of digestive ribonuclease activity coincided with the appearance of difficult-to-digest grasses and cud-chewing digestion<sup>40</sup>.

Table 1 | **Ancestral genes resurrected\***

Extant genes	Ancestral gene resurrected	Approximate age of ancestor (years)	Inference method	Refs
Digestive ribonucleases	Ancestral orthologue in LCA of buffalo and ox	5–10 million	Parsimony	38
<i>L1</i> retroposons in mouse	Ancestral paralogue <sup>†</sup> in mouse genome	“several million”	Consensus	7
Digestive ribonucleases	Ancestral orthologue in LCA of artiodactyls	~40 million	Parsimony	22
Chymase proteases	Ancestral orthologue in LCA of mammals	~80 million	Parsimony	43
<i>Tc1/mariner</i> transposons	Ancestral paralogue in genomes of 8 salmonids	~10 million	Consensus	6
Immune RNases	Ancestral orthologue in LCA of ‘higher primates’	31 million	Parsimony, Bayesian distance	27
Pax <sup>§</sup> transcription factors	Ancestral paralogue (older than the protostome <sup>  </sup> –deuterostome <sup>¶</sup> ancestor)	600–1,000 million	Bayesian distance	26
Vertebrate rhodopsins	Ancestral orthologue in LCA of archosaurs <sup>¶</sup>	240 million	Maximum likelihood	17
Vertebrate short-wave rhodopsins	Ancestral orthologue in LCA of bony vertebrates	>400 million	Maximum likelihood	44
Steroid hormone receptors	Ancestral paralogue (older than the protostome–deuterostome ancestor)	600–1,000 million	Maximum likelihood	18
Elongation factor EF-Tu	Ancestral orthologue in LCA of eubacteria	>1 billion	Maximum likelihood	20

\*Papers that have inferred ancestral sequences and synthesized them for functional analysis. (Studies that used directed mutagenesis to examine the effects of isolated replacements are not included.) <sup>†</sup>Paralogue, evolutionarily related genes that are produced by gene duplication. <sup>§</sup>Pax, paired box protein-encoding gene. <sup>||</sup>Protostome, a bilaterian animal, the mouth of which develops before the anus during embryogenesis. Protostomes include arthropods, molluscs and worms. <sup>¶</sup>Deuterostome, a bilaterian animal, the mouth of which forms after the anus during embryogenesis. Deuterostomes include chordates, hemichordates and echinoderms. <sup>¶</sup>Archosaur, a member of the animal taxon that includes all crocodiles, birds and extinct dinosaurs. LCA, last common ancestor.

The parsimony method has several intrinsic limitations, however, which prevented gene resurrections during the 1990s from reaching back into very ancient evolutionary time. First, the power of parsimony to resolve ancestral sequences declines as terminal sequences become less and less similar<sup>8</sup>. When a sequence site changes more than once on a tree, the method frequently implies several equally parsimonious reconstructions, and there is no way to decide which of these many possible states is the correct one. As a result, only ancestors of extant sequences that are well conserved can be unambiguously reconstructed with parsimony.

A second weakness of parsimony reconstruction is that it does not take account of biases in the evolutionary process. The Fitch algorithm counts all evolutionary changes as equally probable, but it is well known that some kinds of substitution — DNA transitions or conservative amino-acid replacements, for example — occur more frequently than others<sup>10</sup>. A weight matrix can be imposed *a priori* to accommodate a model of evolutionary biases, but there is no way to test or select a model for its fit to a specific data set. Moreover, all parsimony algorithms minimize sequence changes equally on all branches, but substitutions often occur with much greater frequency on branches of a phylogeny that are associated with longer periods of time or accelerated evolutionary rates. When the bias-free assumptions of parsimony are violated, the accuracy of the method declines: simulations have shown that the percentage of correct ancestral state inferences falls as branch lengths (and therefore the number of substitutions on each branch) increase<sup>8</sup>.

Phylogenetic techniques that are based on maximum likelihood (ML) were designed specifically to remedy these shortcomings<sup>11</sup> (BOX 2). ML inference of ancestral sequences was first implemented in 1995 by Z. Yang in his **PAML software** package<sup>12</sup>; others developed various modifications and refinements<sup>8,13–15</sup>. At any internal node in the tree, the ML algorithm evaluates each

possible ancestral state and calculates its likelihood — that is, the probability that the set of present-day sequences would evolve, given that ancestral state, a tree and a statistical model of molecular evolution. The best inference of the ancestral state is the one with the highest likelihood.

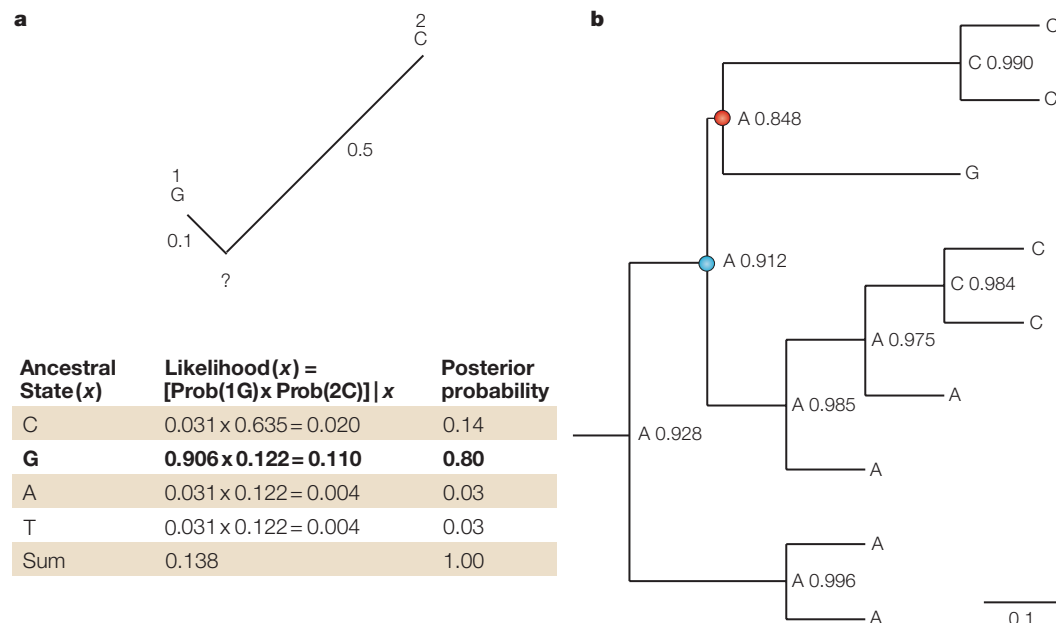
By applying an explicit statistical framework to the reconstruction of evolutionary history, ML has three main advantages over parsimony. First, it allows evidence about the process of molecular evolution, including biases and branch lengths, to inform the inference of ancestral states. This feature allows ML to specifically resolve ancestral states that are ambiguous under the parsimony algorithm. Simulations show that it also makes ML reconstructions more accurate than those inferred by parsimony, and the difference in performance grows as sequences become more diverged<sup>8</sup>. As a result, ancient genes can be inferred more accurately by ML than by maximum parsimony. Second, in an ML framework, an evolutionary model can be chosen not *a priori* but by statistically evaluating its fit to the data: more or less complex models can be selected using a LIKELIHOOD RATIO TEST, and the specific values of the model parameters (for example, the transition/transversion ratio) are selected by finding the values with the highest likelihoods (BOX 3). The third advantage of ML is that it allows the Bayesian posterior probability of all possible states to be calculated, so confidence in the optimal and alternative inferences at each ancestral sequence site can be evaluated statistically (BOX 2).

**More efficient gene synthesis.** The advent of ML methods set the stage for more ambitious applications of the ancestral gene resurrection strategy. Technical advances in molecular and chemical techniques have also made the strategy more practical. In the 1990s, gene construction was a slow and expensive process that required the synthesis of scores of short oligonucleotides, which were then pieced together one-by-one using enzymatic

#### LIKELIHOOD RATIO TEST

A method for hypothesis testing in a likelihood framework. A data set's fit to a more complex model is compared with its fit to a simpler model using the likelihood ratio statistic (twice the ratio of the likelihoods of the two models). The more complex model is adopted if it increases the likelihood more than expected by chance at some critical probability. If the simpler model is a restricted version of the more complex model, the improvement in fit can be evaluated using a chi-square distribution.

## Box 2 | Statistical inference of ancestral states



Ancestral states at any internal node in a phylogenetic tree can be inferred by the maximum likelihood (ML) method, given three pieces of information: a data set of DNA or protein sequences for the terminals, a tree topology with branch lengths and a model that specifies the relative rates of different kinds of substitution<sup>12</sup>. The likelihood of any specific ancestral sequence at an internal node on the tree is defined as the conditional probability that the observed sequence data would have evolved given that ancestral sequence. Sequence sites are assumed to evolve independently, so likelihoods at individual sites can be calculated separately. For each node, the algorithm examines all possible internal states ( $n = 4$  for DNA,  $n = 20$  for amino acids and  $n = 61$  for codons) and calculates their likelihoods. The ML reconstruction for any site is the state that has the highest probability of generating the pattern of observed sequence data at that site. The ML reconstruction of the entire gene sequence is the string of ML states for each site.

One main advantage of the ML method is that it allows the statistical confidence in each reconstructed ancestral state to be calculated. Using BAYESIAN METHODS, the POSTERIOR PROBABILITY of any ancestral state  $x$  is defined as the fractional contribution of  $x$  to the total likelihood over all possible states. Consider the simple case in panel a: a 'tree' of one ancestor (indicated by the question mark) and two descendant 'genes', each one base long. Assuming the branch lengths shown (scaled as the expected number of substitutions per site) and a simple model in which all substitution types have equal rates, the likelihood of each of the four possible ancestral states can be calculated, along with their posterior probabilities (shown in the table). Either C or G would be equally optimal reconstructions if using maximum parsimony, but knowledge of the branch lengths makes G much more likely in a probabilistic framework.

On a real phylogenetic tree, this method can be extended to reconstruct the ancestral states at all nodes. An internal node always connects three branches, so the ML state is the one with the highest probability of generating the states (real or reconstructed, weighted by their probability) at the three neighbouring nodes. The tree in panel b shows the posterior probabilities of all possible states for a single nucleotide position at each internal node, with the ML state indicated (assuming a Kimura-1980 model with a transition/transversion ratio of 10). Using maximum parsimony, the node marked with a red circle would be reconstructed ambiguously as A, C or G, because any of these requires three substitutions on the tree. By accounting for unequal branch lengths and transition/transversion probabilities, ML allows resolution of a single most probable reconstruction. Using a consensus method, the node marked with a blue circle would be reconstructed as C, but parsimony and ML — because they account for tree structure — infer A as the ancestral state. The small bar shows the branch-length scale as the substitution probability per site.

## BAYESIAN METHOD

In phylogenetics, a probabilistic technique for evaluating trees, evolutionary models and ancestral state assignments. Hypotheses are evaluated by their posterior probabilities.

## POSTERIOR PROBABILITY

In Bayesian statistics, the probability that a hypothesis is true after the data have been analysed. The posterior probability is defined as the likelihood of the hypothesis multiplied by its prior probability, divided by the sum of the likelihood multiplied by the prior for all hypotheses.

ligation or PCR. One cause of this inefficiency was the relatively low efficiency of DNA synthesis, which resulted in low purity for long oligonucleotides.

Today, automated synthesizers can readily generate or build oligonucleotides of up to ~125 bases, and it is possible to reach lengths of >200 bp under some circumstances<sup>16</sup>. Although the efficiency of oligonucleotide synthesis declines with increasing length, long oligonucleotides can be amplified by PCR using shorter

end primers to produce high-concentration, high-purity DNA products that can then be assembled by carrying out overlapping PCR reactions or restriction digest/ligation. This advance means that a typical gene can now be reconstructed from just a handful of oligonucleotides in a few sequential PCR reactions. Improvements in techniques for transfecting foreign genes into cultured cells and for characterizing gene function have also increased the efficacy of gene resurrection studies.



Box 3 | **Assumptions and models in ancestral sequence reconstructions**

The maximum likelihood (ML) method calculates the most probable ancestral state given a tree topology, branch lengths and an evolutionary model. Where do these background data come from? In practice, they are usually inferred from the sequence data, although the phylogeny can be taken from the literature if a well-corroborated tree is available. The branch lengths are usually inferred by ML; the set of branch lengths with the highest probability of generating the observed sequence data is the best estimate of the true branch lengths. In the alternative 'Bayesian distance approach', the tree and its branch lengths are inferred by a faster approximation that uses percentage similarity between pairs of sequences as data rather than individual sequence characters<sup>8</sup>.

Various evolutionary models ranging from the simple to the highly complex are available for analysing DNA and protein data. Model choice is important, because using the wrong model can lead to incorrect inferences<sup>41</sup>. The most appropriate model can be chosen using a hierarchical likelihood ratio test, which provides a statistical framework for selecting the model that best fits the data without adding unnecessary parameters<sup>45</sup>. Once a model is selected, the specific values of the parameters that are used for ancestral state inference (such as the transition/transversion ratio) are usually determined by ML optimization; the parameter values used are those that are most likely to have generated the observed sequence data.

The recently developed BAYESIAN MARKOV CHAIN MONTE CARLO (BMCMC) techniques offer an alternative to ML, which relies on a single tree and model<sup>42</sup>. This approach incorporates uncertainty about the tree into ancestral reconstruction by integrating the calculation of probabilities over alternative trees. To infer an ancestral state, the algorithm examines a large ensemble of trees that contain the node and calculates the probability of each possible ancestral state for each tree; the overall posterior probability of an ancestral state is the average of its probability over all trees, weighted by the likelihood of the tree. In a similar way, BMCMC can be used to reconstruct ancestral states over numerous models, thereby incorporating uncertainty about the evolutionary process into the estimate of the ancestral sequence. BMCMC ancestral state reconstruction is expected to be incorporated in future versions of the computer program MrBayes (J. Huelsenbeck, personal communication).

Raising an ancestral gene from the evolutionary dead is still a fairly expensive and time consuming project, but it is rapidly becoming more practical and affordable.

**Peering into the past**

These improvements have made possible a new generation of ancestral gene resurrection studies. The most exciting and ambitious work has used ML-based phylogenetic inference to recreate and characterize ancestral genes that are 240 million to >1 billion years old — far more ancient than the 5–100 million-year-old genes that could previously be resurrected. These projects have addressed fascinating and diverse evolutionary questions, including the visual capacity of ancient dinosaurs, the environmental adaptations of ancestral bacteria and the molecular evolution of hormones and their functions. (Three other recent studies — on the evolution of ultraviolet vision in vertebrates, antiviral RNase activity in the primate immune system and the molecular evolution in the family of Pax transcription factors — are not reviewed in detail here owing to space limitations, but are listed in TABLE 1.)

**Dinosaur nightlife.** In the first of these studies, B. Chang and colleagues<sup>16,17</sup> sought to characterize the vision of the ancestral archosaur as a window on dinosaur lifestyle and the evolution of vision in modern birds and crocodilians. The aim was to resurrect and characterize the **rhodopsin** protein — the pigment that determines the visual quality in dim light — that would have existed in the common ancestor of all archosaurs approximately 240 million years ago (mya). Chang *et al.* used ML to reconstruct the ancestral amino-acid sequence on the basis of the sequences from four extant archosaurs (alligator, pigeon, zebrafish and chick) and 26 other 'outgroup' vertebrates. The data were separately analysed as DNA, protein and codon sequences,

and the best evolutionary model for each type was chosen using a likelihood ratio test (BOX 3; the three analyses gave nearly identical results). The extant sequences differed by a maximum of 16% at the amino-acid level, allowing the ancestral archosaur protein sequence to be reconstructed with little ambiguity using all three data types. The posterior probability of the most likely ancestral reconstruction was >0.90 for more than 97% of sequence sites, with only a few sites dipping below 0.80.

A 1-kb DNA sequence that codes for the ancestral rhodopsin protein was then assembled from five long oligonucleotides and cloned into an expression vector under the control of a constitutive promoter. This vector was transfected into cultured mammalian cells, and the protein was expressed, purified and functionally assayed using the kinds of *in vitro* assay that are typically used to characterize extant rhodopsins.

Despite its great age, the ancestral rhodopsin functioned well, carrying out all the individual steps that are required for visual function in dim light as effectively as the extant proteins in mammals, which generally have good night vision. Specifically, the ancestral protein bound the visual chromophore 11-*cis*-retinal and, when exposed to light, activated the G-protein transducin at a rate similar to that of bovine rhodopsin. These results are consistent with the hypothesis that the ancestral archosaur possessed the ability — at the molecular level at least — to see well in dim light, and might have been active at night. This insight, of course, could never have been drawn from fossils or any other non-molecular evidence about the behaviour of ancient dinosaurs.

**The evolution of hormones.** The second study, by our own group, sought to explain the evolution of function in the STEROID HORMONE RECEPTOR gene family<sup>18</sup>. The human genome contains six closely related steroid

BAYESIAN MARKOV CHAIN MONTE CARLO [METHOD]  
A technique for efficient numerical calculation of Bayesian posterior probabilities.

receptor (SR) genes, the products of which mediate hormonal effects on development, differentiation, reproduction and homeostasis. Each receptor is functionally distinct: it binds a specific hormone, which triggers a conformational change that allows the receptor to bind to specific DNA response elements and then activate transcription of nearby hormone-responsive genes. We sought to explain the evolution of the diverse specificities of SRs for steroid hormones and response elements, so we resurrected the ancestral steroid hormone receptor — the common ancestor of the entire gene family. This gene existed before the split of protostomes from deuterostomes some 600–1,000 mya and is the single progenitor gene from which modern-day receptors descended through a repeated process of gene duplication and sequence divergence.

We inferred the phylogeny of the SR gene family from an alignment of 73 receptor protein sequences (including 18 closely related outgroup genes). We then used ML to reconstruct the sequence of the ancestral receptor protein on this tree, assuming models of amino-acid replacement rates and among-site rate variation that both had 100% posterior probability in a Bayesian Markov chain Monte Carlo analysis (BOX 3). The mean posterior probability of the ancestral sequence was considerably lower than that of the archosaur rhodopsin, reflecting the much greater degree of sequence divergence (up to 75%) among some SRs. Most of the sites at which the reconstruction was less decisive, however, are variable because they are not constrained by crucial contributions to receptor function. By contrast, the sequence sites that are known to structurally confer a receptor's specificity for hormones and response elements were reconstructed with high confidence (mean posterior probability >0.95). Interestingly, these functionally important sites in the ancestor were all identical to those in the extant **oestrogen receptors** but were different from the other members of the family<sup>19</sup>.

We predicted that the resurrected ancestral receptor would have oestrogen receptor-like functions on the basis of the similarity between the ancestral receptor and the modern oestrogen receptors. To test this hypothesis, we used sequential PCR of overlapping oligonucleotides to assemble DNA molecules that would code for the functional domains of the inferred ancestral protein. We cloned these into expression vectors and transiently transfected them into cultured mammalian cells. In reporter gene assays, the DNA-binding domain of the ancestral SR specifically activated transcription almost as effectively as extant oestrogen receptors do from oestrogen response elements, to which the other steroid receptors do not bind effectively. The ligand-binding domain of the ancestral sequence specifically bound oestrogens and activated transcription in the presence of low doses of oestrogens but not the ligands for other receptors.

Together, these data indicate that the ancestral SR had the functional specificity of modern oestrogen receptors, with the other receptors' hormonal partners and target-gene affinities emerging later as derived

novelties. Surprisingly, this finding indicates that the first receptor in the family was activated by the terminal hormone in the steroid-synthesis pathway; in the synthesis of oestrogens, progesterone and testosterone are formed as intermediates. This finding implies that new hormone–receptor pairs emerged when ancient receptors duplicated and evolved increased affinity for steroids that were already present as intermediates, turning biochemical stepping stones into bona fide hormones<sup>19</sup>.

**Hot-living microbes.** In the third recent study, E. Gaucher, S. Benner and their co-workers accomplished the most ancient reconstruction of all, resurrecting the elongation factor **EF-Tu** — a temperature-sensitive GDP-binding protein that regulates the rate of protein synthesis — in the common ancestor of all bacteria, which existed well over one billion years ago<sup>20</sup>. The goal of this work was to understand the environmental conditions in which the earliest life forms evolved — specifically, to test the hypothesis that all bacteria evolved from a thermophilic ancestor that lived in a hot ancient environment. Present-day bacteria live at temperatures that range from 20 to >80°C; previous inferences about the ancestor on the basis of the distribution of thermophily, G+C content and estimates of the temperature of the early environment have been inconclusive and contradictory.

To address this question, Gaucher and colleagues used gene resurrection to characterize the ancestral elongation factor EF-Tu. First, the EF-Tu sequence in the bacterial ancestor was inferred by ML on the basis of a data set of 50 diverse present-day sequences. Because EF-Tu is well conserved (>75% identity across all sequences), the ancestral inference had relatively high confidence: the mean posterior probability of the ML reconstruction was 0.88 per site, and 75% of sites had posterior probability >0.90.

The ancestral gene was synthesized in small steps by overlap PCR using 50-bp oligonucleotides with 15–20 overlapping bases and was cloned into a prokaryotic expression vector. *Escherichia coli* were transformed with the EF-Tu-carrying plasmid, and the protein was expressed and purified. The GDP-binding affinity of the ancestral protein and that of modern-day EF-Tus was assayed across a range of temperatures. All extant EF-Tu proteins showed maximal binding at temperatures close to the optimal growth temperature for the bacteria from which they come. The ancestral EF-Tu had an optimal GDP-binding temperature of 65°C. This result indicates that the ancestor of all bacteria was probably adapted to this temperature, corroborating the hypothesis that bacteria originated in a thermophilic environment.

#### Caveat resurrector

These three studies point to the great potential of gene resurrection for experimentally testing hypotheses about the ancient evolutionary past that would otherwise remain pure speculation. There are important limitations to the strategy, however. In particular, an experiment on a reconstructed gene is only as good as the inferred ancestral sequence. ML provides a great

**STEROID HORMONE RECEPTORS**  
A phylogenetically related family of intracellular transcription factors that mediate the effects of oestrogens, androgens, progestins, glucocorticoids and mineralocorticoids on physiology and development.

advance for ancestral state reconstruction, but several causes of potential error remain. Results from ancestral resurrection studies are therefore most reliable and persuasive when several caveats are kept in mind.

#### *Stochastic errors in ancestral sequence inference.*

Inference of ancestral sequences is never statistically unambiguous; there is always some possibility that a site might have been occupied by an amino acid other than the ML state. The posterior probability that an entire sequence is inferred correctly is the product of the probabilities for all individual sites. For example, if every position in a 500-amino-acid protein is inferred with an impressive 0.95 posterior probability, then the probability that the reconstructed sequence is correct is  $<10^{-11}$ . The ancestral sequence is therefore not the true ancestor but our best approximation. The crucial question is whether errors in reconstruction bias the experimental results and lead to inaccurate evolutionary conclusions.

It is therefore essential to carefully evaluate the statistical confidence in ancestral sequences, particularly at functionally crucial positions. Reconstruction errors can be thought of as mutations in the ancestral sequence. In general, most amino-acid mutations are mildly deleterious; a few knock out function entirely, and a small minority enhance function or confer a new one. This means that reconstruction errors are most likely to reduce the performance of the ancestral gene product in functional assays or eliminate it altogether. From this point of view, results that imply a non-functional or weakly functional ancestor should be more suspect than reconstructed proteins that 'work'.

There are limits to this line of reasoning, however. In some cases, replacing an amino acid at a crucial site can shift the functional specificity of a protein by changing ligand- or substrate-specificity or by crucially shifting protein stability (see, for example, REF. 21). One way to overcome this problem is to specifically evaluate confidence in sequence sites that are known to be functionally vital; erroneous reconstructions at these sites are more likely to introduce artefactual functions than those at other positions. In the steroid receptor reconstruction, for example, the high posterior probabilities at sites that confer on receptors their hormone and response-element specificity — as shown by crystal structures and biochemical experiments — increased our confidence that the experimental result was reliable.

An even better strategy is to directly characterize the robustness of experimental results with respect to uncertainty in the ancestral state inference. This goal can be accomplished by reconstructing and assaying not just the ML ancestor but also alternative ancestral sequences. For example, in their study of artiodactyl ribonucleases (BOX 1), Jermann *et al.* synthesized several different ancestral sequences, each containing alternative states at ambiguously reconstructed sequence positions; they all behaved similarly in the functional assay, increasing confidence in the results<sup>22</sup>.

Ideally, hypotheses about ancestral function should be tested statistically using methods that integrate uncertainty about the sequence into the analysis. This

could be accomplished in a Bayesian framework by treating the posterior probability of ancestral state reconstructions as prior probabilities in the functional analysis. The ensemble of plausible variants would each be synthesized and assayed; confidence limits on the functional characteristics of the ancestral gene could then be estimated by weighting results for each sequence's performance in the assay by its prior probability of being the true ancestor. Further advances in the efficiency and cost of gene synthesis are necessary to make this demanding approach practical for ancestral sequences with more than a few uncertain sites.

**Erroneous assumptions.** A second source of potential error is uncertainty in the background knowledge on which the ancestral inference depends. ML methods calculate the ML reconstruction given a tree topology and an evolutionary model. If tree and model are true, ancestral reconstructions are expected to be unbiased and to converge on the true sequence. Errors in these 'givens', however, can lead to incorrect ancestral states. For example, extreme changes in topology — such as those that change the sequences that descend from the reconstructed node — can result in high-probability but erroneous inferences. Less extreme tree errors that change relationships only within the ingroup or outgroup have a much smaller effect: simulations have shown that the accuracy of ancestral sequences declines only slightly when this type of error is introduced<sup>8</sup>.

To assess the possibility of tree-induced error in an ancestral inference, the degree of confidence in the tree should be characterized using metrics of phylogenetic support, such as BRANCH SUPPORTS, BOOTSTRAP PROPORTIONS, posterior probabilities or PAIRED-SITES TESTS<sup>23</sup>. A weakly supported tree indicates that a crucial assumption on which the ancestral reconstruction is based might be incorrect. Particular attention should be paid to confidence in the node being reconstructed. When a single tree cannot be selected with high confidence, the robustness of the ancestral characterization to uncertainty in the tree should be characterized. Gaucher *et al.*, for example, inferred the ancestral EF-Tu sequence by assuming both the ML tree and a more traditional phylogeny derived from the literature. Both ancestral sequences were synthesized and experimentally assayed: their optimal temperatures were similar, indicating that the hypothesis of ancestral thermophily is robust to plausible errors in phylogeny<sup>20</sup>. Alternatively, recently developed Bayesian Markov chain Monte Carlo methods allow uncertainty about the tree (and the evolutionary model) to be incorporated into the ancestral sequence inference itself (BOX 3).

**Experimental artefacts.** A final caveat is the possibility of misleading results that are caused by the expression and characterization of ancestral proteins in extant assay systems. Ancient proteins functioned in the context of the cells that existed in their day, and they were presumably adapted for interactions with other ancient proteins. In present-day cultured cells or assay systems, the functions of ancestral gene products could be sub-optimal

#### BRANCH SUPPORT

A measure of support in a parsimony context for individual nodes in a phylogeny. The branch support — also known as the decay index or Bremer support — is the number of extra evolutionary changes that are required for a clade not to occur in the most parsimonious phylogeny.

#### BOOTSTRAP PROPORTION

A measure of support for individual nodes in a phylogeny. Sequence sites are sampled randomly with replacement from the original data set, and the optimal tree is inferred. This process is repeated many times, and the bootstrap proportion for a clade is the frequency of bootstrap replicates in which it occurs. A high bootstrap proportion indicates that the clade is not likely to be the result of sampling error in the sequence data.

#### PAIRED-SITES TESTS

A family of statistical methods for comparing two phylogenies as explanations of a data set. The difference in the log-likelihoods of the two trees is calculated separately for each sequence site. If one tree is a better fit to the data than the other, the mean of these differences will be significantly different from zero.



**EVOLUTIONARY RATE SHIFT**  
A change among phylogenetic lineages in the substitution rate for a sequence site or set of sites.

**FITNESS LANDSCAPE**  
A multidimensional plot that shows the fitness (on the vertical axis) for all possible variants of a sequence (occupying the horizontal axis, or sequence space).

or otherwise altered. For example, steroid receptors mediate transcriptional activation by interacting with co-activator proteins. The ancestral steroid receptor would have been optimized to interact with ancient co-activators — possibly explaining, in part, why the ancestral receptor was a less effective transcriptional activator in cultured mammalian cells than are modern-day receptors. As with error in sequence reconstructions, this problem is more likely to bias results towards a partial or total loss of function rather than a gain, so negative functional results should be interpreted with particular caution.

### The future of resurrecting the past

As biologists focus increasingly on the evolution of gene function<sup>24</sup>, more and more ancient genes are likely to be resurrected. There are at least three exciting possibilities for extending the gene resurrection strategy to test hypotheses about protein evolution and function. First, an important goal in molecular evolution is to identify the sequence changes that conferred new functions on evolving proteins. Many studies have proposed a role for specific amino-acid replacements, on the basis of EVOLUTIONARY RATE SHIFTS or amino-acid replacements that occur on the same branches as important functional changes<sup>1,25</sup>. These hypotheses could be experimentally tested by engineering candidate replacements directly into reconstructed genes using site-directed mutagenesis, then assaying the function of both the ancestral and pseudo-evolved gene products<sup>26,27</sup>. By engineering a set of potentially important substitutions singly and in combination, it should be possible to experimentally characterize the FITNESS LANDSCAPE on which specific genes have evolved<sup>28</sup>, an approach that will allow important questions about the dynamics of the evolutionary process to be addressed.

Second, a detailed understanding of molecular evolution can help to explain the mechanistic basis of function in modern-day proteins. Extant proteins have evolved through a massively parallel experiment in functional optimization and adaptation. Ancestral gene resurrection reveals the specific mutational paths that

molecules have taken during this process and allows the function of the intermediate forms — in which various partially optimized amino-acid combinations are present — to be characterized. In this way, ancestral gene studies can shed light on structure–function relationships and the mechanisms by which modern proteins — including those of biomedical importance — carry out their functions.

Finally, it should be possible to study the dynamics and structural basis for the evolution of gene function by experimentally evolving resurrected ancestral genes in laboratory selection systems. Experimental evolution has proved to be an extremely powerful scientific strategy for several reasons: it allows evolution under natural selection to proceed in the controlled environment of the laboratory, the ancestral state is unambiguously known, intermediate evolved forms can be sampled, frozen and retrieved for characterization at any time, and evolution in many replicate lines of selected and control treatments allows for statistical evaluation of apparent patterns in the evolutionary process<sup>29</sup>. It should be possible to transform rapidly growing microbes such as *E. coli* or baker's yeast with resurrected ancestral genes, and impose selection for specific novel functions — including those that evolved from the ancestral gene during the real evolutionary process. The mechanisms and dynamics of this process could then be tracked in detail, allowing such fundamental questions as the inevitability, reversibility, linearity, tempo, mode and context-dependence of the evolutionary process to be rigorously examined.

Ultimately, this strategy — statistically reconstructing ancestral genes, characterizing their functions and manipulating them using the techniques of molecular genetics — could become the gold standard for corroborating evolutionary hypotheses. Inferences from gene resurrections will never be as unambiguous as those that are gleaned by physically examining a fossil preserved in stone. By bringing the reductionist power of molecular biology to bear on the central questions of evolutionary biology, however, the pay-off might be just as high.

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#### Competing interests statement

The author declares that he has no competing financial interests.

#### Online links

#### DATABASES

**The following terms in this article are linked online to:**  
**Swiss-Prot:** <http://ca.expasy.org/sprot>  
EF-Tu | oestrogen receptors | rhodopsin

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<http://abacus.gene.ucl.ac.uk/software/paml.html>

**PAUP software:** <http://paup.csit.fsu.edu/mac.html>

**Peter Wilson's introduction to likelihood-based phylogenetics:** <http://www.bioinf.org/molsys/data/idiots.pdf>

##### Steroid hormone receptors:

<http://nrr.georgetown.edu/NRR/NRR.html>

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### Author biography

After studying English literature at Yale University, USA, Joe Thornton worked for a decade as an environmental activist, ultimately becoming research coordinator for Greenpeace's campaigns on toxic chemicals. Fascinated by the hormone receptors that mediate the health impacts of environmental endocrine disrupters, he returned to the academy for doctoral and postdoctoral training in molecular biology, phylogenetics and evolution with Rob DeSalle at the American Museum of Natural History and Darcy Kelley at Columbia University in New York, USA. His laboratory ([www.uoregon.edu/~joet](http://www.uoregon.edu/~joet)) at the University of Oregon, USA, where he is now Assistant Professor of Biology, seeks to understand the evolution of molecular interactions, with a focus on the evolution of nuclear receptors and their ligands. He is the author of the seminal book on global chemical pollution, *Pandora's Poison: Chlorine, Health, and a New Environmental Strategy* (MIT Press, 2000).

### Online summary

- Hypotheses about molecular evolution can be experimentally tested by resurrecting ancient genes and characterizing their functions.
- An ancient gene is resurrected by phylogenetically inferring its sequence, synthesizing and subcloning it into an expression vector and expressing it in cell culture.
- Maximum-likelihood methods for ancestral sequence reconstruction are an advance over previous methods because they are more accurate for very ancient sequences and they allow statistical confidence in the inference to be calculated at each sequence site.
- Recent studies using likelihood-based phylogenetics have resurrected genes that are far more ancient — up to one billion years old — than was previously possible.
- Ancestral sequence inference can be compromised by erroneous assumptions about the evolutionary process or the phylogenetic tree.
- Studies that use resurrected genes should critically evaluate statistical confidence in ancestral state inferences, with a particular focus on sites that are known to be functionally important.
- Errors in ancestral sequence reconstruction will usually — but not always — bias resurrected genes towards non-functionality.
- In the future, ancestral gene resurrection will be combined with site-directed mutagenesis and experimental evolution systems to determine the specific mechanisms and dynamics by which new protein functions have evolved.

### Online links

Swiss-Prot

EF-Tu

<http://ca.expasy.org/cgi-bin/niceprot.pl?P02990>

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Rhodopsin

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