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Remarkable ancient divergences amongst neglected lorisiform primates

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Lorisiform primates (Primates: Strepsirrhini: Lorisiformes) represent almost 10% of the living primate species and are widely distributed in sub-Saharan Africa and South/South-East Asia; however, their taxonomy, evolutionary history, and biogeography are still poorly understood. In this study we report the largest molecular phylogeny in terms of the number of represented taxa. We sequenced the complete mitochondrial cytochrome *b* gene for 86 lorisiform specimens, including ~80% of all the species currently recognized. Our results support the monophyly of the Galagidae, but a common ancestry of the Lorisinae and Perodicticinae (family Lorisidae) was not recovered. These three lineages have early origins, with the Galagidae and the Lorisinae diverging in the Oligocene at about 30 Mya and the Perodicticinae emerging in the early Miocene. Our mitochondrial phylogeny agrees with recent studies based on nuclear data, and supports *Euoticus* as the oldest galagid lineage and the polyphyletic status of *Galagoides*. Moreover, we have elucidated phylogenetic relationships for several species never included before in a molecular phylogeny. The results obtained in this study suggest that lorisiform diversity remains substantially underestimated and that previously unnoticed cryptic diversity might be present within many lineages, thus urgently requiring a comprehensive taxonomic revision of this primate group.

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INTRODUCTION

Cryptic or sibling species are generally those deemed morphologically indistinguishable from their sister taxa (Mayr, 1963; Mayden, 1997; Bickford *et al.*, 2007; Ceballos & Ehrlich, 2009). Within a broad spectrum of animal groups ranging from tropical frogs (Stuart, Inger & Voris, 2006) to rainforest butterflies (Hebert *et al.*, 2004), deep-sea clams (Vrijenhoek *et al.*, 1994), freshwater fish (Feulner *et al.*, 2006) and mammals (Mayer, Dietz & Kiefer, 2007), advances in molecular methods have resulted in a dramatic increase in the description of cryptic taxa (Bickford *et al.*, 2007). Cryptic species should be more likely to be discovered amongst species that favour mate recognition signals that are less obvious to humans and where convergent and/or parallel evolution have been strong (Mayr, 1963; Bickford *et al.*, 2007). For instance, amongst mammals, nocturnal species may tend towards cryptic species because low or poor light conditions limit opportunities for visual communication and also increase the importance of auditory and olfactory communication. In these taxa, cryptic morphology can also represent an antipredator adaptation by helping the animal hide against a background while resting during the day.

A clade that fits this description and has shown a dramatic increase in recognition of cryptic species in recent years is the nocturnal primates of Africa and Asia. This group comprises the infraorders Lemuriformes and Chiromyiformes of Madagascar, Tarsiiformes of Indonesia and the Philippines, and Lorisiformes of mainland Africa and Asia. Over the last two decades, the Malagasy lemurs have been subjected to extensive molecular and behavioural studies, resulting in an escalation in the number of species from 50 to 101 (Tattersall, 2007, 2013; Mittermeier *et al.*, 2010; Weisrock *et al.*, 2010; Mittermeier, Rylands & Wilson, 2013; Thiele, Razafimahatratra & Hapke, 2013). Similarly, within the Asian tarsiers the number of species has increased from three to 11, and more recently two new genera have been described (Groves & Shekelle, 2010; Shekelle, Gursky-Doyen & Richardson, 2013; Roos *et al.*, 2014). Molecular studies have not only shown higher species diversity within nocturnal primates, but also suggested deep divergences between phenotypically nearly identical taxa. For instance, molecular estimates suggest Late Miocene origins for mouse lemurs (*Microcebus* spp.), dwarf lemurs (*Cheirogaleus* spp.), and tarsiers (*Tarsius* spp.) (Shekelle *et al.*, 2010; Perelman *et al.*, 2011; Springer *et al.*, 2012; Thiele *et al.*, 2013).

Almost completely neglected in terms of taxonomic research are the Lorisiformes, comprising the two families Galagidae (galagos) and Lorisidae, the latter with two distinctive subfamilies: Perodicticinae (angwantibos and pottos) and Lorisinae (lorises) (Groves, 2001; Grubb

et al., 2003). The galagos, pottos, and angwantibos are restricted to Africa and range in body mass from 45–1510 g (Nekaris & Bearder, 2011; Nekaris, 2013a, b, 2014). The lorises are found in Asia and range in body mass from 85–2100 g (Nekaris & Bearder, 2011; Pimley & Bearder, 2013; Nekaris, 2013a). Galagos have one of the widest distributions, across the whole of Africa south of the Sahara, whereas pottos and angwantibos are restricted to Central Africa. Slender lorises are confined to Sri Lanka and southern India, whereas the slow lorises range from northeast India to southern China to Sundaland and the western Philippines (Fig. 1). The lorisiforms occupy a wide range of habitats, from near-desert conditions, through subtropical savannahs, woodlands, and riverine forests to dense tropical rainforests. In altitude they cover the entire range from sea level to upper-montane. Some species live allopatrically but in some areas up to six species occur in sympatry (Grubb *et al.*, 2003; Nekaris & Bearder, 2011; Nekaris, 2013a, b).

Before the 1970s the accepted taxonomy of the Galagidae included only two genera (*Galago* and *Euoticus*) and only six species (Schwarz, 1931). Subsequent revisions expanded the number of genera to five and the number of species to 11 (Nash, Bearder & Olson, 1989; Olson, 1979), 17 (Bearder, Honess & Ambrose, 1995), 18 (Butynski, Kingdon & Kalina, 2013; Nekaris, 2013b), and 25 (Grubb *et al.*, 2003). Bioacoustic studies have been at the forefront of galago taxonomic revision (Zimmermann, 1990; Masters, 1991; Honess, 1996; Bearder, 1999; Ambrose, 2003; Butynski *et al.*, 2006). Taxonomic changes within galagids were also supported by examination of hand, foot, and penile morphology, and comparison of the hair structure (Anderson, 2000; Anderson *et al.*, 2000; Perkin, 2007) and to a lesser extent, genetic research (e.g. Masters *et al.*, 1994; Bayes, 1998).

The systematics within the Lorisidae has been long debated. Currently two subfamilies are recognized: Perodicticinae, including the two African genera *Arctocebus* and *Perodicticus*, and Lorisinae, including the two Asian genera *Loris* and *Nycticebus*. This classification was first proposed by Groves (1971) and was based on craniodental features. In contrast to this hypothesis, Schwartz & Tattersall (1985) suggested that the two robust forms, *Nycticebus* and *Perodicticus*, and the two slender forms, *Loris* and *Arctocebus*, form reciprocal monophyletic clades. Molecular studies based on both sequence data (Chatterjee *et al.*, 2009; Fabre, Rodrigues & Douzery, 2009; Perelman *et al.*, 2011; Springer *et al.*, 2012; Pozzi, Disotell & Masters, 2014a) and mobile elements (Roos, Schmitz & Zischler, 2004) consistently support the former classification. These primates are difficult to trap and occur in areas where genetic sampling is difficult. Our knowledge of their taxonomic diversity has thus been restricted to

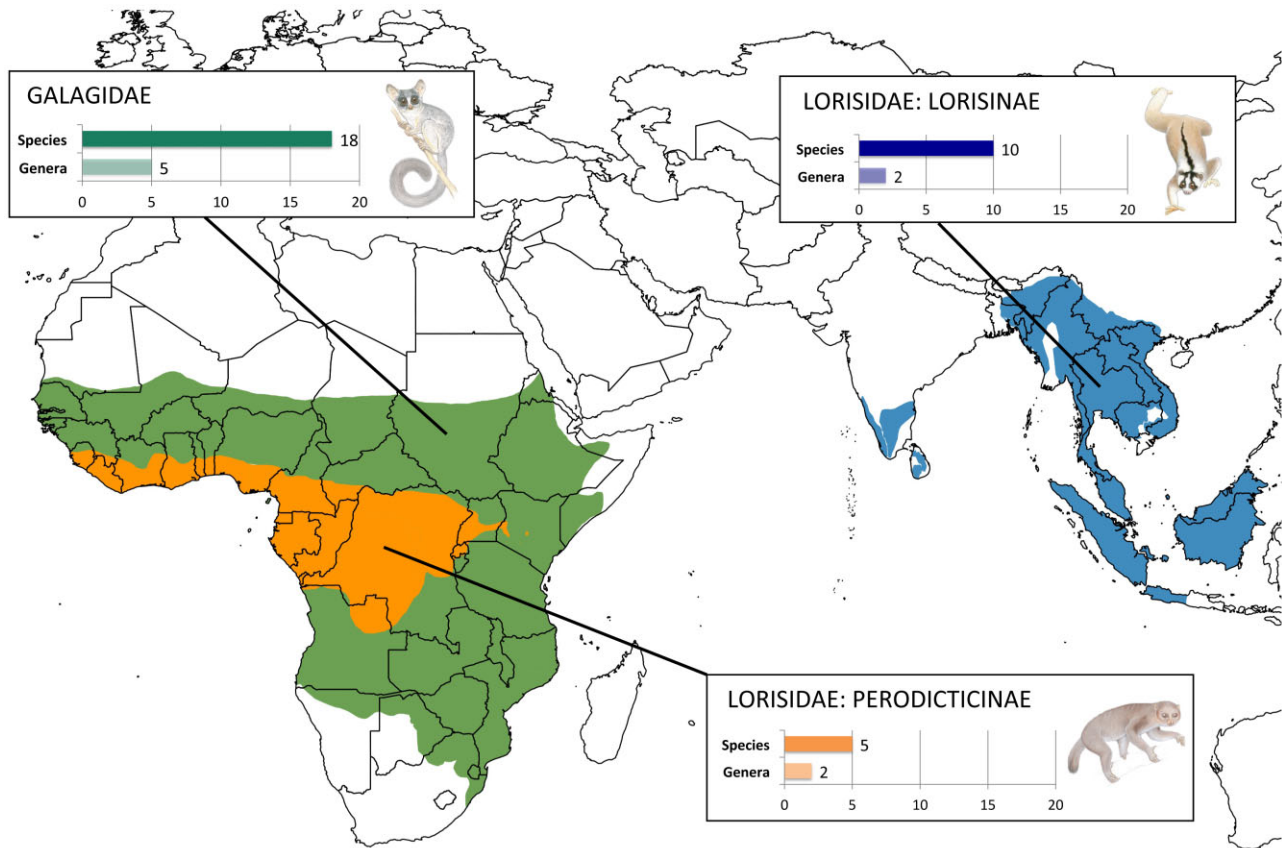


Figure 1. Distribution and taxonomic abundance for lorisiforms, Perodicticinae (or African lorisids), Lorisinae (or Asian lorisids), and Galagidae. Images provided by Stephen Nash and used with permission from the IUCN/SSC Primate Specialist Group.

differences in behaviour, morphology, and facial markings, and limited genetic data (Schulze & Meier, 1995; Nekaris & Jayewardene, 2004; Nekaris & Jaffe, 2007; Nekaris & Munds, 2010; Munds, Nekaris & Ford, 2013). Currently there are five recognized perodicticine species and ten recognized lorisines (Munds *et al.*, 2013; Nekaris, 2013a; Roos *et al.*, 2014).

The evolutionary relationships within lorisiforms are far from resolved (Rasmussen & Nekaris, 1998). Attempts to establish the phylogenetic relationships of the lorisiforms with molecular and morphological evidence have resulted in contradictory results (Yoder, Irwin & Payseur, 2001; Masters & Brothers, 2002; Roos *et al.*, 2004; Masters *et al.*, 2007). Although the standard practice is to consider the Lorisidae (Perodicticinae and Lorisinae) a monophyletic group to the exclusion of the Galagidae, most molecular studies have failed to provide strong support for this clade. No consensus has yet been reached as to whether the pottos and lorises are indeed monophyletic, or if they form one of the most spectacular examples of parallel evolution amongst primates (Yoder *et al.*, 2001; Roos *et al.*, 2004; Masters *et al.*, 2007; Pozzi *et al.*, 2014a).

Recent fossil discoveries have added new vigour to debates regarding the origins of the Lorisiformes. Early evidence for lorisiform origins pointed back to the Miocene of East Africa. Intense debate characterizes the subfamilial designation of the three best-known Early Miocene forms, *Mioeuoticus*, *Progalago*, and *Komba*. Both cranial and postcranial features have allied these genera with either lorisines or galagides (Le Gros Clark & Thomas, 1952; Walker, 1970; Gebo, 1986; McCrossin, 1992). Other authors have suggested that basal lorisiforms may have demonstrated a combination of lorisine cranial characteristics and galagide postcranial adaptations (Rasmussen & Nekaris, 1998). More recent specimens from Egypt suggest that the lorisiforms may be amongst the most ancient of the living primates, with origins extending back to the Late Eocene (Martin, 2003; Seiffert, Simons & Attia, 2003; Seiffert *et al.*, 2005; Seiffert, 2007). A recent molecular study that included most of the lineages within the lorisiformes supported this hypothesis, suggesting a Late Eocene origin for the crown lorisids and an origin for the galagids soon after the Eocene–Oligocene boundary (Pozzi *et al.*, 2014a). True, irrefutable lorisines

(*Nycticeboides simpsoni*) and galagides (*Otolemur howelli*) have been dated to the Late Miocene of Pakistan and Early Plio-Pleistocene of Ethiopia and Kenya (Seiffert, 2007; Harrison, 2010).

In this study, we investigated the evolutionary relationships and divergence times for the loriform primates by using complete mitochondrial cytochrome *b* (cytb) sequences. This gene has been widely used in phylogenetic and phylogeographical studies in mammals, including primates (e.g. Roos *et al.*, 2004; Meyer *et al.*, 2011; Haus *et al.*, 2013; Boubli *et al.*, 2015). Although cytb represents only a relative small fragment of the mitochondrial genome, multiple studies have shown that it can provide good estimates of phylogeny and divergence time when compared with phylogenies obtained by using whole mitochondrial genomes and it also performs better than other mitochondrial genes (Tobe, Kitchener & Linacre, 2010; Nicolas *et al.*, 2012). Moreover, by selecting cytb as the marker for our analyses, we were able to maximize the number of taxa included in the study for two main reasons: (1) for multiple specimens in our study, only low-quality samples (such as faecal or museum specimens) were available, making it extremely difficult to obtain longer fragments or nuclear DNA; and (2) the high number of cytb sequences already available from GenBank for other strepsirrhine primates allowed us to build a large comparative data set for our analyses. We assembled the largest data set for the loriforms to date in terms of the number of taxa represented: overall, 86 specimens representing 26 species (~79% of the species currently recognized) were included in the analyses.

MATERIAL AND METHODS

SAMPLING

In total, 86 specimens of Loriformes were analysed for this study, 37 galagids and 49 lorids. We obtained 59 samples (hairs, tissue, faeces, DNA) from colleagues and various institutions, including both zoos and museums (see Supporting Information Table S1). Another 27 samples were collected from wild populations using Tomahawk and modified Chardonneret live traps as described in previous studies (e.g. Pozzi *et al.*, 2014a) or by hand (cf. Rode-Margono & Nekaris, 2014). Hair and tissue samples were obtained using standard protocols. Hairs were plucked from the dorsum and ventrum and stored in clean dry envelopes; tissue samples – approximately 2 mm² ear biopsies – were taken from each individual and preserved in sterilized 2 mL tubes filled with either RNAlater buffer or 70% ethanol. All the animals were released immediately after sample collection at the exact site of capture. Sample collection was approved by the Animal Welfare Body of the German Primate Center and adhered to the American Society of Primatologists Principles

for the Ethical Treatment of Non-Human Primates (see <http://www.asp.org/society/resolutions/EthicalTreatmentOfNonHumanPrimates.cfm>). No animals were sacrificed for this study. In addition to the above 86 samples, we included 52 cytb sequences available from GenBank (one galago – *Galago matschiei* – and 51 lemurs). All the sequences used in this study, including location, provider, and GenBank accession number are presented in Table S1.

DNA EXTRACTION, AMPLIFICATION, AND SEQUENCING

Total genomic DNA from tissue and faeces was extracted with the DNeasy or Stool Mini Kits from Qiagen, respectively. When hair follicle cells were used, one to five hairs were directly implemented into the PCR. Depending upon DNA quality the complete cytb gene was PCR-amplified either as a single ~1.2-kb-long fragment using the primers 5'-AATGATATGAAA AACCATCGTTGTA-3' and 5'-AACTGCAGTCAT CTCCGTTTACAAGAC-3' or via two to three overlapping fragments (primers available upon request). PCR reactions were carried out in a total volume of 30 µL containing a final concentration of 0.33 µM of each primer, 3 mM MgCl₂, 0.166 mM deoxynucleotide triphosphates, 1× buffer, and 1 U Biotherm Taq DNA polymerase (Genecraft). PCR conditions consisted of a pre-denaturation step at 94 °C for 2 min, followed by 40–50 amplification cycles, each with denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1.5 min. At the end a final extension step at 72 °C for 5 min was added. PCR performance was checked on 1% agarose gels. PCR products were excised from the gel, cleaned with a Qiagen Gel Extraction Kit, and sequenced on an ABI 3130xl DNA sequencer using a BigDye Cycle Sequencing Kit (Applied Biosystems) and the amplification primers. Sequences were assembled and manually edited in SeaView 4.4.0 (Gouy, Guindon & Gascuel, 2010).

PHYLOGENETIC ANALYSES

A total of 138 cytb sequences was aligned using MUSCLE (Edgar, 2004) and the final alignment consisted of 1140 bp. Phylogenetic analyses were conducted using both maximum likelihood and Bayesian algorithms. The data set was partitioned in three different ways: (1) no partition scheme, (2) two-partition scheme, with first and second codon positions together and third separate, and (3) three-partition scheme, with each codon position codified as an independent partition. The optimal substitution model for each partition was selected using the Bayesian information criterion as implemented in jModeltest 2.1.4 (Guindon & Gascuel, 2003; Darriba *et al.*, 2012). Maximum likelihood analyses were run using a randomized accelerated maximum likelihood model in RAxML v. 7.2.6

(Stamatakis, Ludwig & Meier, 2005; Stamatakis, 2006; Stamatakis, Hoover & Rougemont, 2008). Relative support of internal nodes was assessed by a rapid bootstrap (–f a –x option) with 1000 replications. Bayesian phylogenetic analyses were conducted with MrBayes 3.2.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003; Ronquist *et al.*, 2012) using four independent Markov chain Monte Carlo (MCMC) runs for 20 000 000 generations with tree and parameter sampling occurring every 1000 generations. Acceptance rates were in the optimal range of 20–60%. The first 25% of samples were discarded as burn-in and convergence was assessed by checking the log likelihoods, the average standard deviation of split frequencies (< 0.01), and the potential scale reduction factor in MrBayes. We also assessed convergence by visual inspection of the trace and the estimate the effective sample size (ESS > 200) of all parameters using the software TRACER v. 1.6 (Rambaut *et al.*, 2014). Alternative phylogenetic relationships obtained from the analyses were tested with the Kishino–Hasegawa (Kishino & Hasegawa, 1989) and Shimodaira–Hasegawa tests (Shimodaira & Hasegawa, 1989) with full optimization and 1000 bootstrap replications in PAUP 4.0b10 (Swofford, 2003).

To estimate divergence ages we applied the uncorrelated Bayesian relaxed-clock method as implemented in BEAST v. 1.7.5 (Drummond *et al.*, 2012). BEAUTi v. 1.7.5 (part of the BEAST package) was used to prepare the .xml file for use with BEAST v. 1.7.5. Evolutionary rates along branches followed an uncorrelated lognormal distribution, and a birth-death process prior for branching rates was used for all analyses (Gernhard, 2008). Four replicate runs were conducted with four MCMC chains sampled every 1000 generations for 40 000 000 generations, after a 25% burn-in period (10 000 000 generations for each run). Results from the four independent runs were then combined using LogCombiner, and a consensus chronogram with node height distribution and 95% highest probability densities (HPDs) was produced using TreeAnnotator v. 1.7.5 (Drummond *et al.*, 2012). Convergence of all parameters was visually assessed using TRACER v. 1.6 and all BEAST analyses were run to achieve an ESS of at least 200 for all estimated parameters once burn-in was removed. As a calibration point, we used the divergence between Lorisidae and Galagidae (crown Lorisiformes). This calibration point is based on the presence of two stem galagids, *Wadilemur elegans* (Seiffert *et al.*, 2005) and *Saharagalago mirrensis* (Seiffert *et al.*, 2003), around 35–37 Mya. Therefore we applied a normal distribution at 40 Mya (SD = 2.0; 95% range: 36.08–43.92). The data sets supporting the results of this article are available in the TreeBase repository, <http://purl.org/phylo/treebase/phyloids/study/TB2:S17669>.

RESULTS

We sequenced cytb sequences for 86 lorisiform specimens, comprising 26 different species. By combining the 86 newly generated sequences with 52 additional strepsirrhine sequences, the data set represents all seven strepsirrhine families, 23 of the 24 recognized genera (95.8%), and 76 of the 134 currently recognized species (56.7%) (Butynski *et al.*, 2013; Mittermeier *et al.*, 2013; Nekaris, 2013a, b; Roos *et al.*, 2014).

All phylogenetic analyses (RAxML, MrBayes, and BEAST) supported very similar topologies (Figs 2, 3). Within the lorisiformes, Perodicticinae, Lorisinae, and Galagidae were strongly supported (posterior probability or PP > 0.95 and bootstrap support or BP > 0.70) as monophyletic, but the family Lorisidae was recovered as paraphyletic, with the African lorisids (*Perodicticus* and *Arctocebus*) more closely related to the galagids than to the Asian lorisids (*Loris* and *Nycticebus*) (BP = 85% and PP = 1.00). All alternative topologies for this node, including the monophyly of Lorisidae, could not be rejected by either the Kishino–Hasegawa (Kishino & Hasegawa, 1989) or Shimodaira–Hasegawa (Shimodaira & Hasegawa, 1989) tests (Table S2).

Within the galagids, all the analyses strongly supported the basal position of the genus *Euoticus* as sister taxon of all the other galagids. The genus *Galagoides* was not monophyletic: the clade including *Galagoides demidoff* and *Galagoides thomasi* is the sister group to all the remaining galagids, whereas the Eastern clade, including *Galagoides orinus*, *Galagoides rondoensis*, and the Zanzibar complex (*Galagoides zanzibaricus*, *Galagoides granti*, and *Galagoides cocos*), is the sister group to *Galago*. The latter relationship however was only weakly supported by the RAxML (BP = 66%) and MrBayes analyses (PP = 0.94). The genus *Otolemur* was strongly inferred as sister taxon to the genus *Sciurocheirus*, and this clade was recovered to be sister group to the clade including *Galago* and the Eastern *Galagoides* species.

Within the eastern African clade of *Galagoides*, the Zanzibar complex, including *G. granti*, *G. zanzibaricus*, and *G. cocos*, was strongly inferred as monophyletic. However, the relationships between this species complex and the other two members of this clade (*G. orinus* and *G. rondoensis*) were only weakly supported. All of the analyses suggested a sister relationship between the montane dwarf galago (*G. orinus*) and the Zanzibar complex, to the exclusion of the Rondo dwarf galago (*G. rondoensis*). However, the support values for this node were very low in both maximum likelihood (BP = 47%) and Bayesian analyses (PP = 0.70).

Within the lorisids phylogenetic relationships were consistent across analyses. Within *Nycticebus*, the most diverse lorisid genus, all species were inferred to be monophyletic. *Nycticebus pygmaeus* was recovered as

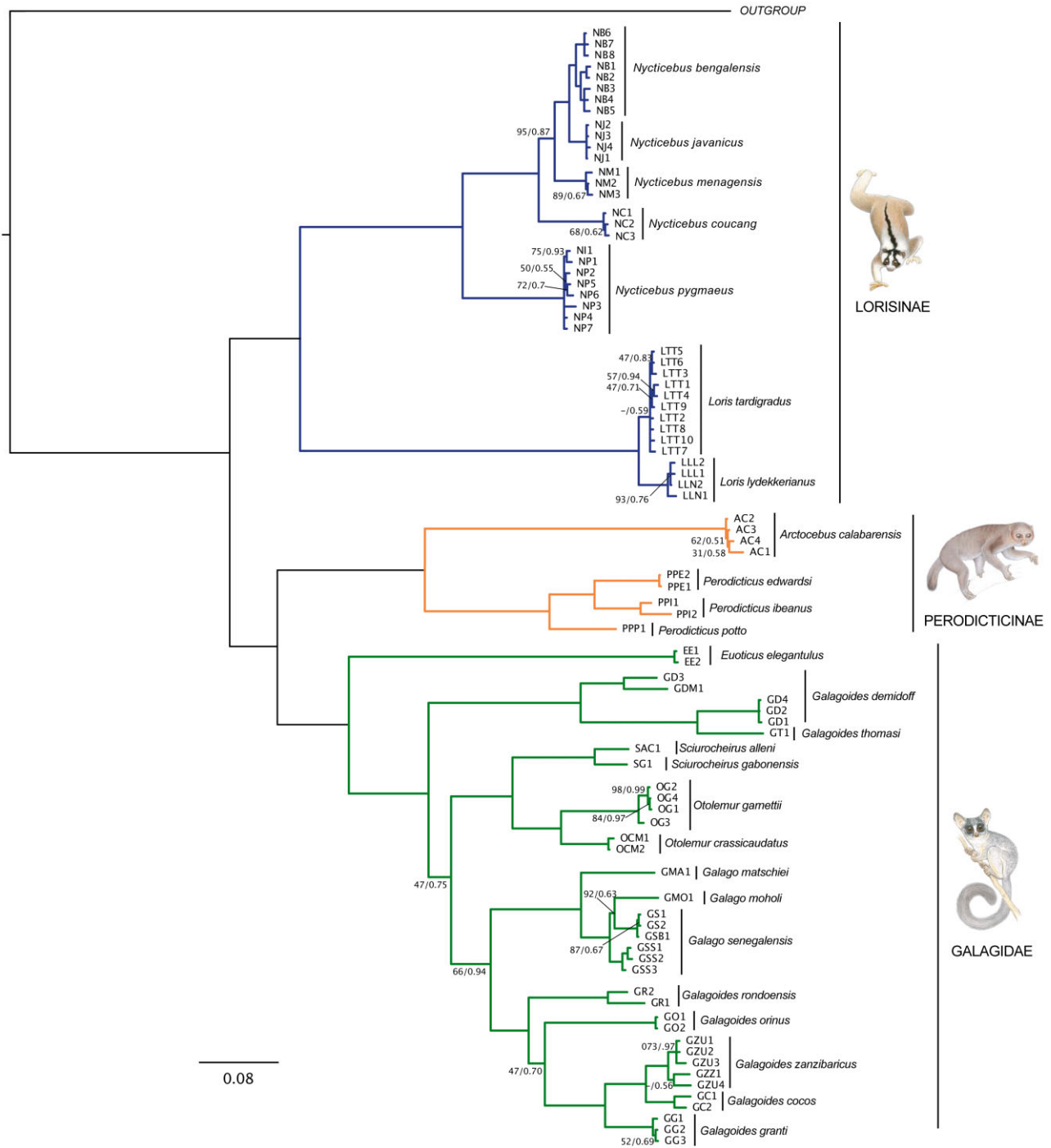
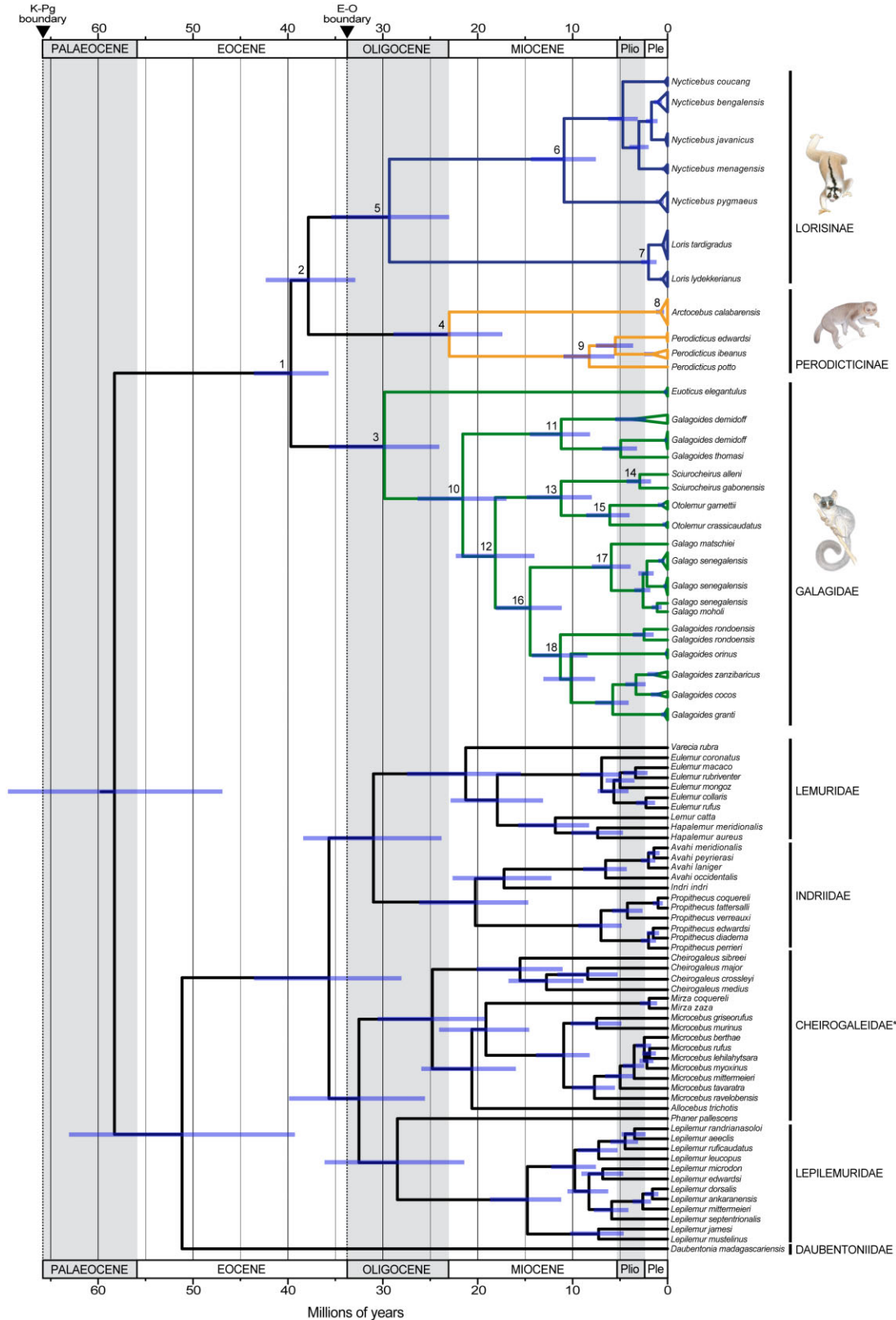


Figure 2. Phylogenetic tree inferred from maximum likelihood (RAxML) and Bayesian (MrBayes) analyses. Specific values for those nodes that were weakly supported in the analyses (bootstrap support < 70% and/or posterior probability < 0.95) are reported in the tree. Scale bar represents substitutions per nucleotide site. Images provided by Stephen Nash and used with permission from the IUCN/SSC Primate Specialist Group.

Figure 3. Estimated divergence ages with 95% highest probability densities (HPDs, blue bars). A geological time scale is given below and above the tree (values are millions of years ago). For detailed information on estimated divergence ages see Table S3. Numbers in the tree refer to the major nodes mentioned in Table S3. Plio, Pliocene; Ple, Pleistocene; K-Pg, Cretaceous–Paleogene; E-O, Eocene–Oligocene. Images provided by Stephen Nash and used with permission from the IUCN/SSC Primate Specialist Group. *The family Cheirogaleidae was not recovered as monophyletic in our analyses.



a relative old lineage (> 10 Mya), and the sister taxon to all the other slow lorises. Within *Perodicticus*, *Perodicticus potto* is basal in the genus, and *Perodicticus edwardsi* and *Perodicticus ibeanus* are sister taxa.

As the monophyly of the Lorisidae was not statistically rejected ($P > 0.05$), divergence ages were also estimated based on an a priori constrained monophyly of the Lorisidae as suggested by retroposon integrations and morphological studies (Yoder *et al.*, 2001; Masters & Brothers, 2002; Roos *et al.*, 2004). The estimates from unconstrained and constrained analyses did not differ significantly (see Table S3); therefore, here we report only the dates for the constrained topology. Age estimates for all the main nodes in the tree are reported in Table S3 and Figure 3. Divergence times within the Lorisiformes are ancient, with the divergence between the two families (Galagidae and Lorisidae) at around 39 Mya (39.70 Mya; HPD = 35.71–43.56), followed rapidly by the split between African and Asian lorisids at 37.85 Mya (HPD = 32.96–42.40). Both the most recent common ancestors (MRCAs) for the Galagidae and the Lorisinae were dated to about 30 Mya (Galagidae: 29.83 Mya; HPD: 23.96–35.55; Lorisinae: 29.32 Mya; HPD = 23.08–35.52), whereas the common ancestor for the subfamily Perodictinae was estimated to be more recent, about 23 Mya (HPD = 17.39–28.87).

Whereas the MRCA for *Nycticebus* was quite old (10.91 Mya; HPD = 7.62–14.46), the one for *Loris* was recent (2 Mya; HPD = 1.21–2.85). Similarly, within African lorisids, *Perodicticus* emerged about 8 Mya (8.25 Mya; HPD = 5.59–10.54), whereas *Arctocebus* diverged only less than 1 Mya (0.77 Mya; HPD = 0.37–1.21). Within the Galagidae, *Euoticus* was the first lineage to emerge in the Early Oligocene (29.83 Mya; 23.96–35.55), whereas the MRCA for all the other genera was dated in the Early Miocene at 21.58 Mya (HPD = 16.92–26.3). Most of the genera within the galagids emerged in the Late Miocene (6–11 Mya), with the only exception being *Sciurocheirus*, for which the MRCA was dated to within the Pliocene, at about 3 Mya (2.93 Mya; HPD = 1.70–4.29).

DISCUSSION

Our study based on cytb data for most of the recognized species of Lorisiformes provided well-resolved phylogenetic relationships and predominantly strong support for most of the nodes. In general, the results obtained herein agree with some recent molecular studies (Springer *et al.*, 2012; Pozzi *et al.*, 2014a). Within galagids, *Euoticus* represents the deepest split, estimated at 30 Mya. This estimate is only slightly younger than the one obtained from nuclear data by Pozzi *et al.* (2014a), and further supports an origin of the living galagids soon after the Eocene–Oligocene boundary. Both

the phylogenetic relationships and the age estimates within the galagids are concordant with the study conducted by Pozzi *et al.* (2014a). The genus *Galago* was not inferred as monophyletic, but it comprises two independent clades, one including *G. demidoff* and *G. thomasi* (Western clade) and one including all the species distributed in eastern Africa (*G. cocos*, *G. zanzibaricus*, *G. granti*, *G. rondoensis*, and *G. orinus*; Eastern clade). This Eastern clade was inferred to be the sister group to the genus *Galago*, but the support values were relatively low in most analyses. This result is generally concordant with nuclear data, where a relatively high level of tree discordance was found across independent loci (Pozzi *et al.*, 2014a). In fact, despite the sister-group relationship between *Galago* and Eastern *Galago* being the most favoured topology, alternative relationships were also recovered, including a sister-group relationship between *Otolemur* and *Galago* and between *Otolemur* and Eastern *Galago* (Pozzi *et al.*, 2014a). It is likely that the relatively small amount of time that passed between the emergence of these three lineages, and the consequent small level of phylogenetic signal, are the main cause of gene tree heterogeneity at this node. Within the Eastern clade of *Galago*, the Zanzibar complex was strongly recovered as a monophyletic group. Our phylogenetic analyses suggested an origin for this group in the Late Miocene (~5.8 Mya) and a sister-group relationship between *Galago cocos* and *Galago zanzibaricus*, to the exclusion of *Galago granti*. Despite the high level of morphological crypsis, species within this complex clearly differ based on species-specific advertising calls (Grubb *et al.*, 2003; Butynski *et al.*, 2006) and penile morphology (Perkin, 2007). This study provides for the first time genetic evidence supporting the taxonomic status of these three species.

The inter-relationships amongst *Galago orinus*, *Galago rondoensis*, and the species belonging to the Zanzibar complex were not fully resolved. More data – and possibly more samples from multiple populations – are needed to better elucidate the phylogeny of the Eastern clade. However, the deep divergence dates within this clade (approximately 10–11 Mya for the split between *G. orinus*, *G. rondoensis*, and the Zanzibar complex) suggest that the diversity within dwarf galagos may be higher than currently recognized. *Galago rondoensis* is the most physically distinctive and smallest (c. 60 g) *Galago* in East Africa supporting this deep divergence date, whereas *orinus* although small (c. 100 g) is phenotypically more similar to the Zanzibar complex (Butynski *et al.*, 2013). As members of *Galago* are almost exclusively forest-dependent species, their radiation was most likely driven by the expansion and later contraction and isolation processes of the equatorial forests of Africa that has occurred over the last 30 Mya.

Over the last two decades, similar primate radiations, such as those of *Lepilemur* and *Microcebus*, have undergone dramatic taxonomic revisions, mainly based on new molecular data. Although some authors argued that the consequent increase in recognized lemur species might be a result of 'taxonomic inflation' (Isaac, Mallet & Mace, 2004; Tattersall, 2007, 2013; Markolf, Brameier & Kappeler, 2011), the actual diversity within nocturnal lemurs is probably much higher than previously thought based on morphological data only (Weisrock *et al.*, 2010; Thiele *et al.*, 2013; Zimmermann & Radespiel, 2014). In contrast to Malagasy lemurs, very little research has been conducted on galagos and very limited genetic data are available for most species. For instance, in this study we presented the first genetic data for the Rondo galago (*Galagoides rondoensis*), one of the most threatened species of primates (Mittermeier *et al.*, 2014). Age estimates indicate a deep split between the southern (Ruawa Forest Reserve) and northern (Pande Forest Reserve) populations (~2.5 Mya). These two populations are separated by the Rufiji river, which has been proposed as a biogeographical barrier for several species of mammals (Kingdon, 1971; Weber, 2001; Butynski *et al.*, 2006). Unfortunately the data set available today for this taxon is still very limited and more samples are required to elucidate the biogeography and taxonomy of this species.

The greater galagos (genus *Otolemur*) were inferred as the sister group to the squirrel galagos (genus *Sciurocheirus*) with high support. This result is in agreement with previous studies on galagid phylogeny (DelPero *et al.*, 2000; Roos *et al.*, 2004; Masters *et al.*, 2007; Chatterjee *et al.*, 2009; Fabre *et al.*, 2009; Springer *et al.*, 2012). However, all these studies – including this one – were based on mitochondrial DNA and no nuclear data are available yet for members of the genus *Sciurocheirus*. Further research should aim to include multiple loci to confirm the sister-group relationship between these two genera.

Interestingly, both *Galagoides demidoff* and *Galago senegalensis* were inferred as paraphyletic in our phylogenetic reconstructions. However, this result is probably a consequence of taxonomic misallocation of members of the two species. Inaccurate classification of specimens in museum collections is very common within galagids (Pozzi *et al.*, 2014a), and in particular members of the species *Galagoides thomasi/demidoff* and *Galago moholi/senegalensis* are commonly misclassified. As a consequence, their taxonomy and biogeography is still poorly known and further studies should be conducted to investigate the genetic variation of these widely distributed species.

In contrast to the strong support for the monophyly of the family Galagidae, the monophyletic status of the family Lorisidae was not confirmed. As in previous studies, the interrelationships amongst Galagidae

(galagos), Perodicticinae (African lorisids), and Lorisinae (Asian lorisids) were not fully resolved. To date the only strong and unambiguous molecular evidence for the monophyletic status of the family Lorisidae is provided by the presence of three mobile elements, short interspersed elements (Roos *et al.*, 2004). Most molecular studies based on sequence data – either mitochondrial or nuclear – have failed to provide strong support for the monophyly of this family, and many studies have proposed alternative topologies, with the galagids more closely related to either the Asian (Goodman *et al.*, 1998; Yoder *et al.*, 2001; Roos *et al.*, 2004; Masters *et al.*, 2007; Chatterjee *et al.*, 2009; Matsui *et al.*, 2009; Pozzi *et al.*, 2014a, b) or the African lorisids (Dene *et al.*, 1976; Finstermeier *et al.*, 2013; this study). Multiple reasons might explain this pattern, including the presence of incomplete lineage sorting or ancient horizontal gene flow. Although further studies including longer (and possibly more informative) sequences might clarify the phylogenetic relationships of these three lineages at the molecular level, it remains likely that the three subfamilies emerged very rapidly after the origins of the Lorisiformes (Pozzi *et al.*, 2014a). This has critical implications in understanding the evolution of this primate group. Given the high level of morphological and behavioural traits shared between Asian and African lorisids, these lineages were either characterized by a relatively rapid evolution of those adaptations or – even more surprisingly – they represent one of the most spectacular examples of parallel evolution amongst primates (Goodman *et al.*, 1998; Yoder *et al.*, 2001). The deep divergence for both the Perodicticinae (23 Mya) and the Lorisinae (29 Mya) suggests that the two subfamilies should be elevated to the family level. Within primates, most families have in fact similar or even younger ages. Future research should better investigate morphological, behavioural, and ecological differences across these two groups, with special attention to the poorly studied pottos and angwantibos, in order to provide support for this revision of lorisiform taxonomy.

In contrast to the ambiguous status of the Lorisidae, our study confirmed the monophyletic status of both subfamilies and all the four lorisid genera with high support values. This result corroborates previous molecular studies based on mitochondrial and nuclear data (Chatterjee *et al.*, 2009; Fabre *et al.*, 2009; Perelman *et al.*, 2011; Springer *et al.*, 2012). Interestingly, whereas *Perodicticus* and *Nycticebus* have early origins in the Miocene, the genus *Loris* originated only quite recently, in the Pleistocene. In our study we included only one species of angwantibo (*Arctocebus calabarensis*) so no conclusions can be drawn regarding the origin of this genus. Within *Perodicticus*, current taxonomy recognizes three different species: *Perodicticus potto* (Western potto), *Perodicticus ibeanus* (Eastern potto),

and *Perodicticus edwardsi* (Milne-Edwards' potto) (Butynski *et al.*, 2013; Nekaris, 2013b). The divergence dates obtained in our study (> 5 Mya) support species status for these three forms of pottos, but to date no obvious ecological and/or behavioural differences have been reported amongst the three putative species (E. R. Pimley, pers. comm.). Long-term studies including radio tracking are needed to discover whether behavioural data elucidate the genetic differences.

With an origin in the Middle Miocene (~11 Mya), *Nycticebus* is the oldest and most diverse genus within the lorises. The lineage leading to the pygmy slow loris (*Nycticebus pygmaeus*) is the first to emerge in this group. Date estimates show a wide gap (roughly 6 million years) between the divergence of *N. pygmaeus* and the radiation of the remaining *Nycticebus* species. This relatively deep divergence between *N. pygmaeus* and other species of slow lorises might explain other stark ecological differences, which may lend support to the distinction of these species at the generic level. Such differences include but are not limited to several unique traits of *N. pygmaeus*, which is the only slow loris known to live sympatrically with other *Nycticebus* spp., to exhibit hairless ears, to show seasonal changes in coat colour, to regularly give birth to twins, and to exhibit a multi-male multi-female rather than a uni-male, uni-female social system (Nekaris & Bearder, 2011).

CONCLUSIONS

In this study we present the largest dated phylogeny for the Lorisiformes. Our results support the general conclusions of previous studies based on nuclear data. Moreover they elucidate phylogenetic relationships for many species never included before in a molecular phylogeny. Overall our results imply that the diversity of the lorisiforms remains substantially underestimated and that previously unnoticed cryptic diversity might be present within many lineages, particularly in Africa. The process of determining true species diversity within the lorisiforms is more than academic. Currently 60% of primate species are threatened with extinction (Mittermeier *et al.*, 2013, 2014). Lorisiform primates suffer from extreme deforestation, bushmeat hunting, and use in traditional medicine and the pet trade (Starr *et al.*, 2010; Nijman *et al.*, 2011; Nijman, Nekaris & Bickford, 2012). Identification of further taxa, either those geographically isolated such as the dwarf galagos of East Africa, or of more diversity within currently widespread 'species' such as *Nycticebus bengalensis*, would dramatically alter the vulnerability of the lorisiform primates to extinction, making an understanding of their taxonomy an area of urgent conservation action.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. List of the specimens used in the present study including specimen source, provider, and GenBank accession numbers.

Table S2. Results from alternative tree topology (Kishino–Hasegawa and Shimodaira–Hasegawa) tests for the relationships amongst Galagidae, Perodicticinae, and Lorisiae. The likelihoods and differences from the most probable topology are shown.

Table S3. Support values and date estimates with 95% highest probability densities (HPDs) for both unconstrained and constrained topologies (see text for details). Analyses were run using BEAST 1.7.5.