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Fossils matter – understanding modes and rates of trait evolution in Musteloidea (Carnivora)

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ABSTRACT

Background: Patterns of change in ecomorphological traits have traditionally been studied using data from the fossil record. Recent advances in molecular phylogenetics created new opportunities for inferring ancestral character states and estimating the modes and rates of trait evolution from phylogenetic hypotheses of extant organisms. However, without fossil taxa useful information is potentially discarded and, in the worst case, results from extant taxa only may be misleading, in particular if extinction rates have been high and directional selection has acted.

Question: How does the integration of fossil information affect our understanding of macroevolutionary dynamics?

Organisms: Extant species of the superfamily Musteloidea (Carnivora – weasels and allies); extinct lineages of this clade (*c*. 30 to 2 Ma) sampled predominantly throughout the northern hemisphere.

Experiments: We focus on the evolutionary dynamics of carnivoran ecometric traits (three index ratios associated with locomotor habit and posture calculated from the osteological measurements), and we highlight the impact of using extant-only phylogenies versus integrated analyses when evaluating modes and rates of trait evolution.

Methods: We integrated extinct taxa into a molecular phylogeny of the extant species based on taxonomic knowledge (association with a particular family/subfamily) and sampling from the estimated times of speciation and extinction of each fossil lineage. We repeated the procedure for each of 500 trees from the posterior distribution of a Bayesian phylogenetic analysis. We compared the fit of different macroevolutionary models (Brownian motion, Ornstein-Uhlenbeck, accelerating/decelerating evolution, and Brownian motion with a directional trend) for all trees (with and without fossils), using AIC regression.

Results: The integration of fossil data into the analyses of trait evolution significantly affected model selection, evolutionary rates, as well as estimated trait values at the root of the phylogeny. In the case of the metatarsal III-to-femur ratio, the integrated analyses provided strong support for a Trend model, whereas the different macroevolutionary models could not be distinguished with strong statistical support when only extant taxa were considered.

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Furthermore, our analyses indicated substantial variation of evolutionary rates across the phylogeny, with high rates of evolution of locomotor traits along the backbone of the Mustelidae tree and within the Lutrinae clade in particular, reconciling complex evolutionary dynamics with more realistic root node estimates than obtained under the Trend model. Thus, the integration of fossils and molecular data has implications not only for our interpretation of evolutionary dynamics, but also for ancestral character reconstructions. Importantly, even in cases where extant-only and integrated analyses are in agreement, fossils remain essential because they provide additional support for inferred patterns of trait evolution.

Keywords: fossil, macroevolution, Musteloidea, phylogeny, traits.

INTRODUCTION

Present-day patterns of biodiversity are governed by biotic and abiotic factors that drive the dynamics of speciation and extinction. Functional traits mediate interactions between organisms and their environment (Tilman *et al.*, 1997; Eronen *et al.*, 2010; Polly, 2010; Schulze and Mooney, 2012; Carmona *et al.*, 2016), and determine how individuals can cope, move or adapt to local conditions (Webb *et al.*, 2010; Violle *et al.*, 2014; Polly *et al.*, 2016). Ultimately, such traits will also govern which species will survive, evolve or go extinct. Traits are thus central to the differential survival and reproduction of individuals in different environmental and geographical contexts (Polly *et al.*, 2011). Patterns of trait change through time have traditionally been studied using data mainly from the fossil record (Fortelius *et al.*, 2002; Hunt, 2007; de Boer *et al.*, 2012; Evans *et al.*, 2012). Recent advances in molecular phylogenetics and the development of new analytical tools have created opportunities for inferring ancestral character states and estimating the modes and rates of trait evolution from phylogenetic trees of extant organisms (Martins, 1994; Butler and King, 2004; O'Meara *et al.*, 2006; Beaulieu *et al.*, 2012). In either case, using only a part of the available data discards potentially useful information and in the worst case may be misleading (Finarelli and Flynn, 2006). Molecular phylogenetic analyses may suffer in particular if extinction rates have been high and directional selection has acted (Fritz *et al.*, 2013; Pyron and Burbrink, 2013). We have argued (Fritz *et al.*, 2013) that including both modern biological data and fossil information may be necessary to fully understand patterns and dynamics of organismal evolution. For example, in a recent study on mammalian body size evolution, Slater *et al.* (2012) demonstrate how molecular phylogenetic data together with information from fossils can be used to infer trait evolution in much greater detail than is usually possible in macroevolutionary studies. Their study shows that fossil data are crucial to identify the appropriate evolutionary model, particularly in cases where directional evolution towards an increased or decreased trait value is acting (i.e. if the evolutionary model deviated from a pure Brownian motion process).

Our focus here is to combine phylogenetic, ecomorphological, and fossil information and to evaluate the potential impact of the incorporation of fossil data on the mode of evolution, evolutionary rates, and character reconstruction, with a focus on locomotor traits. We use functional trait data from the superfamily Musteloidea (Carnivora) due to their high present-day species richness, global distribution, their habitat diversity, and the availability of a well-resolved molecular phylogeny. Specifically, we will (1) compare the fit of different macroevolutionary models with and without the integration of fossil information, and (2) evaluate the impact of integrating fossil information on the parameters of trait evolution models and reconstructed ancestral character states.

MATERIALS AND METHODS

Phylogenetic tree

We used published molecular sequences for Musteloidea: 75 of 86 species (Wilson and Reeder, 2005), from 22 nuclear and 5 mitochondrial genes (e.g. Kurose *et al.*, 2000; Sato *et al.*, 2004, 2012; Koepfli *et al.*, 2007, 2008; Eizirik *et al.*, 2010) (see www.evolutionary-ecology.com/data/3013Appendix.pdf for details). The sequences were aligned using Geneious v.5.1.7 (Kearse *et al.*, 2012), and all alignments were combined into a concatenated matrix (total length 17,972 bp). We built a dated molecular phylogeny of the extant Musteloidea (families Ailuridae, Mustelidae, Procyonidae, and Mephitidae) using MrBayes v.3.2.2 (Ronquist *et al.*, 2012). MrBayes employs a Bayesian MCMC approach to simultaneously infer the tree topology and divergence times under a relaxed molecular clock model [independent gamma rates (Lepage *et al.*, 2007)]. The following nodes were calibrated after Sato *et al.* (2012) using a truncated normal distribution: crown Musteloidea (mean 30.68 Ma), Mephitidae (15.6 Ma), Lutrinae (9.8 Ma), Guloninae (6.51 Ma), Mustelinae (6.56 Ma). The calibration point for the Procyonidae (24.4 Ma) was taken from Koepfli *et al.* (2007). We ran four independent runs (each with six chains) for 10,000,000 generations, sampling trees and parameters every 5000 generations, and combined the resulting data into a consensus tree, discarding the first 20% of the samples as burn-in.

Integration of fossil taxa into the phylogeny

To explore the impact of integrating information from extinct lineages on model selection and character reconstruction, we incorporated four fossil Musteloidea into each of 500 randomly selected trees from the posterior distribution of the MrBayes analysis: *Trocharion albanense* (Leptarctini), *Sivaonyx beyi* (Lutrini), *Teruelictis riparius* (Lutrini), and *Pannonictis* (Mustelidae). Based on the available fossil record of Musteloidea (849 occurrences for 277 species), we first estimated the species-specific times of speciation and extinction using PyRate (Silvestro *et al.*, 2014a, 2014b). PyRate jointly estimates species-specific times of speciation and extinction and the rates of the underlying birth–death process while explicitly incorporating the probability of preservation and sampling. In the case of *Pannonictis*, speciation and extinction times were calculated for the oldest species of this genus. The extinct species were then inserted into the molecular phylogeny using R (R Development Core Team, 2015; A. Michelle Lawing, personal communication). We identified all branches that are in accordance with the age and relationships of each extinct taxon. We selected randomly from each set of appropriate branches and added the fossil as a separate branch, with the ages of the root and tip of these branches being sampled from the posterior distributions of the times of speciation and extinction, respectively. All subsequent analyses were run on these 500 trees.

Morphological data

Locomotor traits were quantified using six osteological measurements (Fig. 1). Measurements of extant Musteloidea species were made on the collection of the Museum für Naturkunde (MfN) Berlin, Germany and added to those previously published by Polly (2010) and Polly and Sarwar (2014). The measurements were taken from the hind limbs of adult specimens (determined based on the closure of the epiphyseal plates). If possible, both male and female individuals were measured for each species. Only undamaged bones were

measured and in most cases the measurements were taken from the right hind limb. All measurements were taken with digital calipers (Promat, Max Schön AG, Lübeck, Germany). Three index ratios associated with locomotor habit and posture were calculated from the osteological measurements (Fig. 1). The first ratio (R1, measurement 1/2), the metatarsal III-to-femur ratio, is a measure of digitigrady and often associated with cursoriality in mammals (Garland and Janis, 1993; Steudel and Beattie, 1993). Ratios R2 and R3 are gear ratios related to the lever mechanics of the foot. Ratio R2 describes the ratio of the in-lever to out-lever of the calcaneum and is calculated from the length of the distal to the proximal calcaneum (measurement 3/4). The third ratio (R3) is calculated from the length of the calcaneum to the position of the sustentacular facet (measurement 5/6). All three index ratios are positively correlated with 'digitigrady' (Polly, 2010). The ratios were first calculated for each specimen and then averaged across species (3013Appendix). We were able to calculate ratio R1 for 58 species, and ratios R2 and R3 for 63 species. In addition, four fossil specimens were integrated into the calculations. Measurements were taken of the extinct otters *Sivaonyx beyi* (Peigne *et al.*, 2008) and *Teruelictis riparius* (Salesa *et al.*, 2013), as well as data for two extinct members of the Mustelidae: *Trocharion albanense* from Spalte von Neudorf, Slovakia (Zapfe, 1950) housed in the Naturhistorisches Museum Wien (Middle Miocene, MN 6, 12.2–15.2 Ma); and *Pannonictis* (CENIEH ATA02-TE9B-I-30-3) from the Sima del Elefante site at Atapuerca, Spain (Early Pleistocene, 1.22 Ma) (García *et al.*, 2008).

Phylogenetic analyses

Modes and rates of trait evolution were analysed using a Markov chain Monte Carlo approach, which identifies the best-fit macroevolutionary model given a phylogeny and a continuous trait, as implemented in the R package *geiger* (Slater *et al.*, 2012; Slater, 2013). For each trait (index ratios R1, R2, R3), we compared the fit of different macroevolutionary models,

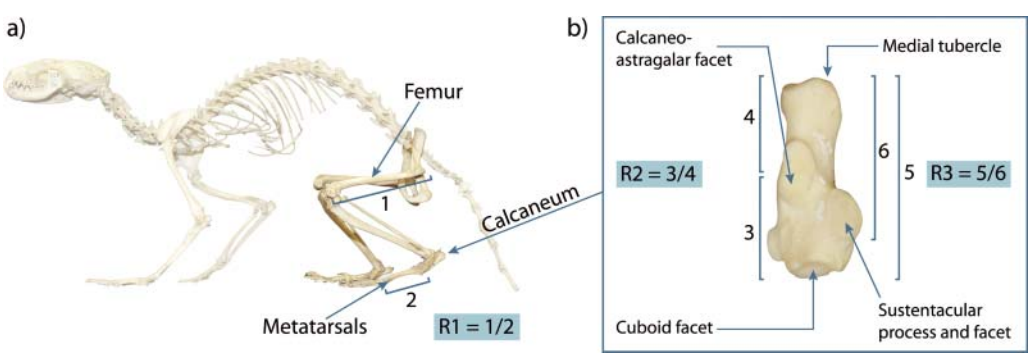

Fig. 1. Osteological measurements. The following measurements were taken: 1, maximum proximodistal length of the femur, from ball to condyle; 2, maximum proximodistal length of metatarsal III; 3, length of the distal calcaneum, from centre of the calcaneoastragalar facet; 4, length of the proximal part of the calcaneum, from medial tubercle to anteroposterior centre of the calcaneoastragalar facet; 5, maximum length of the calcaneum, from medial tubercle to cuboid facet; 6, position of the sustentacular facet, from medial tubercle to distal margin of the sustentacular process where it intersects the body of the calcaneum (see also Polly, 2010, fig. 13.1). Catalogue numbers of the specimen: ZMB MAM 77871 (a), ZMB MAM 77039 (b) from the Museum für Naturkunde, Berlin.

namely Brownian motion (BM), Ornstein-Uhlenbeck (OU), accelerating/decelerating evolution (ACDC; in which the rate of evolution increases or decreases in time), and Brownian motion with a directional trend (Trend) using the Akaike Information Criterion for MCMC samples [AICm (Raftery *et al.*, 2007)]. The median of the AIC scores calculated for each of the 500 phylogenetic trees was used to compare model fit. To assess the impact of integrating fossil data, all analyses were conducted both on the molecular phylogeny of extant taxa and the combined trees (including fossils). Finally, the above models assume equal rates across all branches of the tree (in the case of the ACDC model, the rates change through time, but the same rates apply to all branches present during any time interval). However, evolutionary rates may also vary throughout the tree, with different clades/lineages evolving at different rates (Skinner, 2010; Eastman *et al.*, 2011). Elliot and Mooers (2014) developed a generalization of the Brownian motion model, which incorporates both the drift associated with Brownian motion and occasional bursts across branches in a phylogenetic tree, akin to a punctual model of evolution. Increments of evolving characters are drawn from a heavy-tailed stable distribution allowing rare evolutionary changes of large magnitude ('adaptive' evolutionary shifts). The model is implemented in the software package StableTraits (Elliot and Mooers, 2014). We compared the fit of their stable model to a simple Brownian motion model using the Bayesian Predictive Information Criterion [BPIC (Ando and Tsay, 2010)]. The analysis was run for 1,000,000 generations on the consensus tree of the 500 integrated (combined) phylogenetic trees.

RESULTS

The phylogenetic reconstruction recovered well-supported relationships among the extant members of the Musteloidea (only 9% of the nodes receiving a posterior probability of <0.95) in line with previous studies (Koepfli *et al.*, 2007, 2008; Sato *et al.*, 2009, 2012). The inclusion of fossil information may have a substantial effect on model selection. This is particularly evident for index ratio R1. When only extant taxa were included, the different macroevolutionary models could not be distinguished with strong statistical support (all ΔAICm <2), although Ornstein-Uhlenbeck (OU) was slightly favoured (Table 1). In

Table 1. Model comparison. Median AICm values for all macroevolutionary models analysed for the three ecomorphological traits (R1, R2, R3) using 500 phylogenetic trees containing either only the extant species or combining extant and extinct lineages

	R1		R2		R3	
Model	Extant only	Combined	Extant only	Combined	Extant only	Combined
BM	−161.34	−144.89	−91.980	−95.997	−176.72	−183.69
OU	**−161.59**	−145.31	−103.76	−108.41	**−199.68**	**−207.47**
ACDC	−160.35	−146.36	**−107.07**	**−111.32**	−198.17	−205.80
Trend	—	**−152.16**	—	−94.074	—	−182.02

Note: Lower values indicate a better fit of the model. The best-fit macroevolutionary models are indicated in **bold** type. Note that the Trend model is not applicable in cases where only a molecular phylogeny is available (i.e. without information from the fossil record, either as tips in the tree or prior information on internal nodes). BM, Brownian motion; OU, Ornstein-Uhlenbeck; ACDC, accelerating/decelerating evolution; Trend, Brownian motion with a directional trend.

contrast, the integrated analysis provided strong support for the 'Trend' model (ΔAICm = 5.8). For the other traits, the same models were favoured for both extant-only data and combined data (with fossils included). For R2, the ACDC model was the best-fit model, with a significantly better fit than the OU model (ΔAICm = 3.31 and 2.91 for the extant-only and integrated data, respectively). In the case of ratio R3, the OU model received slightly higher (albeit not significant) support than the ACDC model.

Furthermore, under the different best-fit evolutionary models identified for ratio R1 (with and without fossils), both the model parameters (specifically the evolutionary rate parameter σ^2) and the reconstructed trait value for the root node of the phylogenetic tree differed (Fig. 2). The evolutionary rate was found to be slightly higher in the combined

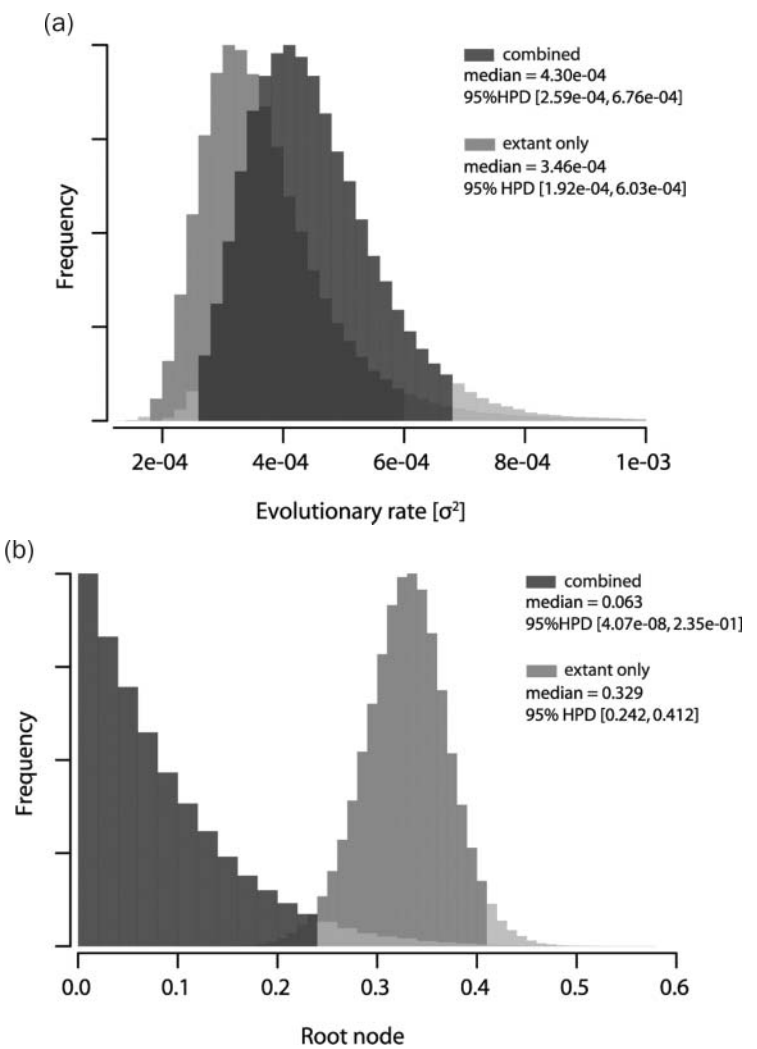

Fig. 2. Posterior estimates of the macroevolutionary model parameters. Posterior distribution of the evolutionary rate parameter (a) and the reconstructed values for the root node (b) calculated over all 500 trees under the best-fit model for the extant-only (light grey) and combined (dark grey) datasets.

analysis (median σ^2: 4.30 vs. 3.45), while the root value differed markedly (median: 0.063 vs. 0.329; Fig. 2). In addition to the selection of macroevolutionary models, the model parameters and trait reconstructions for R2 and R3 did not differ between extant-only and combined analyses.

Finally, analyses under the stable model (Elliot and Mooers, 2014) indicate substantial variation of evolutionary rates across the phylogeny, again rejecting the basic Brownian motion model (BPIC model selection criterion R1: ΔBPIC = 29; R2: ΔBPIC = 5.29; R3: ΔBPIC = 15.1). In fact, for R1 the inferred evolutionary rates vary by several orders of magnitude (95% credibility interval [0.00281, 42.995]; Fig. 3). The inferred root node values under the stable model are similar to those estimated under the best-fit models identified above, except for R1 where the root node value was found to be significantly larger (median: 0.324, 95% credibility interval [0.244, 0.405]; Table 2) than under the best-fit constant rate model of evolution for the combined dataset (Trend; median: 0.063, 95% HPD [4.07E-8, 0.235]; Table 2).

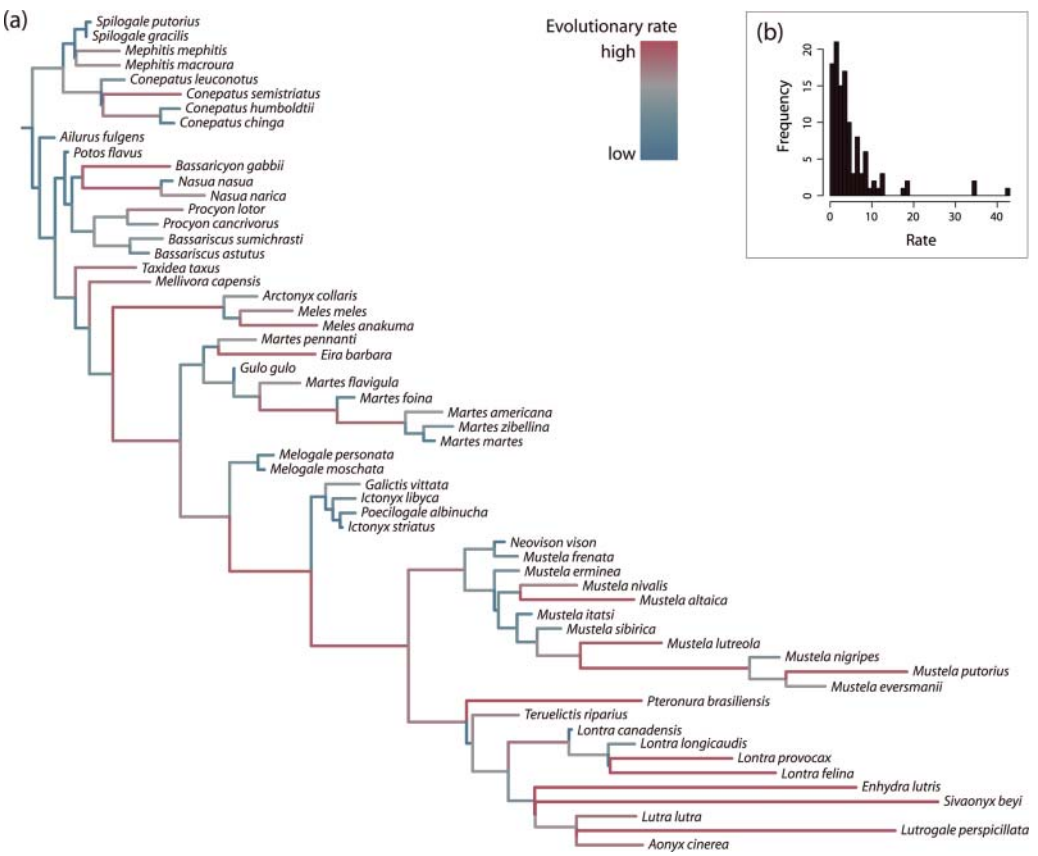

Fig. 3. Rates of locomotor trait evolution in Musteloidea. Phylogeny of Musteloidea with branch lengths scaled according to the inferred evolutionary rate for ratio R1 (a). Specifically, each branch length is equal to the difference in the StableTraits reconstruction divided by the square root of the input branch length. The insert (b) shows the frequency distribution of evolutionary rates.

Table 2. Root node estimates. Estimated trait values (median and 95% credibility intervals) for the root node of the extant-only and combined datasets under the best-fit models of evolution and the stable model

Trait	Dataset	Model	Root value (median)	95% credibility interval
R1	Extant-only	OU	0.330	[0.243, 0.413]
	Combined	Trend	0.063	[4.07E-8, 0.235]
	Combined	Stable	0.324	[0.244, 0.405]
R2	Extant-only	ACDC	0.564	[0.512, 0.622]
	Combined	ACDC	0.579	[0.526, 0.633]
	Combined	Stable	0.586	[0.424, 0.747]
R3	Extant-only	OU	1.209	[1.196, 1.222]
	Combined	OU	1.207	[1.195, 1.221]
	Combined	Stable	1.223	[1.150, 1.298]

DISCUSSION

We have shown how the integration of extant and fossil information might impact macroevolutionary analyses and our understanding of trait evolution. Similar results have been reported previously, for example for body mass reconstructions in Caniforma (Finarelli and Flynn, 2006; Slater *et al.*, 2012), Fereuungulata and Proboscidea (Raia *et al.*, 2013), and Mammaliaformes (Slater, 2013). Problems arising from inappropriate evolutionary models have been highlighted before (e.g. Cunningham, 1999; Oakley and Cunningham, 2000; Rabosky and Goldberg, 2015). Here, we provide further evidence that sometimes even a little fossil information can have a substantial impact on the inference of trait evolution dynamics. This was highlighted in particular by the differences in both model selection and character reconstruction of locomotor ratio R1. In the Musteloidea, the reconstructed root node value for the metatarsal-to-femur ratio differed substantially between combined and extant-only analyses. While the molecular phylogeny, as expected, reconstructed a weighted average of the observed values for the living taxa as root node value (Schluter *et al.*, 1997), the Trend model of the combined analysis led to a root node value that would suggest an extremely short metatarsal III relative to the femur, which is well outside the range of the extant taxa (3013Appendix). In fact, the reconstructed value is not only lower than for any taxon in this study, but also other mammals, where metatarsal-to-femur ratios typically range from <0.1 in some rodents to 1.4 in the giraffe (Garland and Janis, 1993; Carrano, 1999; Lovegrove and Mowoe, 2014), and quite possibly being at odds with carnivoran locomotor biomechanics (Carrano, 2001; Polly, 2007). This is due to the fact that the metatarsal-to-femur ratio for the extinct species is, despite being within the range of the Musteloidea, lower than that of any extant Lutrinae, leading to extremely low values under the Trend model. Among Musteloidea, the metatarsal-to-femur ratio for *Teruelictics riparius* is most similar to that of *Martes foina*, with *Aonyx cinerea* being the most similar Lutrinae species (3013Appendix, Fig. S1) (see also Salesa *et al.*, 2013). *Sivaonyx beyi* is even more different from the extant Lutrinae, being most similar to *Mephitis macoura* (Mephitidae), *Procyon lotor* (Procyonidae), *Spilogale putorius*

(Mephitidae), and *Ailurus fulgens* (Ailuridae; Fig. S1). One possible amendment would be to define informative prior distributions for internal nodes of the phylogeny (see, for example, Slater *et al.*, 2012). However, such priors are not always trivial to define, as true values might lie outside those observed in both extant taxa and the fossil record. Thus, available information might impose specious constraints on character reconstructions. Therefore, such priors should ideally not be based on information from other taxa, but on general morphological/biomechanical constraints (e.g. Allen *et al.*, 2013). Alternatively, it would be beneficial to incorporate more fossil information from across the phylogeny, if possible also for outgroup taxa (Puttick and Thomas, 2015).

The reconstructed root node value for the metatarsal-to-femur ratio under the stable model was found to be significantly larger than under the Trend model, reconciling complex evolutionary dynamics (as documented in the morphologically distinct fossils) with more realistic node estimates. Interestingly, these reconstructions are similar to those found using only the extant taxa, but under very different evolutionary dynamics. While the OU model is characterized by constant evolutionary rates across the phylogeny with the α parameter [often referred to as the stabilizing selection parameter (but see Cooper *et al.*, 2016)] restraining trait divergence away from the global optimum Θ (Hansen, 1997; Butler and King, 2004), the stable model allows for substantial variation in the evolutionary rate across the phylogeny, relaxing the assumption of constant rates across all branches (but without enforcing constraints on trait values). This scenario suggests rapid evolutionary changes along the backbone of the Mustelidae tree and within the Lutrinae clade in particular, while the other clades experienced lower evolutionary rates, remaining closer to the ancestral character states. Thus, in contrast to the Trend model, rapid evolution of locomotor traits occurred predominantly along the branches leading to the Lutrinae as well as within this clade. This is in accordance with a major shift from terrestrial (generalized morphology with a low degree of specialization) to semi-aquatic/aquatic habitats in the Lutrinae, which required a higher degree of adaptation (Estes, 1989; Samuels *et al.*, 2013). In addition, available morphological information for the extinct otters suggests that early members of this clade were predominantly terrestrial with poorly developed aquatic adaptations. Most of the postcranial features seen in extant otters that suggest semi-aquatic adaptations were absent or less pronounced in *Teruelictis riparius* (Salesa *et al.*, 2013) and *Sivaonyx beyi* (Peigne *et al.*, 2008).

Nevertheless, adding fossil information does not always have a substantial impact on either model selection or model parameters, indicating that under some circumstances, extant-only phylogenies may be sufficient to infer evolutionary dynamics. This was evident for locomotor ratios R2 and R3, where no significant effect on model selection could be found (the same macroevolutionary models were preferred with and without fossils), and character reconstructions for the root node were very similar in both cases. Thus, extant taxa may in some cases provide a reasonable approximation of evolutionary dynamics, and available fossil information may not be at odds with character reconstructions. In another example, Puttick and Thomas (2015) have shown that extant taxa and fossil information indicate the same patterns of body mass evolution in Afrotheria, a group that includes elephants, sea cows, and tenrecs. It is, however, also possible that the differences in model selection and reconstructions between extant-only and combined analyses found here for the different locomotor traits (R1 vs. R2 and R3) might, in part, be due to data availability rather than different evolutionary dynamics. Not all osteological measurements (and thus locomotor ratios) were available for all fossil taxa (3013Appendix), presumably because

the relevant structures have either not been preserved or were not accessible. For example, the calcaneal measurements necessary for ratios R2 and R3 were not available for *Sivaonyx beyi and Teruelictis riparius*. Thus, while the reconstructions based on extant species for ratios R2 and R3 are in agreement with available fossil data, the incorporation of further fossil data (e.g. from extinct Lutrini) may still reveal more complex evolutionary dynamics.

The constant-rate, unbounded Brownian motion model, in which the value of a continuous trait evolves by incremental changes drawn from a normal distribution with zero mean and finite constant variance, was not supported in any analysis. Yet, it still is the most commonly applied model of trait evolution, in part due to its simplicity and tractability (Pan *et al.*, 2014). However, many traits will likely have been subject to selection (directional, disruptive or stabilizing), leading to phylogenetic patterns that are at odds with neutral drift as modelled by Brownian motion (Felsenstein, 1985; Losos, 2011). The StableModel (Elliot and Mooers, 2014), along with other recent developments (O'Meara *et al.*, 2006; Eastman *et al.*, 2011; Boucher *et al.*, 2014), offers a useful alternative to constant-rate Brownian motion models by relaxing the assumption of rate constancy and allowing evolutionary rates to vary across the phylogeny.

CONCLUSIONS

Fossils are crucial to our understanding of macroevolutionary dynamics. Our results highlight the importance of evaluating the appropriateness of macroevolutionary models and show how the integration of extant and extinct lineages may impact modes and rates of trait evolution. This has implications not only for our interpretation of evolutionary dynamics, but also for ancestral character reconstructions. We only incorporated a few fossil data points in this pilot study, but clearly incorporating more fossil data across the phylogeny would be desirable. Thus, we now need to utilize the vast amount of information stored in large fossil collections to obtain more data for individual clades. Importantly, even in cases where extant-only and integrated analyses are in agreement, fossils provide additional support. Judging the potential influence of fossils in advance may not always be straightforward. Therefore, we argue that, where possible, fossil information should always be considered when studying macroevolutionary dynamics.

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