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Editor’s Choice Article

Using a multi-gene approach to infer the complicated phylogeny and evolutionary history of lorises (Order Primates: Family Lorisidae)

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ABSTRACT

Extensive phylogenetic studies have found robust phylogenies are modeled by using a multi-gene approach and sampling from the majority of the taxa of interest. Yet, molecular studies focused on the lorises, a cryptic primate family, have often relied on one gene, or just mitochondrial DNA, and many were unable to include all four genera in the analyses, resulting in inconclusive phylogenies. Past phylogenetic loris studies resulted in lorises being monophyletic, paraphyletic, or an unresolvable trichotomy with the closely related galagos. The purpose of our study is to improve our understanding of loris phylogeny and evolutionary history by using a multi-gene approach. We used the mitochondrial genes cytochrome *b*, and cytochrome *c* oxidase subunit 1, along with a nuclear intron (recombination activating gene 2) and nuclear exon (the melanocortin 1 receptor). Maximum Likelihood and Bayesian phylogenetic analyses were conducted based on data from each locus, as well as on the concatenated sequences. The robust, concatenated results found lorises to be a monophyletic family (Lorisidae) ($PP \geq 0.99$) with two distinct subfamilies: the African Perodictinae ($PP \geq 0.99$) and the Asian Lorisinae ($PP \geq 0.99$). Additionally, from these analyses all four genera were all recovered as monophyletic ($PP \geq 0.99$). Some of our single-gene analyses recovered monophyly, but many had discordances, with some showing paraphyly or a deep-trichotomy. Bayesian partitioned analyses inferred the most recent common ancestors of lorises emerged ~42 ± 6 million years ago (mya), the Asian Lorisinae separated ~30 ± 9 mya, and Perodictinae arose ~26 ± 10 mya. These times fit well with known historical tectonic shifts of the area, as well as with the sparse loris fossil record. Additionally, our results agree with previous multi-gene studies on Lorisidae which found lorises to be monophyletic and arising ~40 mya (Perelman et al., 2011; Pozzi et al., 2014). By taking a multi-gene approach, we were able to recover a well-supported, monophyletic loris phylogeny and inferred the evolutionary history of this cryptic family.

1. Introduction

Inferring phylogenetic relatedness in deeply-diverged and cryptic organisms is a major challenge for biologists. Methods that rely on morphology to ascertain differences are useful but limited in scope as many cryptic species closely resemble each other (Bickford et al., 2006; Munds et al., 2013; Pozzi et al., 2015). Our understanding of cryptic species improved with the advent of genetics as many taxa were found to contain distinct genetic lineages. Early phylogenetic studies relied on single genes, often mitochondrial (mtDNA) genes, to analyze relationships (Lavergne et al., 1996; Porter et al., 1996; Rasmussen et al., 1998; Arnason et al., 1999), but more thorough research revealed dissonance in evolutionary rates among genes, emphasizing the need to use more than one gene and one type of gene for phylogenetic reconstructions (Springer et al., 2001; Rokas et al., 2003; Hedtke et al., 2006). What is known is that the incorporation of multiple genes from both the mitochondrial and nuclear genomes are helping researchers gain a clearer picture of the genetic relationships among cryptic species and their evolutionary histories, yet many taxa remain unexamined. Here, we adopt the use of multi-gene analyses to provide better insight to a primate family with an unresolved phylogeny, the lorises.

In addition, phylogenetic analyses are being improved by concatenating genes or through the use of a partitioned analysis which allows for the ideal model of molecular evolution for each individual locus (Springer et al., 2001; Rokas et al., 2003; Hedtke et al., 2006; Drummond et al., 2012). But studies have found that concatenation and partitioned analyses can be biased towards a single locus that overwhelms the phylogeny. Often these methods result in discordance

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between the designed gene-trees and the accepted species-tree (Pamilo & Nei, 1988; Kubatko, 2007; Heled & Drummond, 2009). To overcome for these incongruences, gene-tree species-tree analyses were developed. Unlike concatenation analyses that can be influenced strongly by one locus, the use of a multispecies coalescent or gene-tree species-tree reconciliation model has been demonstrated to provide a more robust phylogeny (Heled & Drummond, 2009; Larget et al., 2010; Pozzi et al., 2014). Specifically, reconciliation analyses do not average all gene trees together to create a species tree, but instead recognize the gene trees are rooted in the species tree and work back in time from the present to the past (whereas concatenation analyses work from the past to the present) (Heled & Drummond, 2009). Gene-tree species-tree reconciliation analyses are still new and not widely used, as concatenation and partitioned analyses still can produce well supported trees (Rokas et al., 2003; Heled & Drummond, 2009; Pozzi et al., 2014). To attempt to infer the most robust phylogeny for lorises we will employ both a partitioned analysis and a gene-tree species-tree reconciliation analysis. This type of methodology has been used on Lorisiformes (galagos and lorises) (Pozzi et al., 2014).

Galagos and lorises are the non-Malagasy radiation of strepsirrhine primates. There are five genera of galagos (family: Galagidae): *Galago, Galagoides, Euoticus, Otolemur,* and *Sciurocheirus*. All galagos are nocturnal, primarily vertical clingers and leapers or arboreal quadrupeds, omnivorous, and are only found in Africa (Nash et al., 1989; Bearder, 1999; Nekaris & Bearder, 2007; Pozzi et al., 2015; Svensson et al., 2017). Within lorises (family: Lorisidae) there are two genera in Africa (*Arctocebus* and *Perodicticus*) and two genera in Asia (*Loris* and *Nycticebus*). Lorises are nocturnal, omnivorous, arboreal quadrupeds that cannot leap (Nekaris & Bearder, 2007). All lorises share a suite of traits, such as cryptic locomotion in which they move steadily and quietly throughout the forest making them difficult to detect (Charles-Dominque, 1977; Nekaris & Bearder, 2007), and some are similarly built: robust (*Nycticebus* and *Perodicticus*) or gracile (*Loris* and *Arctocebus*). All lorises possess a strong grasp facilitated by a highly-extended hallux and pollex and a reduced second digit on their hands and feet (Rasmussen & Nekaris, 1998; Yoder et al., 2001; Harrison, 2010). This grasp can be kept tight for an extraordinarily long amount of time because of their unique circulation system (Harrison, 2010). Their crania are highly similar, with all genera having a diastema, and raised temporal lines. They also share specialized features in their post-crania, such as an elongated lumbar, a reduced tail, and practically equal lengths of their fore- and hind limbs (Cartmill, 1975; Schwartz & Tattersall, 1985; Masters et al., 2005). In general, this shared loris morphology is a common argument for their proposed monophyly.

It is widely accepted that galagos and lorises (African and Asian) comprise a monophyletic infraorder (Lorisiformes) distinct from the Malagasy lemurs (Pozzi et al., 2014; Pozzi et al., 2015), but it is not as widely accepted that galago and loris families are monophyletic. Phylogenetic studies routinely distinguished the galagos, the African lorises, and the Asian lorises as three monophyletic groups, but the relationship among these groups remains a subject of debate due to differing interpretations of molecular, morphological, and biogeographic data (Yoder et al., 2001; Masters et al., 2005; Seiffert, 2007; Pozzi et al., 2014; Pozzi et al., 2015). A multi-gene approach clarified genus-level and species differences, and confirmed the monophyly of galagos (Pozzi et al., 2014; Pozzi et al., 2015). Such work has underscored the importance of using multiple genes for phylogenetic reconstruction, and the value of such research in interpreting the evolutionary histories of cryptic species. Although our understanding of galagos has improved, the same is not true for lorises. Much of the issue in interpreting loris phylogeny is due to a poor understanding of the relationship between the African and Asian lorises; without an improved understanding of their phylogeny we cannot adequately interpret their evolutionary history or dispersal events.

Currently, there are several commonly proposed phylogenies for the loris family, with the first being loris monophyly (Fig. 1A; Schwartz & Tattersall, 1985; Roos et al., 2004; Harrison, 2010). It has also been suggested they are paraphyletic/diphyletic with an African loris-galago clade with an independent Asian loris group, or vice versa, an Asian loris-galago clade with the African lorises forming their own clade (Fig. 1B; Yoder et al., 2000; Seiffert et al., 2003; Roos et al., 2004; Masters et al., 2005; Masters et al., 2007; Seiffert, 2007). Additionally, some propose all three primate groups (galagos, African lorises, and Asian lorises) are equally related to each other, forming an unresolvable trichotomy (Fig. 1C; Pickford, 2012; Pozzi et al., 2015). In addition, there is debate on how the genera are related to each other. Commonly, it is accepted that there are African (subfamily Perodictinae: *Arctocebus*, and *Perodicticus*) and Asian (subfamily Lorisinae: *Loris* and *Nycticebus*) subfamilies (Rasmussen & Nekaris, 1998), but other topologies have been put forth. Based on morphology, it has been suggested that robust lorises (*Perodicticus* and *Nycticebus*), and gracile lorises (*Arctocebus* and *Loris*) form different groups (Schwartz & Tattersall, 1985). Karyotype studies have found *Nycticebus* and *Arctocebus* to be more closely related, with *Loris* and *Perodicticus* excluded (de Boer, 1973; Petter and Petter Rousseaux, 1979). Lastly, some have found *Perodicticus* to be an outgroup of the other lorises, based on cranial differences (Yoder, 1994). These various phylogenies are mainly based on morphological, fossil, and historical biogeographic analyses, although some have used molecular analyses too (de Boer, 1973; Petter and Petter Rousseaux, 1979; Yoder, 1994; Roos et al., 2004; Masters et al., 2007).

The geographic separation of the African (*Arctocebus* and *Perodicticus*) and Asian (*Loris* and *Nycticebus*) lorises, in which the African lorises share a continent with the closely-related galagos, suggest a complicated evolutionary history that is poorly represented in the fossil record. There are three, well-confirmed loris and galago fossils that have been discovered: the galagos *Saharagalago misrensis* and *Wadilemur elegans* and the loris *Karanisia*. All three are North African and have been dated to the Eocene (~35–41 million years ago (mya)) (Seiffert et al., 2003; Seiffert, 2007, 2012; Harrison, 2010). Additionally, there are three younger loris fossils dated to the Miocene (~6–10 mya). From Pakistan, a partial skeleton was attributed to *Nyticeboides simposoni*, and dental remains were attributed to *Microloris pilbeami* (Harrison, 2010). Finally, a 6 mya snout from Kenya was attributed to a primate related to *Arctocebus* (Pickford, 2012). Based on the fossil record, some researchers have suggested that lorises have an Afro-Arabian origin (Roos et al., 2004; Masters et al., 2007; Seiffert, 2012; Pozzi et al., 2015). Others suggest that galagos evolved in Africa and lorises in Southeast (SE) Asia, and from there *Perodicticus* and *Arctocebus* spread to Africa during the late Miocene (Pickford, 2012). Such a proposal would explain why galagos are not present in SE Asia, but this proposal is not well supported by the Eocene fossil record (Seiffert, 2007; Seiffert, 2012).

Additionally, tectonic events inform our understanding of loris dispersal and evolution. During the Eocene (~40 mya), a land bridge formed connecting Africa to Asia, and opening a possible route of dispersal to Asia. During this time, the Indian plate was moving away from Africa and towards Asia, which could have facilitated loris movement to Asia. The land bridge and movement of the Indian plate to Asia are estimated to have occurred from 29 to 55 mya (Chatterjee & Scotese, 1999; Ali & Aitchison, 2008). This timeline matches well with galago-loris and African-Asian loris divergences, which are estimated to 40 mya and 38 mya, respectively (Roos et al., 2004; Masters et al., 2007; Seiffert, 2007; Pozzi et al., 2015). Yet, it remains unclear as to the manner in which the African and Asian lorises split. Some have suggested lorises are exhibiting an amazing form of parallel evolution. This hypothesis is supported by past molecular studies that found lorises to be either paraphyletic or polyphyletic, even though morphologically they appear very similar. Through parallel evolution these cryptic primates could have evolved similar morphologies, even similar robust (*Perodicticus* and *Nycticebus*) and gracile (*Arctocebus* and *Loris*) morphs between the two African and Asian groups (Yoder et al., 2001; Masters et al., 2007). But, it is not unreasonable to propose that these primates