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Studying the effect of environmental change on biotic evolution: past genetic contributions, current work and future directions

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Evolutionary geneticists currently face a major scientific opportunity when integrating across the rapidly increasing amount of genetic data and existing biological scenarios based on ecology, fossils or climate models. Although genetic data acquisition and analysis have improved tremendously, several limitations remain. Here, we discuss the feedback between history and genetic variation in the face of environmental change with increasing taxonomic and temporal scale, as well as the major challenges that lie ahead. In particular, we focus on recent developments in two promising genetic methods, those of 'phylochronology' and 'molecular clocks'.

With the advent of ancient DNA techniques, we can now directly sample the recent past. We illustrate this amazing and largely untapped utility of ancient DNA extracted from accurately dated localities with documented environmental changes. Innovative statistical analyses of these genetic data expose the direct effect of recent environmental change on genetic endurance, or maintenance of genetic variation. The 'molecular clock' (assumption of a linear relationship between genetic distance and evolutionary time) has been used extensively in phylogenetic studies to infer time and correlation between lineage divergence time and concurrent environmental change. Several studies at both population and species scale support a persuasive relationship between particular perturbation events and time of biotic divergence. However, we are still a way from gleaning an overall pattern to this relationship, which is a prerequisite to ultimately understanding the mechanisms by which past environments have shaped the evolutionary trajectory. Current obstacles include as-yet undecided reasons behind the frequent discrepancy between molecular and fossil time estimates, and the frequent lack of consideration of extensive confidence intervals around time estimates. We suggest that use and interpretation of both ancient DNA and molecular clocks is most effective when results are synthesized with palaeontological (fossil) and ecological (life history) information.

Keywords: climate; serial coalescent; microevolution; Lamar Cave; calibration; macroevolution

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Table 1. *Relevant genetic studies with respect to testing evolutionary impact of environmental change on an increasing geological timescale*

time period	lineage	method	climate effect	representative reference
century (100 a)	*Peromyscus*	aDNA	human impact	Pergams *et al.* (2003)
millennium (1000 a)	*Ctenomys*	aDNA	climate, human impact, volcanism	Hadly *et al.* (2003)
Holocene (1000–3000 a)	*Thomomys* *Microtus*	aDNA aDNA	warming, cooling	Hadly *et al.* (1998); Hadly *et al.* (2004)
Pleistocene (10 000 a–1 Ma)	*Pygoscelis*	aDNA	glacial cycles	Lambert *et al.* (2002)
Miocene (10 Ma)	*Sciuridae*	clock	sea-level drop	Mercer & Roth (2003)
Eo–Oligocene (30–40 Ma)	birds, mammals	clock	global cooling	van Tuinen *et al.* (2004)
K–T (60–70 Ma)	birds, mammals	clock	impact, volcanism, global cooling	van Tuinen *et al.* (2004); Springer *et al.* (2003)

1. Introduction

Inference of the past from both genes and fossils gives us a history of a particular species or population. In order to address more significant issues, such as whether evolution is stimulated by environmental change, we must investigate not only genetic variation and fossil history but also the associated perturbation events. In this paper we present examples of two such uniquely integrative approaches, which allow us to infer how (or if) evolution is driven by changes in the environment. It has long been documented that changes in the environment, when severe enough, may have long-term evolutionary impacts. This is not surprising, since the environment generally determines where individuals live or cannot reside. At the population scale, environmental change may impact the abundance and geographic range of individuals and can direct towards speciation through the creation of reproductive barriers. Above the species scale, the environment may cull (sets of) lineages through extinction (Vrba 1993). The notion used throughout this paper is that environment-based alteration of species and population dynamics can be documented through genetic signatures on both microevolutionary and macroevolutionary scales. The procedure we employ attempts to reconstruct evolutionary history of populations and species back in time. Instead of knowing the mechanism of environmental change on biotic evolution at different established times in the evolutionary trajectories of lineages, we document and compare genetic differences within or among species. Translation of genetic variation into geological time allows correlation of the origin or diversification of evolutionary lineages to the nearest major perturbation events approximating this geological time. This method, based on a 'molecular clock', is commonly used in molecular biology and its progress is reviewed below (see also table 1). However, because this method is based on correlation, it cannot be used to discern exactly how changes in the environment shape evolution. We have recently developed novel meth-

ods with genetic data that directly investigate the above question. 'Phylochronology' (Hadly *et al*. 2004) uses ancient and modern DNA data from ecologically different species in combination with fossil abundance data and historical climate records (table 1) to infer microevolutionary processes. Placed in the context of historical climate change, these methods provide great promise for investigating the role that environment plays in shaping species evolution. Therefore, we begin our journey at the microevolutionary scale by describing the utility of phylochronology and the statistical analysis of temporal genetic data.

2. Understanding the evolutionary process in populations: why is it challenging?

Evolutionary history impacts genetic variation through the balance between four processes: mutation, migration, drift (random sampling effects) and selection, such that, over time, separation of populations or species will result in an increasing number of differences across the genome. Given a theoretical framework quantifying these processes, we can infer the evolutionary histories of species and populations using modern molecular genetic data. With the proliferation of molecular genetic techniques, studies have been conducted on many species and results have provided valuable insight into the past. For example, many different genes point to a recent origin of our ancestors (modern humans) in Africa, and a subsequent population expansion in the rest of the world (reviewed in Excoffier (2002)). Additionally, molecular analysis coupled with behavioural data has helped elucidate the patterns of species diversification triggered by environmental change in the cichlid fish in African lakes (Meyer *et al*. 1990).

(*a*) *The uses of 'ancient' genetic data*

Phylogeography has advanced our understanding of the spatial distribution of modern genetic diversity within and between species (Avise 2000). However, temporal change in genetic diversity in a single locality over time has not yet been placed in a phylogeographic framework over ecologically long periods of time. Genetic variation (nucleotide and gene diversity) of a sample from a given location does not necessarily reflect the history of populations that have previously occupied that location. Empirical evidence of this dynamic process remains difficult to obtain, because it requires a longer temporal record than typically is available. After the death of an organism, its DNA begins to degrade immediately. However, under certain conditions (low temperature, high salt concentration and rapid desiccation), the enzymes that degrade DNA are slowed down (Hofreiter *et al*. 2001). This special set of circumstances allows us to extract DNA from some individuals and more rarely from populations that lived in the past. Such information is very valuable, as it allows an additional glimpse into the evolutionary trajectory of populations and species. So far, many ancient-DNA studies have been used to establish phylogenetic relationships among extant and extinct species (see, for example, Shapiro *et al*. 2002), but new work on unique fossil assemblages allows us to sample populations at several times in the past few millennia (Hadly *et al*. 1998; Leonard *et al*. 2000; Lambert *et al*. 2002; Hadly *et al*. 2003). When available, the combination of temporal genetic data and modern spatial genetic data provide a unique opportunity to assess the history of a population (or

populations) in a given locality. We view this approach as 'phylochronology', or the study of populations in space and time using phylogenetic and population genetic methods (Hadly *et al*. 2004). This approach will be particularly valuable given our emphasis on the response of species and communities to past climatic change, in the hope of making population-level, species-level and community-level predictions for future environmental perturbations.

This type of research highlights the influence of environmental change on the population dynamics and phenotypic response for the study species. Advancement of ancient DNA techniques allows us to investigate directly the impact of prehistoric environmental perturbations on population size and genetic diversity. We have also derived independent estimates of population size through time, using relative fossil abundance (Hadly *et al*. 1998, 2004 and references therein). This type of species comparison will allow us to assess in unusual detail the contributions of past processes to the formation of modern genetic variation across the landscape (Avise 2000).

3. Using the coalescent and serial coalescent to reconstruct history from genetic data

Biological sciences are driven primarily by the experimental process. For example, to investigate the effect of a drug on disease resistance, we would conduct an experiment where the drug or a 'control' (placebo) are administered to a cohort of patients, and the resulting changes in patient condition (with respect to the disease) are followed. Such an experiment would allow us to quantify both the effect of the drug (positive or negative) relative to the 'control' and the varying level of drug response between patients. Reconstructing population history is fundamentally different from such studies. The data we observe are the result of an experiment that has already occurred and that biologists do not control: the evolution of a population through time. Our goal is to understand what the experiment was. We do this by setting up alternate 'experimental conditions' and 'controls' and developing evolutionary hypotheses that have led to the observed results. The genetic data we 'observe' correspond to one realization of this experiment, equivalent in our analogy to data from a single patient. In other words, for the same evolutionary history, the process of evolution can result in a range of possible genetic outcomes. Our data correspond to the one, realized outcome and if we are lucky we might be able to collect data from a 'control'. Thus, reconstructing past history of populations consists of two steps: the construction of appropriate models that mimic processes active in the past, and ascertainment of how the observed data are concomitant with the proposed models. The presence/absence of species through the fossil record and the changes in species' abundance through time allow us to set up models that mimic the past. Coalescent simulations help us to statistically parametrize how likely these competing models of evolution are in producing the data we observe. The coalescent (Kingman 1982; Hudson 1990; figure 1*a*) is a modelling framework that allows us to predict the outcome of evolution on a set of genes in a population for a given population history. Creating a set of computer simulations based on the coalescent thus allows us to create possible genetic outcomes of a given evolutionary history, many, many times! We can then compare these simulated, artificial data to investigate which model of evolution our data seem to represent the best.

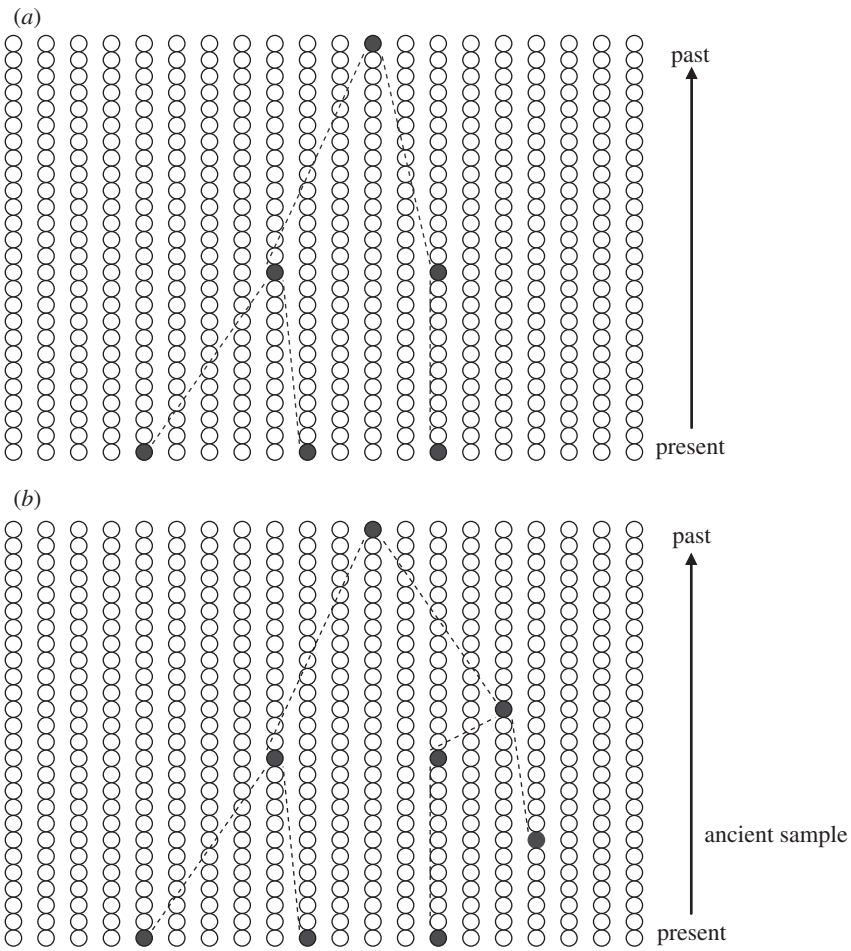

Figure 1. (*a*) Using the coalescent as a framework to model a population through time. The empty circles represent all the individuals in a population, the filled circles the sampled individuals. All individuals in the present are derived from a single ancestral lineage in the past. This figure shows a constant population of 20 individuals over 30 generations. (*b*) The serial coalescent demonstrated with one ancient sample collected six generations ago.

The coalescent modelling framework has been used extensively in population genetics, both as an analysis tool to understand real data and as an exploratory tool to investigate the possible impact of complex evolutionary histories. For example, the coalescent was used to infer the population history of the serial introduction of the South-Central American cane toad in the Caribbean and Pacific islands (Estoup *et al*. 2001). Analyses demonstrated initial genetic admixture between the introduced and native toads, followed by a population crash of the introduced toads, then population expansion and finally a stabilization of population size, all over a period of about 20 000 generations. Using the coalescent in an estimation framework allowed evaluation of the timing and intensity of these different events.

Similar to using the coalescent for modelling current genetic variation, a modified version, the serial coalescent (Rodrigo & Felsenstein 1999; figure 1*b*), can be used

to model ancient genetic data. As its name suggests, the serial coalescent models samples through time. This is very important because it allows us to treat both present and ancient samples as part of the same evolutionary process and promises to give a deeper insight into evolutionary processes. Thus far, the serial coalescent has been used to analyse ancient data in two ways: Bayesian analysis to estimate variation in mutation rate, and a simulation-based approach to explore summary statistics, which are the baseline data enabling evolutionists to compare populations and species with each other.

When analysing population genetic data, we construct possible evolutionary histories and investigate how well the observed data match predictions from competing hypotheses. Likelihood (Edwards 1992) is a statistical framework that allows us to formalize the above comparison. In likelihood analysis, we investigate the probability of the observed data given a set of parameters, which is equivalent to a quantitative description of evolutionary history. This results in a probability distribution, which we then can explore for the best fit for the observed data. In Bayesian analyses, we start with initial distributions for the parameters of interest and evaluate these parameters in the context of the likelihood function. Using bones that were up to 7000 years old, Bayesian analysis was used to estimate mutation rate of a segment of the mitochondrial genome, and results revealed a surprisingly high mutation rate (Lambert *et al*. 2002). These analyses were also used to investigate past evolutionary history of penguins in Antarctica using ancient DNA, and the estimated hypothesis matched well with predictions made based on glacial cycle (Ritchie *et al*. 2004). In some cases, we may have information that gives us a hint about the history (or 'prior'), such as data on environmental change or population fluctuation. For example, if the fossil data suggest a population crash in a stratigraphic layer dated between 1000 and 2000 years before present, we could incorporate this information into a Bayesian model. There are few studies using Bayesian analysis for fossil data, and of those that do use it, most are based on an assumption of constant population size or exponential growth of a single population. For example, the estimates of mutation rate arrived at in the penguin study would have been lower if the models had allowed for the possibility of multiple penguin populations with migration between these multiple populations. What this means is that models with different parameters could lead to the same observed results. Although the Bayesian methods allow estimation of parameters, they do not allow us to investigate the impact of a particular evolutionary hypothesis or history on the data. To date, the only method we have for doing so is the serial coalescent. Using the serial coalescent as a phylochronologic model, we have developed a simulation tool that allows us to model both present and ancient DNA data, and to investigate the effects of evolutionary history and sample size on possible outcomes of a particular history (Hadly *et al*. 2004). We used this approach to demonstrate that the changes in ancient genetic variation observed in voles in Lamar Cave (Yellowstone National Park) could not be explained solely by local changes in population size as suggested by fossil abundance. These results indicate that migration from neighbouring populations was an important part of this species' response to climatic change and became most significant during local population decline.

Also, we have used the above simulation-based methods to investigate the effects of sampling, a factor of critical importance in ancient DNA studies, where sample sizes will always be limited. Results from simulations demonstrated that the pay-off

from sampling is not linear, i.e. doubling the number of samples does not double our ability to discern the past evolutionary history. In some cases, especially where there is extraordinarily high diversity, we need very few samples to deduce this. Our analyses also demonstrated that gene flow from neighbouring populations increases the statistical power to choose between evolutionary hypotheses, whereas growth following a population crash has the opposite effect. Most importantly, results reveal that using ancient DNA samples in combination with modern genetic data always results in a better ability to detect population history (Ramakrishnan *et al*. 2005). This study clearly validates the temporal sampling approach. The above simulation-based approach allows us to model various hypotheses (i.e. model flexibility), but it does not allow us to estimate parameters. The most we can do with the serial coalescent alone is to determine the probability of a given hypothesis for the set of observed data. However, the ultimate goal is to reconstruct history accurately, and be able to reconstruct the presence, intensity and timing of different events in the population.

(*a*) *Future developments in ancient DNA studies: sampling, methods and analysis*

So far, most ancient DNA studies have concentrated on (maternally inherited) mitochondrial DNA, because there are many mitochondria (and consequently many mitochondrial genes) in the cell and only one nucleus. However, recent studies (see, for example, Bunce *et al*. 2003) have successfully extracted information on the gender of individual moa fossils by targeting sex-related genes. As molecular methods get more sophisticated, we hope to gain the ability to extract nuclear DNA from fossils. Such methods in combination with data from additional fossil assemblages (Appendix A) will allow a much more complete representation of the evolutionary history of populations.

The analysis of temporally spaced samples is a relatively new field (reviewed in Drummond *et al*. 2003). We predict that, in the future, these methods will continue to be computationally intensive. Theoretical challenges will involve developing methods to combine data from multiple spatial populations, multiple points in time and multiple molecular markers, and developing methods to chose between various models that match the observed data. In our next stage of model development, we hope to use the simulation-based data in the context of estimating evolutionary history. We will do this using a rejection algorithm, a computational approach used in combination with the coalescent theoretical framework. Although the rejection algorithm has been used in other studies (Pritchard *et al*. 1999; Estoup *et al*. 2001), this will be its first application to serial sampling. The rejection algorithm iteratively finds the evolutionary history parameters that best fit a set of statistics that summarize the observed data.

4. Genetic variation beyond speciation: how useful is it for reconstructing macroevolution?

After speciation, genetic variation (between species) continues to accrue with time. If genetic mutations occur at a reasonably constant rate through time, a simple positive relationship exists between evolutionary time and genetic difference. The amount of

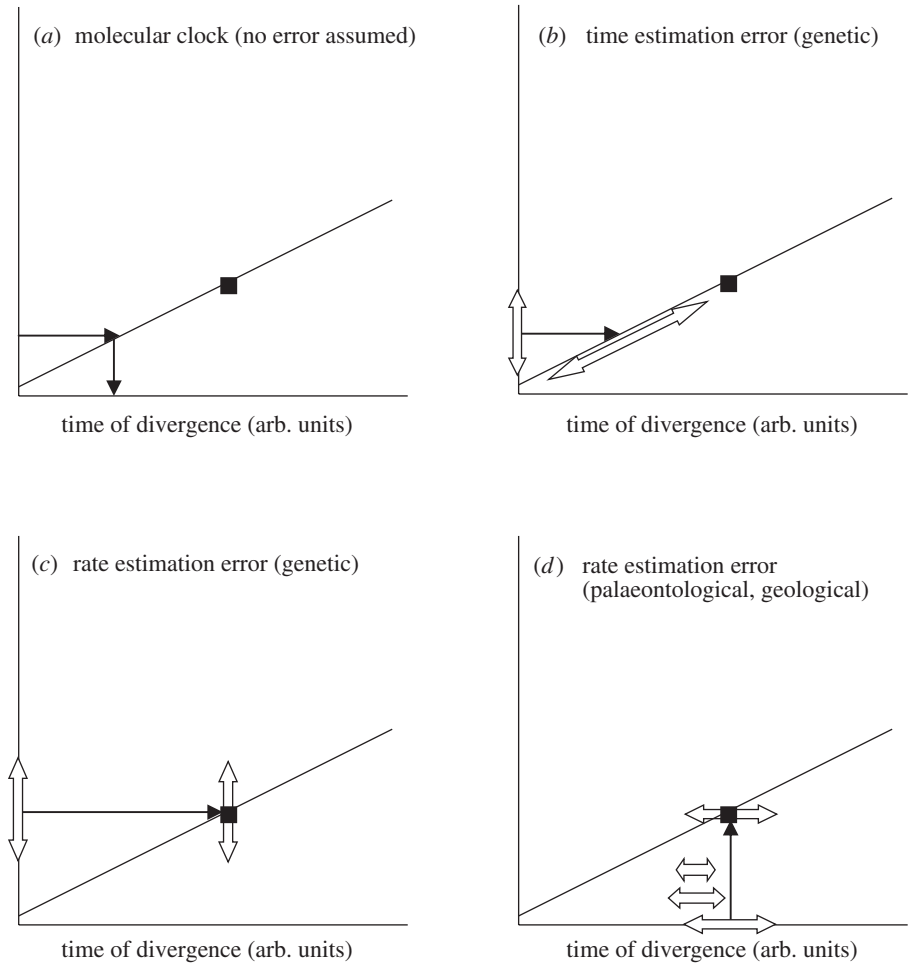

Figure 2. Concept of the molecular clock. (*a*) Clock calibration is performed with a known fossil divergence time. (*b*) Error in estimation of genetic distance from divergence of interest affects the intersect with the slope. (*c*) Error in estimation of genetic distance from calibration divergence affects the value of the slope (or 'rate'). (*d*) Error in estimation of calibration divergence time from the fossil records also affects the value of the slope ('rate').

genetic difference between species generally increases with time and will thus carry genealogy information. Information about this genetic 'distance' is fundamental for reconstructing evolutionary relationships. A related and more controversial method of studying genes through time (Zuckerkandl & Pauling 1965) is the strict assumption of a linear relationship between genetic distance and evolutionary difference (figure 2*a*). This interpretation of molecular data leads to the corollary that genetic variation also describes the timing and pattern of diversification. Indeed, like phylochronology, the 'molecular clock' may be an effective means to study whether past environmental change coincides with major evolutionary transitions.

A breadth of molecular studies using phylogenetics to study species' relationships has also included a molecular-clock component. The addition of estimation of divergence times has allowed testing of several palaeoclimatic or palaeontological hypothe-

ses with molecular data. Representative studies in birds and mammals include the investigation of potential causes of recent population bottlenecks (Paxinos *et al.* 2002; Hadly *et al.* 2003), time of speciation caused by Pleistocene glacial cycles (Hewitt 2004; Zink *et al.* 2004) and divergences between groups of species related to time of mountain uplift (Smith & Patton 1999; Randi *et al.* 2000), sea-level changes (Cooper & Cooper 1995; Mercer & Roth 2003), aridification (Riddle 1995; Douady *et al.* 2003), global cooling and volcanism (Springer *et al.* 2003; van Tuinen *et al.* 2004).

Because DNA degrades with time, it is likely that there is a limit to aDNA studies contained well within the Quaternary (Hofreiter *et al.* 2001). Events from the pre-Quaternary are reconstructed only from fossils themselves or from modern DNA with genetic differentiation between the organisms coalesced to their most recent common genetic ancestor. This approach can be enhanced through repeated investigations using different molecular markers, and is strengthened when agreement is reached between genetic data and the fossil record. The optimum approach to reconstructing evolutionary events from deep time is to find consensus among geological dates, different molecular datasets, among palaeontological datasets and between molecules and fossils. While progress continues towards this optimum, certain discrepancies persist and links to climate often remain based on analyses of few loci.

(*a*) *The value of palaeontology in a molecular world*

Although we are currently in the genomic era, it can be argued that geological expertise is needed more than ever (Hadly 2003). In part, this follows because palaeontology and geology continue to provide the macroevolutionary framework for hypotheses actively tested with genetic data. The synthesis between palaeontology and neontology remains one of the most exciting platforms for current and future evolutionary research, provided it is conducted with care and a clear understanding of the data and their limitations (Smith 1998; Smith & Peterson 2002; Hadly 2003). Clearly, palaeontology has contributed greatly to our understanding of organismal evolution and patterns of speciation. The fossil record is used frequently to test whether biotic extinction and origination are correlated in time with particular environmental changes (Vrba 1993; Meng & McKenna 1998; Janis *et al.* 2000). Since the advent of the molecular-clock principle 40 years ago (Zuckerkandl & Pauling 1965), geneticists have refined the time-scale of organismal evolution while vastly improving our knowledge of molecular evolution. The molecular-clock principle asserts that the number of molecular character substitutions or phylogenetic distance between two organisms is roughly proportional to time since divergence (Zuckerkandl 1987). To use a molecular clock, the slope of the linear molecular distance–geological time function is estimated ('calibrated') from a node with a known divergence time and this slope ('molecular rate') is then applied to estimate divergence times of groups with scant fossil records (figure 2*a*).

(*b*) *Conflicts between fossil ages and molecules' time estimates: when and why do they occur?*

The partnership between palaeontology and neontology is perhaps closest in the study of dating the branches on the 'tree of life' (Benton & Ayala 2003; Conroy &

van Tuinen 2003), yet debate in this area is also strongest regarding the relative merits of fossils and modern genetic records. This debate is fuelled by major discrepancies observed in the evolutionary time of first appearance of well-known groups such as animal phyla (Doolittle *et al*. 1996; Wang *et al*. 1999; cf. Conway Morris 1998), and major mammalian (Benton 1999; Foote *et al*. 1999; Novacek 1999; cf. Easteal 1999; Hedges & Kumar 1999) and bird (Feduccia 2003; cf. Hedges *et al*. 1996; Cooper & Penny 1997; van Tuinen & Hedges 2001; Paton *et al*. 2002; van Tuinen *et al*. 2003) lineages. Molecular time-scales frequently have suggested that lineage origination may be twice as old as fossils imply. In these examples, molecular estimates suggest that a considerable part of the early history of major animal lineages is missing from their fossil record. According to these estimates, metazoan phyla originated several hundred million years (*ca*. 750–1200 Myr) before the Cambrian explosion (*ca*. 560 Ma) of these animals in the fossil record. Additionally, most orders of birds and mammals appear to have originated within the mid-late Cretaceous (*ca*. 80–100 Ma), while the fossil record suggests a sudden appearance of modern lineages in the early Tertiary (65–54 Ma). All too often, the debate among palaeontologists and molecular biologists has stagnated into concluding that either clocks or fossils must be severely biased (Cooper & Penny 1997; Benton 1999; Feduccia 2003), which has not helped in further developing the interface between palaeontology and genetics.

In the last decade, research on evolutionary time-scales has considerably progressed with new fossil discoveries or reinterpretations (see, for example, Bajpai & Gingerich 1998), and with an improved understanding and modelling of molecular evolution (see, for example, Conroy & van Tuinen 2003). Some consensus is now emerging between datasets (Benton & Ayala 2003; Springer *et al*. 2003). Of particular interest is the congruence along several parts of the vertebrate time-scale (Kumar & Hedges 1998). Examples of agreement within mammals now abound in Artiodactyla (the even-hoofed mammals), rodents and several other orders (Goodman *et al*. 1998; Adkins *et al*. 2003; Springer *et al*. 2003). Within birds, although fewer studies exist, molecular time estimates also frequently agree closely with the fossil record or biogeographic expectations (Cooper *et al*. 2001; Cracraft 2001; van Tuinen *et al*. 2004). However, none of the palaeontological and molecular developments have eliminated entirely the existing discrepancy regarding the evolutionary divergence times of major modern bird and mammal lineages. This discrepancy complicates assessment of potential relationships between environmental change and vertebrate evolution. Hence, the effect of major environmental change on macroevolution at major points in vertebrate evolution remains unclear.

Several explanations of why molecular time estimates often precede those of the oldest fossils have been proposed in the literature. First, several fossil biases most likely exist, such as a bias against fossilization in certain habitats (Smith & Peterson 2002). Second, certain molecular biases are relevant, including the overestimation of molecular time when calibration is performed on saturated data, or occasions of strong lineage-specific rate heterogeneity. Third, the fossil record will never capture the exact time of divergence, and additionally the time of gene divergence may precede the time of lineage divergence. While these biases undoubtedly are at play in creating temporal discrepancies, molecular biologists do not believe that any genetic bias could be so consistent and strong and yet still be undetected. Likewise, palaeontologists find it hard to believe that the fossil record significantly and consistently underestimates time of divergence or that palaeontological biases are so prevalent

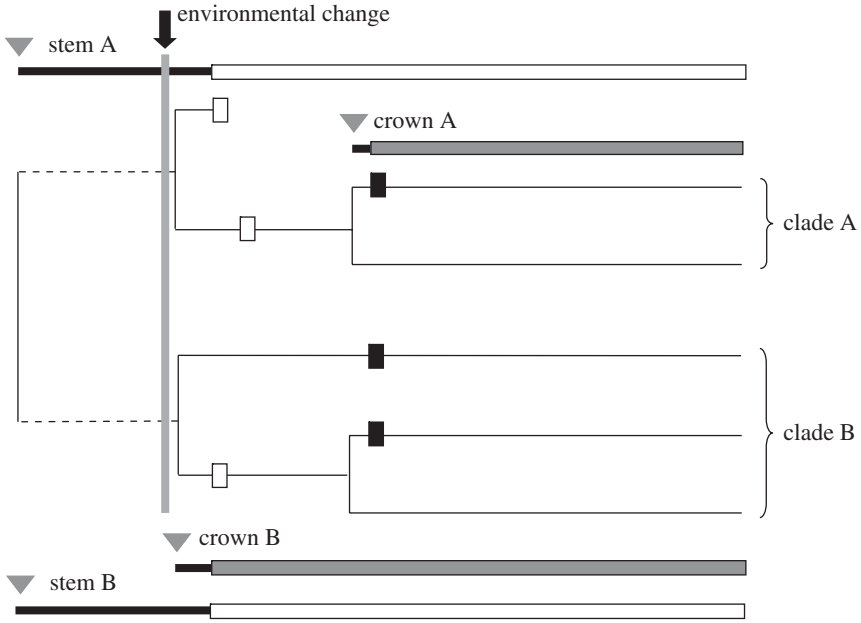

Figure 3. Concept of crown–stem in taxonomy, and its effect on interpreting the comparison of molecular time estimates against fossil ages. The crown of a lineage defines the diversification among extant species, while the stem points to the older origination event. Filled squares illustrate the oldest fossils relevant to a clade including only extant species; this age may be preceded by ancestral fossils (open squares) that do not fall within this clade and represent lineages now extinct. See text for discussion.

that they are the sole reason for the discrepancy with molecules. To better understand the reasons for the described conflict it is important to restate that agreement is frequently found as well, and that conflicts appear to occur mostly in describing the timing of the earliest history of major phyla and vertebrate classes. Communication between these fields will produce deeper insights into both molecular and fossil evolutionary patterns and processes. Without the integration of these two fields rigorous testing of either field would not be possible. Two additional causes that may underlie the conflict continue to be little recognized. Hence we will elaborate on these further. These are the past emphasis of molecular and fossil studies on different parts of the tree, and the extensive uncertainties intrinsic to both records.

(*c*) *Lineage origination and diversification*

So far, it has been unclear to what extent the fossil record underestimates time of lineage divergence or whether consistent biases are pervasive in estimating ages through the assumption of a molecular clock. What has not been well established is whether this conflict is a general one, with increasing taxonomic depth. The conflict between molecular and fossil ages within vertebrates often has centred on the origin of orders of mammals and birds. For example, the molecular age of the primate–rodent split falls well into the Cretaceous (Kumar & Hedges 1998), but no fossil rodents or primates have been found from the Cretaceous. Is this discrepancy truly a conflict between methods? We need to remember that a molecular age for the divergence of

two clades A and B (figure 3) defines the time of origin of both clades A and B. This time can significantly precede the diversification within clade A and within clade B as defined by the deepest split among extant A or B members. This initial split among extant members is known as the crown of a clade, and the origin of a clade is designated as its stem. Therefore, to assess the true extent of temporal conflict among molecular and fossil ages, we should also ask whether molecular crown estimates also predate crown ages based on fossils. In the case for modern birds, most of the fossil record may better reflect crown group divergences than stem group divergences (figure 3). In contrast, most molecular-clock studies have emphasized stem lineage originations of ordinal and supraordinal clades (Hedges *et al*. 1996; Cooper & Fortey 1998; van Tuinen & Hedges 2001; Paton *et al*. 2002) such as the age of divergence between model systems, including the primate–rodent, cat–horse, ostrich–zebrafinch or duck–chicken split. Unfortunately, comprehensive molecular studies on crown-family time-scales are lagging behind because it requires large taxon sampling. Thus, cross-validation with the existing fossil record may yet be unjustified (Feduccia 2003; van Tuinen *et al*. 2003). Most recent analyses in unison of genetic and fossil data from mammals (Archibald 2003; Springer *et al*. 2003) and birds (van Tuinen *et al*. 2004) indeed argue that a significant portion of the observed disparity between divergence dates may be caused by their differential resolution of the origin of stem groups and crown groups. This work suggests that molecular clocks and fossil records of birds and mammals tell a similar story about time of diversification of orders and families, but that the fossil record remains quiet on the time of origination of many major lineages (figure 3). If this is true, effects of the environment on shaping bird and mammal biodiversity through time are optimally testable for diversification, not origination. Interestingly, the time of diversification within avian order and family appears to correlate with the two largest environmental changes in Tertiary history (van Tuinen *et al*. 2004): the transition from the Cretaceous to the Tertiary (K–T: global cooling, volcanism, meteorite impact), and from the Eocene to the Oligocene (global cooling).

(*d*) *Accounting for error in molecular-clock research*

How does the assessment of error affect resolution in time-scale studies? Surprisingly little is known about the size of error in molecular time estimation (Hillis *et al*. 1997), and the importance of fossil error is only beginning to be addressed in more detail (Hedges & Kumar 2004; Reisz & Müller 2004; van Tuinen & Hadly 2004*a*, *b*). To explore the historical usage of molecular clocks, documentation of error and palaeontological calibration, we performed an extensive survey of vertebrate molecular systematics literature. This survey covered 40 years of molecular evolutionary literature and yielded more than 500 different fossil-based calibrations. Furthermore, calibration error related to the dating of fossils has been either consistently unrecognized or avoided and we did not detect a single study that quantified this error. This review indicates that, although commonly recognized as critically important, evolutionary research is yet devoid of a detailed investigation on optimizing molecular-clock calibration with fossils. It thus remains to be determined how calibration error translates to other clock errors (figure 2*b*–*d*), how it can be quantified and how it may impact the power of the molecular clock. We recently addressed these issues quantitatively for the first time with emphasis on the modern bird (van Tuinen & Hadly 2004*a*)

Table 2. *Effect of error by genetic and fossil component on interpretation of molecular evolutionary divergence times for the major groups of placental mammals and extant birds*
($error_g$, genetic error; $error_{g+c}$, genetic plus calibration error; est_f, fossil age estimate.)

Placentalia				Neornithes			
estimate point[a]	$error_g$	$error_{g+c}$	est_f	estimate point[a,b]	$error_g$	$error_{g+c}$	est_f
Placentalia	+	+	+	Neornithes	+	+	+
Boreoeutheria	+	+	+	Neognathae	+	+	+
Laurasiatheria	+	+	+	Palaeognathae	+	−	−
Euarchontoglires	+	+	−	Galloanserae	+	+	+
Afrotheria	+	+	−	Neoaves	+	−	+
Xenarthra	−	−	−	Galliformes	−	−	−
Primates	+	−	−	Apodiformes	−	−	−
Rodentia	−	−	−	Charadriiformes	−	−	+
Eulipothyphla	+	−	−	Passeriformes	−	−	−

[a]95% confidence interval precedes K–T boundary for both origination and diversification (+) or neither (−).
[b]Point estimates also support a Cretaceous diversification of Anseriformes, Caprimulgiformes, Psittaciformes, Coraciiformes and Gruiformes.

and mammal (van Tuinen & Hadly 2004*b*) evolutionary time-scales in relation to testing the evolutionary impact of the K–T events, and proposed a standard method of calibrating clocks with fossils (see also Appendix B).

From the pertinent molecular, geological, palaeontological, biochronological and biostratigraphical literature we documented that several errors are involved in molecular estimation of time. Several uncertainties arise alone in assignment of fossil age to a calibration point, and may be more significant than commonly accepted due to the inability to date directly most fossils (figure 2*d*). As an example, we focused on the palaeontological age of the often-used bird–mammal calibration based on North American Carboniferous. Ages of these fossils are inferred from global stratigraphic correlation with European strata based on similarities of the plant faunas. The ages of the European Upper Carboniferous strata derive from radiometric dating of available volcanic ash material. However, several major boundaries still can be dated only through interpolation between geological strata due to lack of datable material. Each of these steps incurs error. Compared with the Carboniferous geological time-scale, the Cenozoic is better known. Partly this is true because of the additional tool that magnetic stratigraphy brings at this time level. However, the dating of popular Cenozoic calibrations such as the whale–artiodactyl divergence still suffers somewhat from certain geological limitations, such as the indefinite relative stratigraphic merits of benthic to planktonic foraminifera or to terrestrial mammals and the evaluation of information on eustatic sea-level changes in biozonation of marine strata at the foothills of the Himalayas (Thewissen *et al*. 2001).

These errors, when considered simultaneously with molecular error (Appendix B), may lead to compounded error of more than 10% of the age of the calibration. If true, the statistical power of molecular clocks to test macroevolutionary hypotheses may be significantly weaker than has been claimed with a confidence interval based

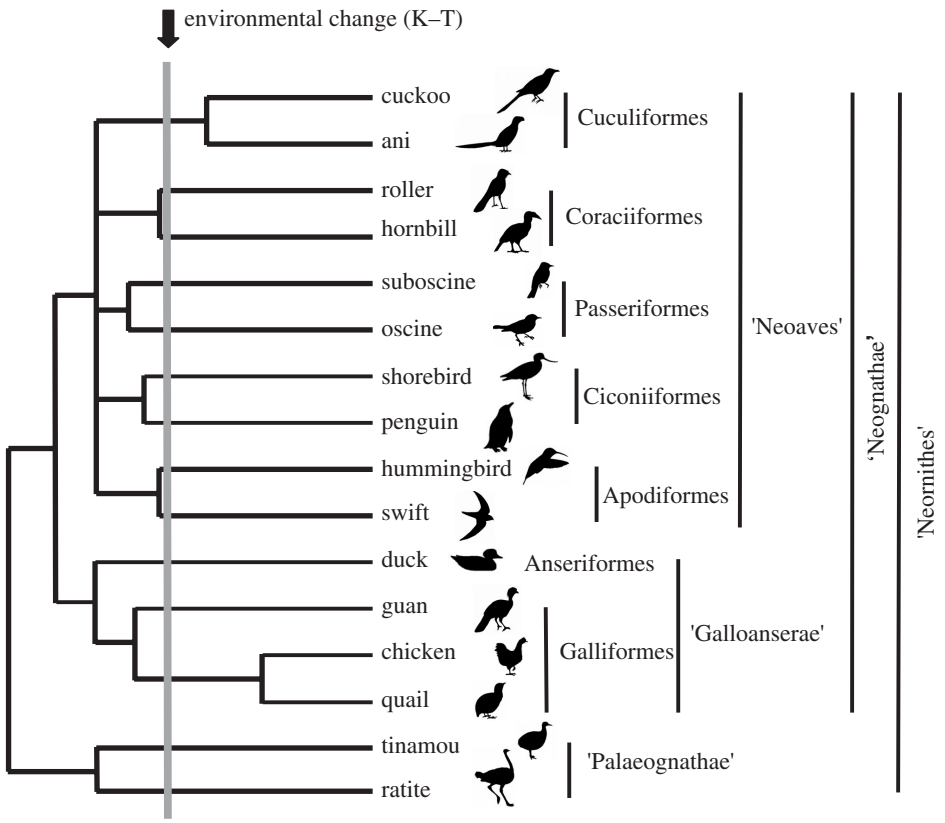

Figure 4. Evolutionary time-scale with nomenclature of the major lineages of birds. (Adapted from van Tuinen & Hedges (2001).) Note that only point estimates are shown.

solely on assessment of genetic error. For example, when compounded with genetic uncertainty ('error in time estimation', figure 2*b*), calibration uncertainty ('error in rate estimation', figure 2*c*, *d*) itself can alter the power of the molecular clock in interpreting diversification of bird (figure 4) and mammal (figure 5) orders around the K–T extinction event. After taking into account calibration error, none of the molecular clock estimates from representative molecular studies for the diversification of placental mammals and neornithine bird orders significantly predates the K–T boundary (table 2). Thus, some molecular- and fossil-based divergence times may have less accuracy and precision than described thus far.

Our survey demonstrates that overlooking calibration error (type-1 error) may be part of the cause of the discrepancy observed in the tempo of vertebrate diversification (figure 6). This assessment, however, is statistically based and it remains to be confirmed on a biological basis. This can be accomplished by sampling across more genes but also by further refinement of molecular-clock calibration and error assessment. Regardless of the outcome, it is clear that discrepancies between molecular time estimates and the K–T events remain. For the early evolution of birds, the fossil record does not reach as far back in time as molecular studies suggest, and bird fossils crossing this gap are all based on fragmentary material. However, for placental mammals the story is altering and fossils now are beginning to fill some of these

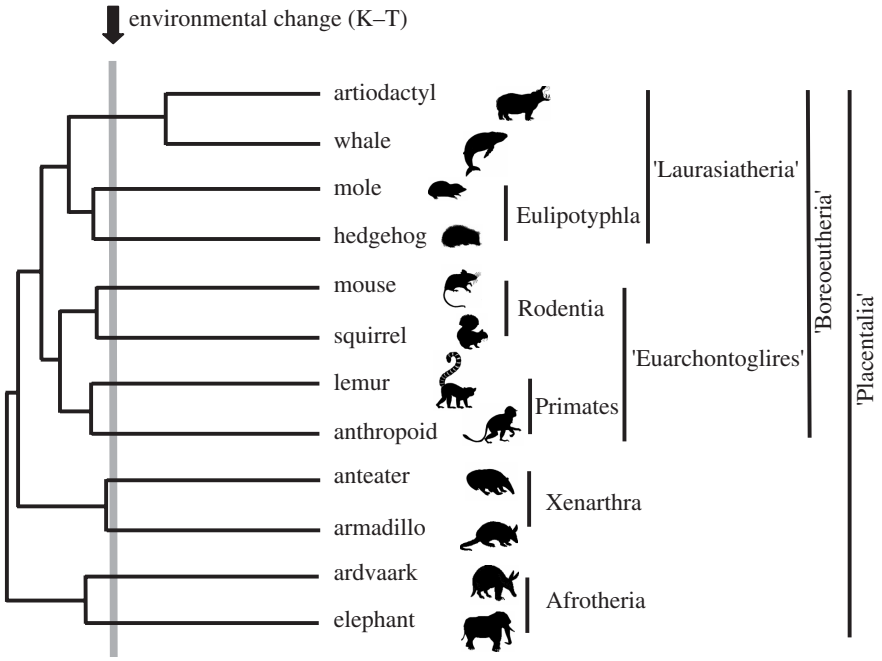

Figure 5. Evolutionary time-scale with nomenclature of the major lineages of placental mammals. (Adapted from Springer *et al*. (2003).) Slightly older ages for some of these groups have been found in another multi-gene analysis (Kumar & Hedges 1998). Note that only point estimates are shown.

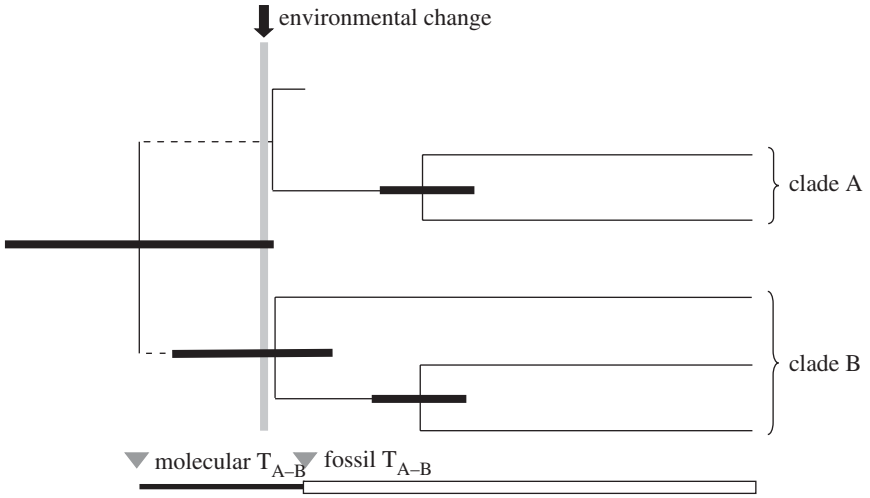

Figure 6. Effect of molecular uncertainty on testing environmental or palaeontological hypotheses. See text for discussion.

gaps (Archibald 2003). All in all, the remaining anomalies suggest that the fossil record may better reflect lineage diversification (figure 3): a hypothesis supported by the general congruence of clock estimates with fossil ages for the diversification of placental mammal and neornithine bird orders and families (Springer *et al*. 2003;

van Tuinen *et al*. 2004). Such a result is perhaps not surprising considering that the earliest stem lineages are defined by more uncertain characters and fewer species.

(*e*) *Quantification of molecular and fossil time uncertainty*

The minimum approach is one way to deal with multiple uncertainties at once (Appendix B). This method yields a single propagated error around each (average) time estimate. Comparison of molecular time-scales with fossil ages is performed in a conservative way by comparing the upper 95th percentile (equal to the youngest) of the molecular estimate to the age(s) of the oldest diagnostic fossil(s). Although total error is smaller than the sum of the individual errors, this propagated error value may have a low probability because of considerable temporal overlap of the distribution for several uncertainties. Therefore, to estimate more realistic uncertainty intervals, we could employ a random sampling method that estimates the most likely distribution of final age uncertainty (Ramakrishnan *et al*. 2005). This sampling process essentially runs many times through a series of steps, each of which incurs an uncertainty. The series of steps starts with choosing which fossils to use. Once a decision is made (e.g. guided by the minimum approach), we then assign the fossil to a geological stratum using an assumed probability distribution. After this step, we assign a time-interval to this stratum, based on picking a lower and upper boundary age from a probability distribution of time extracted from direct radiometric age and corresponding error estimates. Once a time-interval is chosen, we assign the age of this fossil randomly within this interval (unless we have more specific information). This step yields an absolute fossil age. This age then is assigned to a node on a molecular phylogenetic tree, and the molecular rate of this dataset is inferred from the estimated genetic distance defining this calibration node. Genetic distance then is estimated for the divergence that we would like to date. Finally, a single divergence time is estimated by dividing the genetic distance by the estimated molecular rate. By running through these steps, say 1000 times for each calibration model, we produce 1000 divergence times for the same node. The distribution and range of these times for each node concurrently reflect the underlying net geological and genetic uncertainties. For cross-validation with the palaeontological record, we can compare these divergence time-intervals to the available fossil data for each node (table 2). A comparison often is performed in the literature between molecular and fossil point estimates but without accounting for error in either record. Therefore, the quantification of multiple errors and resulting time confidence intervals will be a significant advance towards molecular appraisal of evolutionary hypotheses based on climatic and/or fossil data.

5. Quantification of congruence between molecules and fossils

The addition of uncertainty ranges to molecular-based time-scales allows us to better investigate the amount of congruence between fossil and genetic time estimates across clades. We currently are developing a novel method that quantifies the congruence across the entire tree. This method estimates the index of time-interval congruence (TIC) that takes advantage of our modelled uncertainties in both fossil and molecular times. Here, TIC index $= 1 - x$, where x is the proportion of nodes on the entire tree where the known fossil record is younger than the 5th percentile of the estimated

molecular time-interval. TIC is the proportion of nodes on the entire tree that contain overlap of fossil age interval with the $p > 0.05$ of the molecular time range. The closer the TIC index is to 1, the greater the overall congruence between fossils and genetics. For example, of the 10 nodes in the bird tree in figure 4, only four nodes (the three basal nodes plus the arboreal songbirds) show that the molecular ages statistically continue to precede the existing fossil record. Hence, the TIC index is 0.6. For comparison, the congruence between bird fossils and molecular clocks only would be 0.1 when not considering geological or genetic uncertainty. Worse, for the early history of placental mammals all 10 nodes in figure 5 yield molecular estimates older than the available fossil record. A TIC value of zero for mammals can be revised to 0.4. Considering that the minimum molecular estimate for all placentals only predates the oldest 'placental' fossils by 4%, conflict may be considerably smaller than is generally assumed. Thus, our approach underscores the need to acknowledge that molecular-clock-based divergence times come with extensive uncertainty, and that it is difficult to evaluate truly where we stand in the current fossil–clock debate without accounting for error (figure 4).

(*a*) *Future developments in molecular-clock research*

What are the respective roles of molecular biology, palaeontology and geology in the twenty-first century? It is clear that estimation of an evolutionary timeline will be furthered by integration of these fields. Increasingly relevant to the construction and interpretation of this timeline are not only genetic themes such as molecular phylogenetics, genomics, bioinformatics, population genetics, molecular evolution and developmental biology (Carroll 2000), but also palaeontological themes such as biostratigraphy, geochronology (Armentrout 1994), geobiology, taphonomy, palaeoclimatology, macroevolution, functional morphology and systematics (Lane *et al.* 1997). Surprisingly, one of the underdeveloped and understudied interfaces between palaeontology and genetics that remains in the twenty-first century is the joint role both fields play in constructing accurate evolutionary time-scales. Thus far, discussion has centred on the temporal usefulness of fossils and molecular clocks and their inherent biases. The increased interest in construction of a 'tree of life' will naturally be followed by development of a 'time-scale of life' (Benton & Ayala 2003). Since molecular time-scales must always be calibrated with fossils, continuing research on the qualities and errors of the fossil and genetic record is required. For temporal calibration of molecular clocks, fossils are needed that are easily diagnosed morphologically, that approach a major extant evolutionary node in age, but that also are tightly constrained in geological age. Geologists and palaeontologists can supply these data to geneticists. Unfortunately, this information transfer has not been straightforward or optimized yet, and understanding the errors involved in such a transfer has hardly been addressed. Future attempts to prioritize certain fossil records for use in molecular-clock calibration will need to reflect both the benefits and limitations of molecular, palaeontological and geochronological data. The provision of increased temporal stringency and resolution will be of tremendous scientific value because only accurate evolutionary time-scales will allow understanding of the relationship between climatic, geological and evolutionary trends. Ultimately, such insight will tell us how, why and not just when major organisms evolved on our planet. We suggest that a more conservative approach in calibrating molecular clocks with the

fossil record may be required, demonstrating that molecular time estimation must remain firmly rooted in palaeontology and hence continuously updated. The realization of such an integrative evolutionary time-scale will most likely be recognized as a Rosetta Stone to the tree of life and hence a most valuable source to all of biology.

(*b*) *Final thoughts on bridging the gap*

Increased geographical and temporal sampling and increased number and type of molecular markers coupled with the appropriate analysis techniques and estimation of uncertainty in these techniques will allow us to determine a much more accurate picture of the evolutionary past of populations, and its links to the environment. Most progress will likely be made at the population level, but insights must eventually be coupled to patterns and processes above the species level. This coupling will remain a challenging prospect for years to come, partly because phylochronology does not extend to beyond the species scale and divergence-time estimation is confounded below the species level. A legitimate option of studying the interface between micro and macro occurs at the community scale, where surprising stability within communities is now being discovered in species composition both in time and space (Hadly & Maurer 2001). Species composition may be maintained by the microevolutionary processes underlying species abundance and appears robust to macroevolutionary changes such as extinction.

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Appendix A. Taphonomic requirements of appropriate assemblages for phylochronology

We suggest the following set of requirements for future scientific opportunities in phylochronology.

(i) For DNA preservation, the assemblages must be of Holocene or Late Pleistocene age.

(ii) The assemblages should be accurately dated, well stratified and have good age association.

(iii) The assemblages preferably should contain a high diversity of species.

(iv) The assemblages preferably should contain hundreds of individuals per species.

(v) Each assemblage must contain many time units for the purpose of serial comparison.

(vi) For best interpretation of obtained genetic data, information must exist about external environmental data.

(vii) There must be accompanying evidence of demonstrated response of species.

Appendix B. Uncertainties in estimation of molecular divergence times, and the minimum approach

There are several errors inherent to use of molecular clocks. These are broadly separated into molecular error and error associated with the fossil calibration. Assessment of molecular errors (e.g. in branch length estimation and deviation from substitution rate homogeneity) is increasingly common in molecular-clock studies (Bromham *et al*. 1999; Nei & Kumar 2000; Rodriguez-Trelles *et al*. 2002). In sharp contrast to this welcome development, once a particular fossil lineage is picked for calibration, molecular biologists consider the age of these fossils to be without error. This ignorance is in part due to the lack of collaboration between palaeontologists, geologists and geneticists (Reisz & Müller 2004). Below we summarize probable uncertainties that may need to be considered for most common fossil calibration points.

(*a*) *Fossil choice (phylogenetic assignment uncertainty)*

Most researchers use the literature age assigned to the fossil specimen(s), and apply this age to the node that defines the most recent common ancestor of the extant lineages displaying the diagnostic macroevolutionary characters. This strategy makes an important assumption: that the earliest fossils from an extant lineage provide accurate measures of the divergence and are not significantly biased towards the present. Uncertainty arises when the stem and crown group cannot easily be distinguished from the fossil record, when the selected minimum age is far from the true divergence time, and when the phylogenetic placement of the fossil on the tree is unclear.

(*b*) *Stratigraphic uncertainty*

Stratigraphic uncertainty is the uncertainty involved in assigning a fossil to a geological stratum. Stratigraphic positioning of fossils may be difficult in certain cases.

(*c*) *Geological boundary age uncertainty*

A stratigraphic interval is characterized by the ages of the upper and lower boundaries, both of which contain radiometric-dating-associated uncertainty. This uncertainty is likely to be normally distributed around the mean radiometric age inferred from, for example, the $^{40}Ar/^{39}Ar$ and $^{40}K/^{39}Ar$ plateaus (Hess & Lippolt 1986; Menning *et al*. 2000).

(*d*) *Geological boundary age interpolation uncertainty*

In order to estimate the final absolute age of the fossil lineage and the calibration node on the tree, the fossil has some probability of falling at any point between the upper and lower boundaries of the designated stratum. However, these boundaries have not always been directly dated. Indirectly dated boundaries are based on interpolated ages from averaging between the nearest directly dated boundaries, assuming constant sedimentary deposition rate.

(*e*) *Correlation uncertainty*

This error may be the most difficult to quantify. It is the uncertainty in inferring geological age by aligning identical strata across continents.

Once an age has been assigned to a node in the phylogenetic tree, we can estimate the genetic distance or branch length between the terminal living lineages describing that node. From this calibration distance and the assigned age, we then estimate the molecular rate. The final step towards time estimation is to take observed genetic branch lengths elsewhere on the tree and convert these into evolutionary divergence times using this rate. Here again, several uncertainties should be acknowledged.

(*f*) *Quantifying molecular uncertainty in pairwise distance estimation*

Phylogenetic models often are selected to optimize particular genetic parameters given the data related to the phylogenetic tree. Such choices may impact the ratio between pairwise branch lengths of the calibration to that of other nodes. However, since most researchers focus on optimizing the tree and not the individual branch lengths, the impact of model assumptions on this uncertainty has not been explored exhaustively. For example, where parameter-poor models perform nearly as well as models with multiple parameters, simpler models may be preferred since they carry less variance.

(*g*) *Stochastic variation among different partitions of the genome*

Error derives from varying representation of genomic rate in single gene estimates (Kumar & Hedges 1998; Nei & Kumar 2000). Generally, sampling across many clock-like genes can reduce this stochastic error, which is easily quantified and often reported. For multi-gene studies it currently is debated whether one should take the mean (or mode) of single gene estimates or use a concatenated gene approach (Nei *et al*. 2001). Both methods average the variety of molecular behaviour across genes, but differ in the weighting approach.

(*h*) *Targeting for minimum molecular divergence time estimates*

A problem with quantifying phylogenetic error is that either an older fragmentary fossil is part of the clade of interest or it is not. As a possible solution, we recently proposed the use of only those fossils for calibration that carry diagnostic characters for the calibration clade and called this the minimum approach because it is based on estimation of minimum, not true or likely, divergence times (van Tuinen & Hadly 2004*a*, *b*). There are several reasons for using a minimum approach despite its overly conservative nature. First, minimum estimates are readily provided by the age of the earliest undisputed fossils, and not based on fragmentary remains of earlier fossils carrying unknown phylogenetic and temporal error. Furthermore, molecular clocks are most often used to test evolutionary scenarios derived from the fossil record, and the nature of the fossil record makes these scenarios one-tailed. This is because a known stratigraphic range based on undisputed fossils can only err on the lower bound. We acknowledge that strict cladistic interpretation based on critical characters combined with a minimum-age approach necessitates frequent revision of calibration age and amended molecular estimates.

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