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Patterns of mammalian diversification in recent evolutionary times: global tendencies and methodological issues

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Abstract

Changes in diversification patterns estimated from phylogenetic trees are an important source of information about the dynamics of evolution. To study the diversification of mammals, we reconstructed phylogenetic trees of 29 families and fitted both constant-rate and variable-rate models of diversification. In addition, we investigated the effect of clock models and phylogenetic reconstruction problems on diversification analyses. We observed, first, that none of the families increased its diversification rate during the last few million years, including the Pleistocene. Furthermore, we detected a decrease in diversification that, after application of different tests, was significant only for a minority of families. However, when diversification variation was analysed in a combined tree of all families, a global decline in diversification became significant. Therefore, although distorted by some methodological artefacts, we found an underlying signal of gradually decreasing diversification that suggests that ecological factors may have shaped the recent diversification of mammals.

Introduction

The dynamics of origination and extinction of species has been a long-studied topic in evolutionary biology, mainly by examination of taxa in the fossil record (Sepkoski, 1998; Benton, 2009), but also using phylogenetic methods. Indeed, phylogenetic trees contain crucial information regarding the patterns of diversification of a group of organisms (Hey, 1992; Nee *et al.*, 1992; Kirkpatrick & Slatkin, 1993; Harvey *et al.*, 1994; Paradis, 1997; Pybus & Harvey, 2000; Nee, 2001, 2006). Diversification is the net generation of lineages in a phylogeny, and it is therefore equivalent to the effect of speciation minus extinction. One of the fundamental questions about biodiversity is why, after balancing speciation and extinction, some lineages are composed of so many species whereas others are very species poor (Bokma, 2003; Alfaro *et al.*, 2009). In addition, it is crucial to understand why the growth in species number in some lineages has been constant through time, whereas others reveal changes in diversification at different time points. Recently developed likelihood methods can fit different models of constant-rate and variable-rate diversification in a phylogeny, allowing the selection of those that better explain the branching patterns of a phylogeny (Nee *et al.*, 1994; Paradis, 1997; Rabosky, 2006b; Stadler, 2011). Detection of these phylogenetic patterns in diversification rates is particularly interesting, as they may provide clues about the mechanisms involved in the generation of biodiversity (Sanderson & Donoghue, 1996; Moore *et al.*, 2004; Rabosky, 2006b).

Factors that affect diversification rates can be broadly classified as biotic and abiotic (Barnosky, 2001; Benton, 2009). Biotic factors include, for example, the appearance of key adaptations that may trigger the rapid generation of new lineages. Other important biotic factors are the saturation of ecological niches occupied by a group of related species and the subsequent competition for resources, which may slow down the diversification possibilities for the group (Rüber & Zardoya, 2005; Kozak *et al.*, 2006; Weir, 2006; McPeek, 2008; Phillimore & Price, 2008; Rabosky & Lovette, 2008a,b). In principle, these biotic factors should be

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specific to certain taxa, and they do not necessarily coincide in time. Among the abiotic factors are large climate changes, tectonic processes, and catastrophic events, and they are supposed to affect the phylogenetic patterns of different groups at approximately the same time. Particularly interesting among the abiotic factors are the Pleistocene glaciations, which have been proposed to increase the rate of diversification in different taxa. According to this hypothesis, speciation would have been promoted as a consequence of the fragmentation of the geographical distribution of faunas at both temperate and tropical latitudes, and the separation of populations in glacial refugia (Hewitt, 2000; Lister, 2004).

Studies of diversification should benefit from the comparison of a large number of groups to reveal general tendencies as well as factors that may have had a greater impact in the generation of current biodiversity. In mammals, works on diversification have been performed using supertree approaches, but they have been restricted to certain groups like the orders Carnivora (Bininda-Emonds *et al.*, 1999) and Chiroptera (Jones *et al.*, 2005), or to the oldest evolutionary events (older than 25 Myr) in the whole mammalian phylogenetic tree (Bininda-Emonds *et al.*, 2007). In addition, a recent work used the same mammalian supertree to study the recent diversification of mammals using a novel method that estimates diversification rate shifts along a phylogeny (Stadler, 2011). In this work, several shifts, with abrupt changes in diversification rates, were found in the recent evolution of mammals. However, this particular supertree is very poorly resolved at the species level and contains many polytomies in the last 25 Myr (Bininda-Emonds *et al.*, 2007), which could affect diversification analyses.

In this study, we used the mitochondrial cytochrome *b* gene to generate species-level phylogenetic trees of mammalian families that had enough available sequence information. We compiled nucleotide sequences for 29 families, estimated phylogenetic trees, made them ultrametric, and studied the variation in diversification rates along the phylogenies. We also paid particular attention to the effects that methodological artefacts could have on diversification analyses. Thus, we used different molecular-clock models to analyse whether they affected diversification analyses and devised a new diversification test that includes the potential effect of phylogenetic reconstruction problems. After these analyses, several important patterns in the diversification of mammals emerged. First, although none of the families showed an increase in diversification during the last few million years of evolution, we detected a significant decrease in diversification in five families. Furthermore, when all families were analysed in a single composite phylogenetic tree, a gradual and significant decrease in diversification rate along evolution with no diversification rate shifts could be observed. We propose that this diversification pattern could be due to saturation of ecological niches.

Materials and methods

We retrieved cytochrome *b* sequences from GenBank (Benson *et al.*, 2009) and followed a thorough process of curation and alignment (see Data S1 in Supporting Information for details). Only families with eight species or more and a coverage > 50% were used, resulting in a final data set of 29 families. Alignment length was between 384 and 1146 base pairs, with an average of 1060 base pairs.

Reconstruction of maximum-likelihood phylogenetic trees and estimation of relative divergence times

Model selection was assessed with jModeltest (Posada & Crandall, 1998; Posada, 2008) using the Akaike Information Criterion (AIC) (Akaike, 1974). Twenty-three families (of a set of 29) followed a General Time Reversible (GTR) model with a gamma distribution of evolutionary rates with or without a proportion of invariable sites (Table S1 in Supporting Information), indicating that this model is the most appropriate to describe the molecular evolution of cytochrome *b* in mammalian families. As we wanted to calculate evolutionary parameters in a comparable way across mammals, we used this model for all families. We also checked that the use of the most general model in the families where this was not the best-fit model did not affect the diversification analyses (not shown).

Phylogenetic trees were reconstructed by maximum-likelihood estimation using PHYML 2.4.4 (Guindon & Gascuel, 2003) with a GTR evolution model, a gamma distribution of substitution rates with four rate categories, and a proportion of invariable sites. The parameters of the GTR model, the gamma parameter and the proportion of invariable sites were estimated by the program.

Diversification analyses require ultrametric trees. We did not calibrate the maximum-likelihood trees with multiple fossils to avoid that incongruent combinations of calibration points could alter the tree shape. Rather, we estimated ultrametric trees using the NonParametric Rate Smoothing (NPRS) method, as implemented in r8s 1.71 (Sanderson, 1997, 2003). For this purpose, each maximum-likelihood tree was rooted with the outgroup, which was later removed, and the age of the root was arbitrarily fixed to 1. Next, the scale factor that best approximated the ultrametric tree to the maximum-likelihood tree was calculated using Ktreedist 1.0 (Soria-Carrasco *et al.*, 2007). Then, the ultrametric tree with arbitrary scale was multiplied by this scale factor to obtain a genetic distance scale. In this new arbitrary timescale, ultrametric trees have an overall mean evolutionary rate of 1. Given that we do not assume constant evolutionary rates in the process of constructing these ultrametric trees, their branches are not directly proportional to the substitution rate. They are rather proportional to time. However, this scale allows an

appreciation of the relative differences in the ages of families (provided that the variation in evolutionary rates among families is moderate) as well as the conversion to a timescale in Myr by using an overall evolutionary rate, which was necessary for the combined analyses of trees. Moreover, it allows a direct comparison with BEAST trees, where the mean evolutionary rate is also fixed to 1.

Bayesian inference of phylogenetic trees assuming an uncorrelated relaxed clock

Bayesian phylogenetic reconstructions were carried out using BEAST 1.5.2 (Drummond & Rambaut, 2007). In these analyses, outgroups were removed and the root position was estimated by the program. A GTR model with four substitution rate categories for the gamma distribution was used along with a proportion of invariants estimated by the program. An uncorrelated relaxed molecular clock fitted by a lognormal distribution (Uncorrelated Clock Log-Normal) with the mean evolutionary rate fixed to 1 was assumed. All priors were left to default settings, except that a speciation Yule process was set as a tree prior. BEAST was run for a different number of generations depending on the number of species in the analysed alignment in order to ensure that effective sample size values for all parameters were higher than 200. In addition, we did several independent runs to prevent incorrect results derived from Markov chain Monte Carlo (MCMC) chains being trapped in local maxima. Thus, we did two runs of 15 000 000 generations for alignments of 100 species or less, and four runs of 15 000 000 generations for alignments over 100 species. Multiple runs were combined with LogCombiner. In all cases, the chains were sampled every 1000 generations and the first 10% of the samples was removed as burnin. We initially checked that the default 10% threshold was adequate. We obtained the subsequent maximum clade credibility summary tree with median node heights using the TreeAnnotator program (also included in the BEAST package) setting the posterior probability limit to 0.5.

General diversification analyses

The methodology of Rabosky (2006b) was initially followed to perform the diversification analyses. This approach was designed to find out if diversification rates have changed through time. Thus, the null hypothesis is that a phylogeny follows a constant-rate model. The analyses are based on the difference in the AIC values (Akaike, 1974) between constant-rate and variable-rate models of diversification (ΔAICrc). First, the vector of branching times (separation times between successive nodes) of a given tree was fitted by maximum likelihood to a set of diversification models, and the model with the lowest AIC value was chosen as the best-fitted model. The set of diversification models consisted of two constant-rate and four variable-rate models. The constant-rate models were a Yule speciation process (pure birth) and a constant speciation and extinction rates model (birth–death). The variable-rate models comprised a pure birth model with a shift of the speciation rate at certain time (yule2rate), a birth–death model with a shift of the speciation and extinction rates at certain time but maintaining the extinction fraction (extinction/speciation) constant (rvbd), an exponential density-dependent speciation rate model (DDX), which models the speciation rate as a function of the number of extant lineages at any time point and may be increasing or decreasing, and a logarithmic density-dependent speciation rate model (DDL), in which the number of lineages increases until reaching a K limit analogous to the 'carrying capacity' parameter of population ecology.

To avoid false positives in the detection of variable diversification, it is necessary to know whether the difference in AIC between the best variable-rate model and the best constant-rate model (ΔAICrc) is statistically significant (Rabosky, 2006b). In addition, most families had incomplete sampling of species (average species coverage = 75% in our data set; Table 1), and therefore, their phylogenetic trees may show a variation in diversification due to missing species (Heath *et al.*, 2008). Thus, when the highest-likelihood model for a phylogenetic tree was a variable-rate model, a null distribution was generated to verify it. Taking the highest-likelihood constant-rate model (decided on AIC) as the null model, 500 ultrametric tree simulations were generated with Phylogen (Rambaut, 2002), using the estimated speciation rate (and the estimated extinction rate when the resulting highest-likelihood constant-rate model was birth–death). Each simulation was run until the number of species of the Wilson & Reeder (2005) mammalian taxonomy for the family was reached. To consider the effect of incomplete species sampling, the tips of trees were randomly pruned in the corresponding proportion to reproduce the same species coverage observed in the original family tree. Afterwards, the set of models was fitted to every simulated tree, generating a ΔAICrc distribution from which a one-tail critical value at a 0.05 significance level was used to determine whether to discard the constant-rate model. For constructing the plots of the simulated trees, they were scaled to the genetic distance of the original tree.

In addition to the previous test, we calculated for each phylogenetic tree the γ statistics (Pybus & Harvey, 2000), which reflect whether diversification in a phylogeny has been increasing or decreasing through time, together with the critical value at a 0.05 significance level obtained from the null distribution of the γ statistics. This distribution was constructed, for all families, from a set of tree simulations as described earlier.

All of the diversification calculations were carried out with the APE 1.9.4 (Paradis *et al.*, 2004) and LASER 1.0

Table 1 Total number of known species in each family (*N* total), number of species in the phylogeny (*N*), % taxon sampling, and diversification model selected by the ΔAICrc test and γ value for the NPRS and BEAST trees. In the ΔAICrc tests, CR and VR indicate constant-rate and variable-rate models, respectively.

Order	Family	*N* total	*N*	% taxon sampling	ΔAICrc model NPRS	γ NPRS	ΔAICrc model BEAST	γ BEAST
Artiodactyla	Bovidae	142	119	83.8	DDL (VR*)	−5.68*	yule2rate (VR*)	−3.20*
	Cervidae	51	30	58.8	DDX (VR)	−2.34	DDX (VR)	−1.71
	Suidae	19	11	57.9	DDL (VR)	−1.22	Pure birth (CR)	−0.80
Carnivora	Canidae	35	23	65.7	Pure birth (CR)	−0.20	Pure birth (CR)	−0.27
	Herpestidae	33	20	60.6	DDL (VR*)	−4.39*	DDL (VR*)	−3.65*
	Mustelidae	59	37	62.7	DDX (VR*)	−3.85*	yule2rate (VR)	−1.67
	Otariidae	16	15	93.8	rvbd (VR)	−0.49	Pure birth (CR)	−0.28
	Phocidae	19	18	94.7	DDL (VR)	−2.22*	DDL (VR)	−1.16
	Ursidae	8	8	100.0	DDL (VR)	−1.24	Pure birth (CR)	−0.40
	Viverridae	35	27	77.1	DDL (VR)	−1.58	Pure birth (CR)	−0.15
Cetacea	Delphinidae	34	33	97.1	yule2rate (VR)	−1.91*	DDL (VR)	−1.56
	Ziphiidae	21	19	90.5	DDL (VR*)	−3.18*	DDL (VR*)	−3.98*
Chiroptera	Mormoopidae	10	8	80.0	DDL (VR)	−1.39	DDL (VR)	−1.04
	Phyllostomidae	160	100	62.5	yule2rate (VR*)	−3.95*	yule2rate (VR)	−0.56
Dasyuromorphia	Dasyuridae	69	60	87.0	DDL (VR)	−2.34*	DDL (VR*)	−6.01*
Diprotodontia	Potoroidae	10	9	90.0	DDL (VR)	−0.92	DDL (VR)	−0.88
Lagomorpha	Leporidae	61	38	62.3	DDL (VR)	−1.93	Pure birth (CR)	0.41
	Ochotonidae	30	26	86.7	rvbd (VR*)	−2.88*	yule2rate (VR*)	−2.60*
Primates	Cheirogaleidae	21	14	66.7	Pure birth (CR)	−0.11	Pure birth (CR)	−0.02
	Hylobatidae	14	10	71.4	DDX (VR)	−1.02	DDL (VR)	−0.95
	Lemuridae	19	10	52.6	DDL (VR)	−0.93	Pure birth (CR)	−0.11
	Lepilemuridae	8	8	100.0	DDL (VR)	−1.05	DDL (VR)	−0.95
Rodentia	Bathyergidae	16	11	68.8	Pure birth (CR)	0.02	Pure birth (CR)	0.45
	Cricetidae	681	360	52.9	DDL (VR*)	−8.29*	DDL (VR*)	−5.97*
	Ctenomyidae	60	38	63.3	DDX (VR*)	−2.86*	DDL (VR)	−1.14
	Geomyidae	40	32	80.0	DDL (VR)	−2.38*	DDL (VR)	−1.93
	Heteromyidae	60	53	88.3	DDL (VR*)	−3.17*	DDL (VR)	−2.32*
	Octodontidae	13	8	61.5	DDL (VR)	−1.67	DDL (VR)	−1.40
Soricomorpha	Talpidae	39	21	53.9	DDL (VR*)	−3.09*	DDL (VR*)	−2.56*

DDL, logarithmic density-dependent speciation rate model; DDX, exponential density-dependent speciation rate model; NPRS, nonparametric rate smoothing.
*ΔAICrc or γ values were significant.

(Rabosky, 2006a) packages for the R statistics programming environment (R Development Core Team, 2010). The calculations necessary to construct the null distributions were performed with the help of a grid of computers using a shell script that splits the sets of simulations to analyse them in different processors.

Diversification analysis taking phylogenetic reconstruction problems into account

We modified the test of Rabosky (2006b) described earlier to explore the possibility that phylogenetic reconstruction problems (including the transformation of trees with NPRS) could generate false positives in the standard diversification analysis. As before, we generated, for each mammalian family, 500 ultrametric tree simulations under a constant-rate model. The simulated trees were scaled to the genetic distance of the original tree. Then, we generated alignment simulations from the simulated trees with Seq-Gen 1.3.2 (Rambaut & Grassly, 1997). The program options were set to a GTR model with relative rate of substitutions between nucleotides, α shape parameter of the gamma distribution of substitution rate categories, proportion of invariants, and nucleotide frequencies taken from the previous maximum-likelihood estimations of the corresponding mammalian phylogeny. The number of substitution rate categories was set to 4. As before, phylogenetic trees were reconstructed from these alignments using PHYML with the same options previously used. These maximum-likelihood trees were midpoint rooted using the RETREE program included in the PHYLIP package (Felsenstein, 1989) as they were generated assuming a strict molecular clock. Subsequently, rooted trees were made ultrametric using the NPRS algorithm implemented in the r8s program. Finally, these trees were used to construct the ΔAICrc statistics distributions, and to calculate the critical values as above.

Diversification analysis using a combined tree of mammalian families

To have a global view of the diversification of mammals through time, we constructed a combined tree that included the 20 phylogenies that spanned more than 0.125 substitutions/positions from root to the tips. This threshold was equivalent to 10 Myr assuming an approximate evolutionary rate of 0.0125 substitutions per position per Myr (Pereira & Baker, 2006; Bininda-Emonds, 2007; Weir & Schluter, 2008), meaning that we had 20 families older than 10 Myr. We selected this threshold for combining trees in order to recover as much recent evolutionary history as possible without eliminating too many families (with a higher threshold we could cover more time but with a lower number of families). For the construction of the combined tree, we joined the phylogenies in such a way that all tips aligned to present time and the root branch of every tree was prolonged to the same time in order to form a basal polytomy. This operation was performed with a Perl script that used Bioperl subroutines (Stajich *et al.*, 2002). Then a lineage-through-time (LTT) plot, which represents the growth of the number of lineages in logarithmic scale, and a rate-through-time (RTT) plot, which represents the diversification rates at different time points, were drawn to graphically observe the global diversification trends in mammals over the last approximately 10 Myr. For the RTT plot, diversification rates were calculated by fitting a pure-birth model in windows of 0.2 Myr. In this small interval, a constant-rate model is a reasonably good approximation and fitting a pure-birth model allows the calculation of the absolute diversification rate at a given time period. Subsequently, we fitted a regression line to the diversification rates and calculated its slope as a measure of the diversification rate variation. Thus, negative values of the slope represent an increase in diversification rate and positive values a decrease (taking into account the reverse direction of the time axis in the corresponding figure).

To analyse whether the rate variation observed in the combination of mammalian trees was significant, we constructed combined trees as above with simulated trees including the effect of phylogenetic reconstruction problems and random sampling. Simulations were generated using Phylogen with the speciation rate (birth rate), number of species, species coverage, alignment length, and substitution model parameters sampled from the empirical distributions derived from the reconstructed trees of mammalian families. In order to reproduce the effect of sampling, tips of the simulated trees were randomly pruned in the corresponding proportion to reproduce the same species coverage observed in the original family tree. We also investigated the effect of nonrandom sampling by pruning trees following a scheme of overdispersed sampling with a moderate degree of sampling bias towards the root ($\alpha = 1$) (Brock *et al.*, 2011), which rendered qualitatively similar results (not shown). We assumed as before an evolutionary rate of 0.0125 substitutions per position per Myr. Simulations that yielded trees whose root was under 10 Myr or trees with fewer than eight species were discarded in order to reproduce the original process. When the sum of species in the simulated trees reached the number of species in the real combined tree, all the trees were joined as in the real data set. Then, we calculated diversification rates in windows of 0.2 Myr and estimated the slope of the regression line. We repeated this process 500 times to construct the null distribution of global rate variation.

Results

Temporal variation in diversification rates

We were interested in detecting possible shifts in diversification rates along the mammalian phylogenies. For this purpose, we used cytochrome *b* sequences of 1166 species belonging to 29 mammalian families and 10 different orders. All these families had more than 50% species coverage and they would include, with complete coverage, 1783 species (Table 1). In addition, sequences come from different works for each family, contributing to a random coverage of different phylogenetic depths in most families. We reconstructed maximum-likelihood phylogenetic trees of these families and converted them to ultrametric trees using the NPRS algorithm. Using these trees, we fitted different models of constant and variable diversification rates to the corresponding trees (Table 1). In three families, a model of constant rate (pure birth) was selected by the AIC. For the remaining families (26), one of the four variable-rate models was selected: the DDL model in 18 families, the DDX model in four families, the yule2rate model in two families and the rvbd model in two families. In all cases, the temporal variation implied a decrease in diversification rate towards present time, either gradual (DDL and DDX models) or through an abrupt transition point that divides the phylogeny into two periods with different diversification rates (yule2rate and rvbd models).

In order to test the significance of ΔAICrc and to take incomplete taxon sampling into account, we followed a protocol of tree simulations to construct a null distribution of ΔAICrc values (see Materials and methods). Table 1 shows that only for 10 mammalian families, the ΔAICrc value was significant with this test ($P < 0.05$). Examples of phylogenies with different diversification patterns, together with the LTT plots of the simulated phylogenies, are in Fig. 1. (Fig. S1 in Supporting Information shows the diversification analyses for all families.)

Of the 10 families with a significant variation in diversification, only two followed a model with a shift in diversification: the chiropteran family Phyllostomidae (yule2rate) and the lagomorph family Ochotonidae (rvbd). Thus, the number of families showing an abrupt

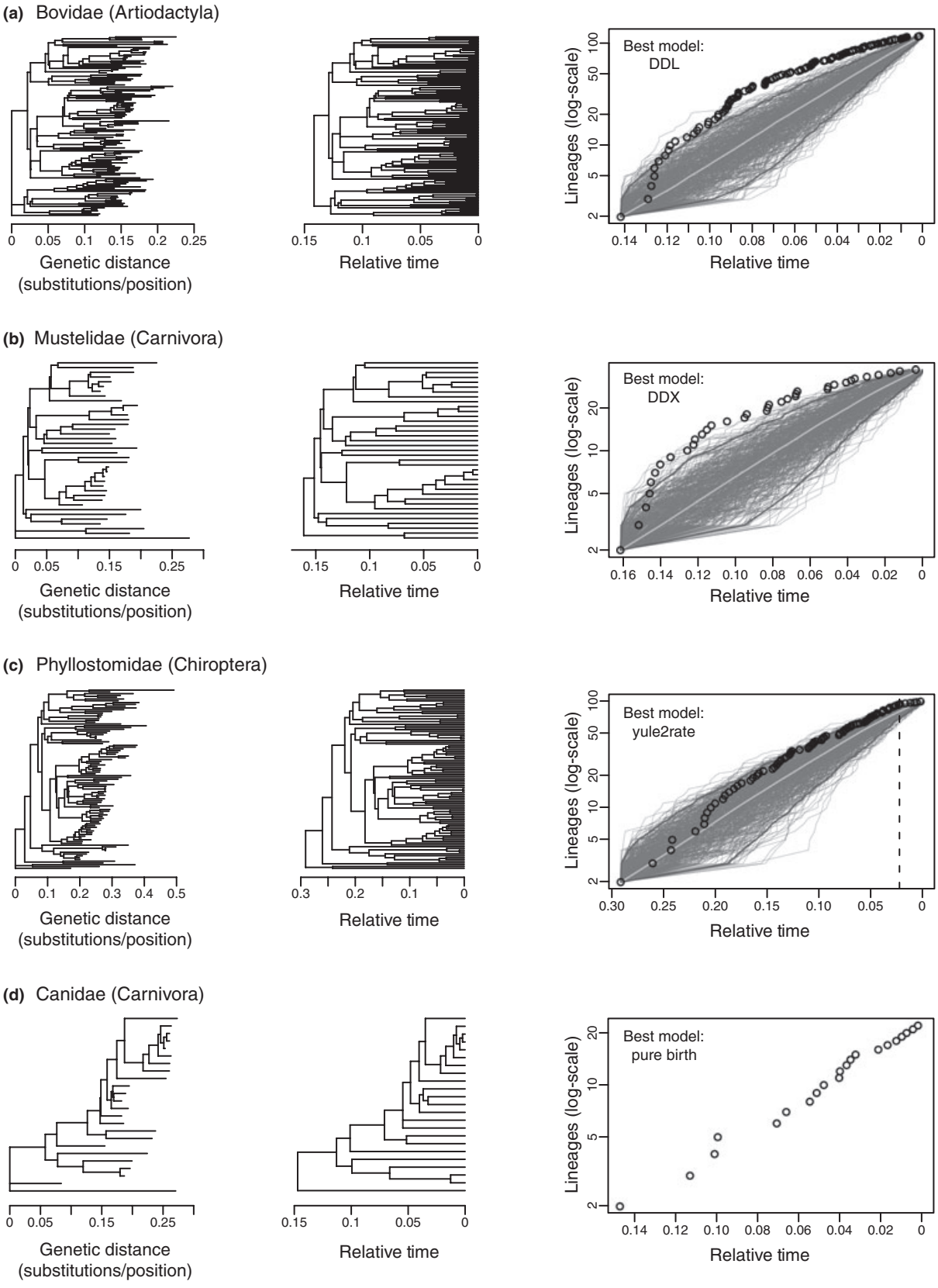
(a) Bovidae (Artiodactyla)
0 0.05 0.1 0.15 0.2 0.25
Genetic distance
(substitutions/position)
0.15 0.1 0.05 0
Relative time
Best model:
DDL
Lineages (log-scale)
2 5 10 20 50 100
0.14 0.12 0.10 0.08 0.06 0.04 0.02 0
Relative time
(b) Mustelidae (Carnivora)
0 0.05 0.15 0.25
Genetic distance
(substitutions/position)
0.15 0.1 0.05 0
Relative time
Best model:
DDX
Lineages (log-scale)
2 5 10 20
0.16 0.14 0.12 0.10 0.08 0.06 0.04 0.02 0
Relative time
(c) Phyllostomidae (Chiroptera)
0 0.1 0.2 0.3 0.4 0.5
Genetic distance
(substitutions/position)
0.3 0.2 0.1 0
Relative time
Best model:
yule2rate
Lineages (log-scale)
2 5 10 20 50 100
0.30 0.25 0.20 0.15 0.10 0.05 0
Relative time
(d) Canidae (Carnivora)
0 0.05 0.15 0.25
Genetic distance
(substitutions/position)
0.15 0.1 0.05 0
Relative time
Best model:
pure birth
Lineages (log-scale)
2 5 10 20
0.14 0.12 0.10 0.08 0.06 0.04 0.02 0
Relative time

Fig. 1 Phylogenetic trees reconstructed from cytochrome *b* sequences by maximum likelihood (left panel), ultrametric trees obtained by the NonParametric Rate Smoothing method (middle panel), and lineage-through-time (LTT) plots (right panel) of four representative mammalian families: (a) Bovidae, with a logarithmic density-dependent speciation rate model, (b) Mustelidae, with a exponential density-dependent speciation rate model, (c) Phyllostomidae, with a yule2rate model, and (d) Canidae, with a pure-birth model. On the right panel, open dots represent the LTT plot of the ultrametric tree of the family, and grey lines are the LTT plots of each of the 500 simulated trees. White and black lines represent the mean of the simulated LTT plots and the 2.5% and 97.5% percentiles, respectively. In the phylogeny with the yule2rate model, the shift time is graphically represented with a vertical dotted line. In Canidae, the selected model by the ΔAICrc test was a pure birth model, and therefore, the diversification simulations were not performed.

shift in diversification was very small and not concentrated in a particular period (Fig. S1 in Supporting Information).

We also calculated for all families the γ statistics (Pybus & Harvey, 2000), and tested its significance with tree simulations as above (Table 1). All 10 families for which ΔAICrc was significant also gave rise to a significantly negative γ value, which indicates a decrease in diversification through time. Four additional families were significant for the negative γ value. Thus, although there was an overall good agreement between the ΔAICrc and γ criteria, the former was slightly more conservative, as previously shown (Rabosky, 2006b).

To test the effect of different clock models in diversification analyses, we also reconstructed molecular-clock trees with the BEAST program using an uncorrelated relaxed clock model. The diversification analyses with BEAST trees indicated, again, a consistent decrease in diversification (Table 1). The number of families that significantly rejected the constant-rate model was 7, six of which coincided with significantly variable-rate families in the NPRS trees. The γ parameter was negative for most BEAST trees (and significant for eight of them), also indicating a tendency of most families to decrease in diversification. The correlation of the γ parameter between NPRS and BEAST trees was high (Pearson's $r = 0.74$, $P < 0.0001$), but it also showed certain spread, indicating that the shape of both types of trees was different in some families as a consequence of using different clock models. In summary, BEAST trees also showed a decrease in diversification, although this tendency was significant for a smaller number of families than with NPRS trees. In no instance, a significant increase in diversification towards present time was observed.

Methodological factors affecting the analysis of diversification variation: effect of phylogenetic reconstruction problems

Using the NPRS trees and the more conservative ΔAICrc statistics, we evaluated whether the decrease in diversification observed in several families could be due to some type of phylogenetic reconstruction problems that could generate false positives in the diversification analyses.

The process of reconstruction of ultrametric trees involves the determination of the topology and branch lengths by a maximum-likelihood method as well as the transformation of trees with NPRS. Biases in some of these steps could lead to an artifactual alteration of the tree shape that could in turn affect the diversification analysis. In particular, it is well known that nucleotide substitution saturation may lead to shorter branch lengths towards the root of the tree (Philippe & Laurent, 1998), which can alter the observed diversification pattern (Revell *et al.*, 2005). Thus, sequence saturation may produce an artifactual tendency of decreasing diversification along a phylogeny. To analyse this possibility, we devised an extended protocol to test the significance of ΔAICrc by which we first simulated phylogenetic trees with parameters obtained from the tree of each family, then simulated sequences along these phylogenies and, finally, estimated maximum-likelihood trees from such sequences. These estimated trees, reconstructed with the same methodology than the real tree, were then used to calculate the null distribution of ΔAICrc values (see Materials and methods). The LTT plots constructed this way widen due to different sources of stochasticity associated with the phylogenetic reconstruction, making it more difficult to attain significance. Using this test, significance was lost for the families Phyllostomidae, Cricetidae and Ctenomyidae (Table 2 and Fig. 2). Thus, in these three families, the observed decrease in diversification could be due in fact to some type of bias in phylogenetic reconstruction such as substitution saturation.

After the application of these tests, seven families remained with a significant decrease in diversification: Bovidae (Artiodactyla), Herpestidae (Carnivora), Mustelidae (Carnivora), Ziphiidae (Cetacea), Ochotonidae (Lagomorpha), Heteromyidae (Rodentia) and Talpidae (Soricomorpha). Of these, Mustelidae and Heteromyidae did not show significantly variable diversification with BEAST trees and thus, in the end, only five families passed all the tests. These five families, which have a strong signal of decreasing diversification, do not seem to have any particular feature in common: they belong to different mammalian orders and do not share any obvious pattern related to life-history variables such as body size, habitat or distribution range (not shown).

Global diversification trends in mammals

Our results show that in most mammalian phylogenies, there is a tendency towards a decreasing diversification. It seems, however, that most individual phylogenies do not have enough power to show a significant pattern, and this could be mainly due to incomplete sampling. To

Table 2 Best diversification model for families with a rate-variable model of diversification, ΔAICrc and critical value (CV) at a 0.05 significance level of the standard diversification analysis and a test that takes phylogenetic reconstruction problems into account. An asterisk indicates that ΔAICrc is bigger than the critical value and thus it is significant. In the standard analysis, the asterisk in parenthesis indicates that the test was not significant when using BEAST trees.

Family	Best model	ΔAICrc	CV standard	CV phylogenetic
Bovidae	DDL	33.21	6.76*	27.41*
Herpestidae	DDL	25.96	6.71*	9.14*
Mustelidae	DDX	17.95	8.01 (*)	10.37*
Ziphiidae	DDL	13.71	5.83*	12.74*
Phyllostomidae	yule2rate	16.53	10.48 (*)	17.77
Ochotonidae	rvbd	11.99	6.20*	7.53*
Cricetidae	DDL	69.63	26.61*	103.24
Ctenomyidae	DDX	9.12	8.62 (*)	12.53
Heteromyidae	DDL	9.90	6.53 (*)	9.73*
Talpidae	DDL	11.20	7.46*	8.34*

DDL, logarithmic density-dependent speciation rate model; DDX, exponential density-dependent speciation rate model.

overcome the possible lack of data for individual families and to visualize the general pattern of diversification variation that arises in mammals, we merged ultrametric mammalian phylogenies into a single combined tree with all tips aligned to present time (Fig. 3a). This phylogenetic tree contains all branching points of the constituent mammalian families from the most recent common ancestor of the youngest family to the present. Many of the older divergences are not present in this tree as we did not try to reconstruct the relationships among mammalian families as in a typical tree. To recover as much recent evolutionary history as possible without eliminating too many families, we combined the phylogenetic trees of families older than 10 Myr (assuming an evolutionary rate of 0.0125 substitutions per position per Myr). This set included 993 species belonging to 20 families. In this tree, the LTT plot can be constructed for the last 10 Myr and it should be a straight line if all trees followed a pure-birth model. However, as expected from the pervasive decline in diversification rates found in most mammalian families, this plot also showed a gradual decrease in diversification (Fig. 3b). We estimated in this plot the diversification rates at different time points by fitting a pure-birth model in windows of 0.2 Myr. The RTT plot constructed this way (Fig. 3c) clearly shows the actual decrease in diversification values from approximately 0.19 Myr^{-1} at 10 Ma to approximately 0.08 Myr^{-1} at present time. The slope of the regression line, representing the diversification rate variation, was 0.0113. Some points, particularly in the beginning of the plot, deviate from the central tendency due to the small window selected to construct the RTT plot and to the stochastic variation caused by the lower number of splits in this part of the tree, but they do not imply any particular trend. Thus, the LTT and RTT plots of the combined mammalian tree indicate that diversification has been gradually decreasing without showing abrupt shifts during the last 10 Myr. However, as shown above for individual families, these plots may reflect the effects of both real biological phenomena as well as the spurious decline due to a number of artefacts. Therefore, we evaluated the combined effect of phylogenetic reconstruction errors and random sampling by constructing, through simulation, the null distribution of the slopes of the RTT plots for a similar combination of pure-birth trees and conditions of phylogenetic reconstruction and incomplete sampling. Using this stringent test, the one-tail critical value at a 0.05 significance level was 0.0052. Thus, the estimated slowdown in diversification rate for the combined families was significant even in the presence of these possible artefacts.

Discussion

Diversification models in mammalian families

The most important conclusion of our analysis is that a constant diversification model cannot be rejected for most mammalian families. In addition, when the phylogenetic trees of the mammalian families were combined, we observed a gradual and significant decrease in diversification. These results are in disagreement with a recent analysis on mammalian diversification using a supertree, where four shifts in diversification rates were observed during the last 33 Myr of mammalian evolution, all of them involving abrupt changes (even with a three-fold change in diversification rate in some shifts) (Stadler, 2011). In particular, two of these shifts occurred in the last 10 Myr, a period in which we do not observe any large diversification shift in our composite tree (Fig. 3). Also, our analysis of individual families did not support the existence of generalized shifts in diversification rates. How can these different results be explained? First of all, the method of Stadler (2011) only allows the fitting of birth–death processes with shifts in diversification rates, and therefore it is still unknown whether a gradual increase or decrease diversification model would fit better the supertree. The other important difference is the data set used. Whereas we used phylogenies resolved at the species level, Stadler (2011) used a supertree where many recent groups were unresolved and therefore represented as polytomies in the supertree, as described in the original publication (Bininda-Emonds *et al.*, 2007 and supplementary information therein). According to the authors of this supertree, this mammalian phylogeny is increasingly unresolved after 50 Myr ago and they actually avoided any interpretation on the diversification patterns over the last 25 Myr (Bininda-Emonds *et al.*, 2007). Our data set is less complete, but it is fully resolved and therefore it should be more appropriate to answer evolutionary

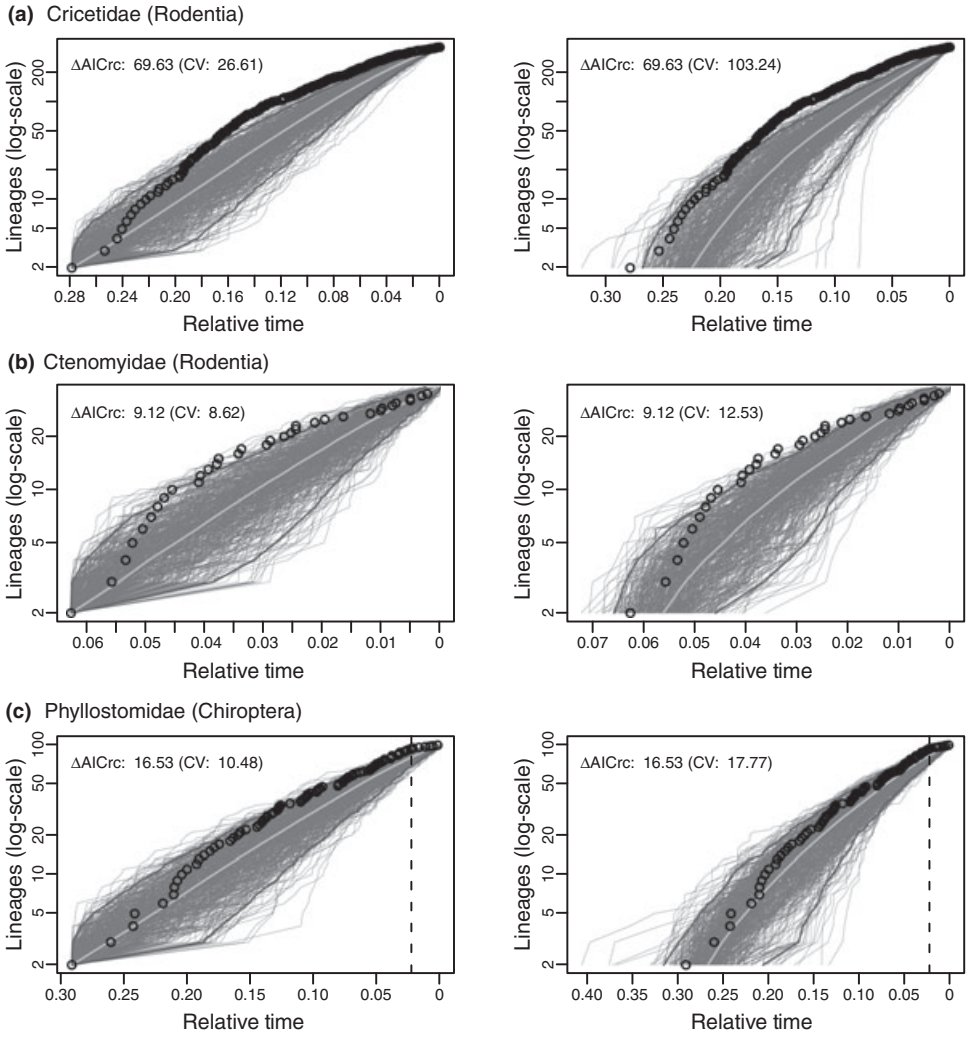

Fig. 2 Standard test of diversification analysis (left) and the extended test that includes the effect of phylogenetic reconstruction problems (right) for three families: (a) Cricetidae, (b) Ctenomyidae and (c) Phyllostomidae. Open dots represent the lineage-through-time (LTT) plot of the ultrametric tree of the family, and grey lines are the LTT plots of each of the 500 simulated trees (left) and of each of the 500 trees reconstructed from simulated alignments (right). White and black lines represent the mean of the simulated LTT plots and the 2.5% and 97.5% percentiles, respectively. The values of ΔAICrc for the tree of each family are indicated on the top left part of each plot, along with the critical values (CV) for rejection obtained through simulation.

questions at recent periods if incomplete sampling is taken into account. However, only a robust tree with complete sampling will allow to definitely determine the general diversification pattern of mammals.

A striking result of our analyses was that most diversification models selected for mammalian families did not include extinction. The only exceptions were Ochotonidae and Otariidae, where a model that includes extinction (rvbd) was selected. This was remarkable given that, according to fossil data, extinction rates have been shown to be high along most periods of mammalian evolution (Alroy, 1996). As already pointed out for other phylogenies where a similar situation was observed (Rabosky & Lovette, 2008b), this contradiction can be explained if a great majority of the extinct fauna belonged to clades not represented in the reconstructed phylogenies, that is, to crown groups. Thus, our phylogenies could be biased towards the most successful groups. Additionally, it has been recently shown that, when diversification rates vary among lineages, estimators of extinction based on simple models like the birth–death model are unable to recover true extinction rates (Quental & Marshall, 2010; Rabosky, 2010). This is a topic that deserves greater attention in the future, particularly by expanding the range of diversification models that include extinction as well as by integrating direct fossil information in order to obtain more realistic estimates of extinction rates.

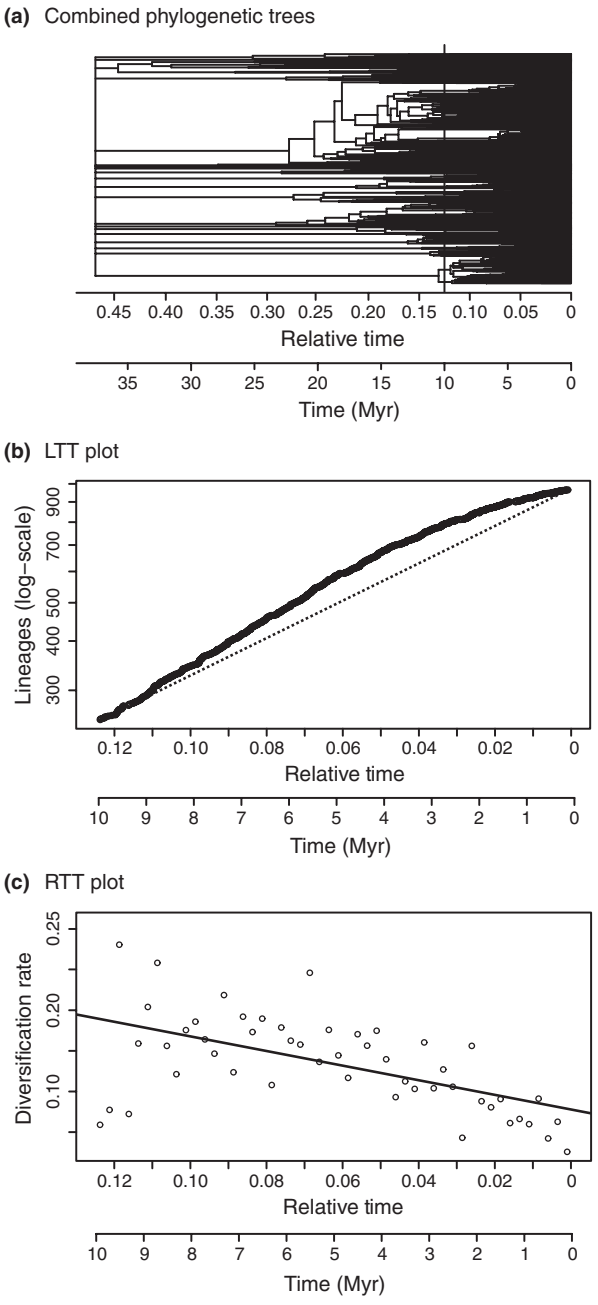

Fig. 3 (a) Phylogenetic tree formed by the combination of 20 phylogenetic trees of mammalian families that spanned more than 0.125 substitutions per position from root to the tips (approximately 10 Myr assuming a rate of 0.0125 substitutions per position per Myr) where all tips are aligned to present time. (b) Lineage-through-time plot derived from the last 10 Myr of the combined phylogenetic tree. The straight line that would be expected under a pure-birth model is shown as a dotted line. (c) Rate-through-time (RTT) plot constructed by calculating the diversification rate in windows of 0.2 Myr. A straight line has been fitted to the RTT plot (slope = 0.0113).

Diversification during the recent evolution of mammals: did Pleistocene glaciations affect diversification?

Our analyses indicated that all mammalian families showed a constant or a decreasing diversification, but never an increase. Therefore, an important consequence of our analyses of diversification variation in mammalian families is that, contrary to previous viewpoints, the Pleistocene glaciations did not trigger an increase in evolutionary diversification in mammals. Although these glaciations probably separated populations in different refugia (Hewitt, 2000; Lister, 2004), this effect was apparently not enough to increase the net rate of generation of new species. Previous works that addressed this problem were based on the comparison of the split times of sister species after and before the start of the Pleistocene epoch (Zink & Slowinski, 1995; Klicka & Zink, 1997; Avise & Walker, 1998; Avise *et al.*, 1998; Johnson & Cicero, 2004; Weir & Schluter, 2004). Although some of the earlier works on this topic did not show a great effect of Pleistocene glaciations on speciation (Zink & Slowinski, 1995; Klicka & Zink, 1997; Avise & Walker, 1998; Avise *et al.*, 1998), the most recent analyses detected such an effect (Johnson & Cicero, 2004; Weir & Schluter, 2004). However, branching times should be compared against a neutral model of lineage growth in a phylogeny (Zink & Slowinski, 1995), which could be a pure birth model or a birth–death model if extinction has been important. Using this approach for the diversification analyses of both the individual families and the composite plot, we observed that mammalian diversification decreased slightly during the Pleistocene epoch and it never increased. However, this effect was not different from the more general tendency of most families to decrease in diversification rate during their evolutionary history, so the Pleistocene was also not exceptional in this trend. Therefore, our analyses do not support any global change in rates and patterns of diversification in mammals during the Pleistocene glaciations. Given the low extinction levels that, except for megafauna, existed in the Pleistocene (Bennett, 2004), this would indicate that speciation events were not particularly favoured in this epoch. Nonetheless, these results do not contradict the possibility that glaciations had an important effect at the micro-evolutionary level and that the isolation of populations in glacial refugia promoted the subdivision of species into phylogroups with reduced gene flow between them (Avise & Walker, 1998; Avise *et al.*, 1998). It is also possible that some of these phylogroups, mostly those that separated as a consequence of the earliest glaciations, correspond to incipient species. However, the lag time required to generate new species is still largely unknown, so more data should be analysed to address these issues, particularly at the intraspecific level. In addition, it is possible

that analyses based on more restricted geographic areas (for example, boreal biomes or highland regions) may show local effects of the Pleistocene glaciations on the diversification of particular faunas (Weir, 2006).

Diversification decrease along mammalian evolution: artefact or real phenomenon?

The most frequent pattern that we found in the phylogenetic trees of mammalian families was a gradual decrease in diversification along the phylogeny, so that lineage generation appears to become slower towards present time. A similar decrease in diversification has been observed in many other vertebrates (Rüber & Zardoya, 2005; Kozak *et al.*, 2006; Weir, 2006; Phillimore & Price, 2008; Rabosky & Lovette, 2008a). A possible mechanism to explain this repeated pattern of slowdown in diversification, particularly for those families with density-dependent diversification, is an initial burst of speciation close to the origin of a group followed by saturation of ecological niches (McPeek, 2008; Rabosky & Lovette, 2008a). According to this model, after a certain time of evolution of a clade, competition for resources and saturation of the niches occupied by the species reduce the chances of successful speciation until reaching a state of species turnover equilibrium. However, this pervasive trend requires extensive testing to understand whether some artefact is involved in generating these results. We therefore checked several potential phylogenetic artefacts that could contribute to this decline in diversification.

The estimation of molecular-clock trees used in the diversification analyses requires the transformation of branch lengths, for example through methods that estimate evolutionary rates in a phylogeny (Sanderson, 1997, 2002) or with the use of a Bayesian model that incorporates evolutionary rate parameters in the phylogeny estimation (Drummond *et al.*, 2006). These methods may have a critical effect on the relative branch lengths of the final phylogeny. Although recently developed Bayesian methods may have a clear advantage because they allow using uncorrelated relaxed-clocks and can estimate ultrametric trees in a single step, there may be problems with overparameterization in small alignments (Wertheim *et al.*, 2010) and with the setting of priors, which could particularly affect branch lengths (Brown *et al.*, 2010; Marshall, 2010; Schwartz & Mueller, 2010). Additionally, it is not clear how the use of a Yule process as a tree prior might affect the downstream diversification analyses. Maximum-likelihood methods do not have these problems for branch length estimation but methods that estimate evolutionary rates from a reconstructed phylogeny such as NPRS assume autocorrelated evolutionary rates (Sanderson, 1997, 2002). Thus, it is possible that these algorithms could give rise to cumulative errors that potentially would create an artifactual variation of diversification. However, autocorrelated rates may be a reasonable assumption at the family level, where no large jumps in mutational mechanisms are expected (Welch *et al.*, 2008). To test the effects of these different methods, we used both strategies to obtain molecular-clock trees and, as expected, there were some differences in the shape of the trees obtained with NPRS trees and the Bayesian trees estimated with BEAST. However, the diversification analyses rendered similar results with both methods, with many families showing a decrease in diversification and never an increase. Although the conclusions of our work were not affected by the use of these different methods to estimate molecular-clock trees, it is clear that the diversification pattern of some families could change, and therefore, it might be advisable to use both methods in studies of specific families.

The process of phylogenetic reconstruction can be affected by other numerous artefacts that can alter not only the topology but also the tree shape. One of the most important problems that we expected with diversification analyses was sequence saturation, as this effect can lead to the underestimation of basal branch lengths and may seriously bias diversification analyses (Revell *et al.*, 2005). This effect could be particularly important for mitochondrial sequences, whose mode of evolution is more prone to sequence saturation (Kocher *et al.*, 1989). To try to estimate the magnitude of these problems, we designed a diversification test that takes phylogenetic reconstruction problems including substitution saturation into account. This test indicated that, for a few families (three out of 10; Table 2), a diversification slowdown could indeed be due to phylogenetic reconstruction problems, presumably sequence saturation.

After application of these tests, only five families (Bovidae, Herpestidae, Ziphiidae, Ochotonidae and Talpidae; Table 2) remained with a significantly variable diversification model. This is a very small number compared with the 26 initial phylogenies that showed a slowdown in diversification. In the other 21 phylogenies, different tests indicated that this pattern was not significantly different from a neutral model of lineage growth. However, despite the small number of phylogenies with a significantly variable pattern, it is intriguing that none of them showed a pattern of increasing diversification. This may indicate that the tendency for decreasing diversification is important but individual phylogenies did not have enough sampling to show this pattern as significant. The combined LTT plot of all phylogenies that we introduced here is very informative in this respect as it points to a gradual decrease in diversification along the last 10 Myr with no abrupt shifts in diversification rates. Furthermore, we have shown that this decrease cannot be artifactually produced by incomplete sampling or phylogenetic errors. Thus, this pattern may be more important than suggested by the individual phylogenies. All this would indicate that niche saturation could have had a genuine role in shaping the diversification patterns of a considerable number of mammalian groups.

Finally, it is important to emphasize that our study was designed to reveal general tendencies in the diversification of mammals by means of the analysis of a large number of species. However, it is clear that not all groups have necessarily been equally affected by the same evolutionary forces and that many other factors might have influenced the diversification of mammals. Groups that deviate most from the generalities are particularly interesting and surely deserve further studies.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1 Additional methods.

Table S1 Best-fit substitution model selected for the different mammalian phylogenies.

Figure S1 Diversification rate tests for 29 mammalian families.

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