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Molecular Phylogeny, Taxonomy and Conservation of Slender Lorises

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Abstract: Traditionally, two species of slender loris have been recognized, viz. the red slender loris *Loris tardigradus*, with two subspecies from Sri Lanka and the grey slender loris *L. lydekkerianus*, with four subspecies from Sri Lanka and India. In 2017, two more subspecies were described from Sri Lanka (*L. t. parvus* and *L. l. uva*) on the basis of morphological data, a conclusion purportedly supported by mtDNA sequence data. We conducted phylogenetic analyses of available cytochrome *c* oxidase subunit 1 (*co1*) sequences from 25 individuals of both the Sri Lankan slender loris species and found: (1) there is reasonable support for a two-species arrangement; (2) there is only a small amount of genetic differentiation within species; and (3) there is no support for the recognition of distinct, monophyletic taxa (subspecies or otherwise) that are genetically differentiated from other such taxa within each species. The management and practical conservation of the two species of slender lorises requires on-the-ground efforts in both India and Sri Lanka, and the availability of data on the distribution, morphology and genetics of the two species of slender loris needs to be taken into account when planning and executing conservation measures.

Keywords: *Loris*, Lorisidae, taxonomy, Sri Lanka, conservation

Introduction

The slender lorises comprise two species, namely the red slender loris *Loris tardigradus* (Linnaeus, 1758), endemic to the southern part of the island of Sri Lanka, and the grey slender loris *Loris lydekkerianus* Cabrera, 1908, that occurs in northern Sri Lanka and large parts of southern India. Partially reflecting the differences in distribution ranges, the conservation status of the red slender loris has been assessed as "Endangered" and that of the grey slender loris as "Least Concern" on the IUCN Red List of Threatened Species (Nekaris 2008; Nekaris *et al*. 2008). While originally described as three, four or even more species, for most of the 20th century, the slender lorises were considered one species (Osman Hill 1953). During this time, research on slender lorises was conducted mainly on captive animals kept in facilities outside Asia (Phillips 1931; David *et al.* 1974; Izard and Rasmussen 1985; Müller *et al*. 1985; Goonan 1993; Sellers 1996). These animals originated from India and Sri Lanka, and almost certainly comprised a combination of the two species we now recognize; retrospectively assigning the individuals used in these studies to either red or grey slender lorises, Indian or Sri Lankan, however, is problematic. Until the late 1990s, only one 16-day study had been published on wild slender lorises (Petter and Hladik 1970). Initially the first proper longer-term field studies were restricted to the grey slender lorises in southern India (for example, Nekaris 2001; Radhakrishna and Singh 2002; Nekaris and Rasmussen 2003). In the early 2000s, the grey and red slender lorises were studied at several sites in Sri Lanka (for example, Nekaris 2003; Nekaris and Jayewardene 2004; Nekaris *et al.* 2005) and these field studies continued (Kumara *et al*. 2009; Kumara and Radhakrishna 2013; Nekaris and Stengel 2013; Nekaris *et al*. 2013; Gamage *et al.* 2014). The result of the preponderance of studies being conducted on captive slender lorises, often with limited information on provenance, and the delay in field studies, was (and is) that the true morphological and evolutionary diversity of slender lorises, and hence their taxonomy, has yet to come to light.

A comprehensive overview of the taxonomy of slender lorises, particularly those from Sri Lanka, is provided by Gamage *et al.* (2017). Osman Hill (1953) recognized six slender loris taxa of just one species, *Loris tardigradus*—two in India and four in Sri Lanka—but they are now grouped into two species, the grey slender loris with four subspecies (*L. lydekkerianus lydekkerianus*, *L. l. grandis*, *L. l. malabaricus*

and *L. l. nordicus*) and the red slender loris with two subspecies (*L. tardigradus tardigradus* and *L. t. nycticeboides*) (Table 1). Gamage *et al.* (2017) added two subspecies from Sri Lanka (*L. t. parvus* and *L. l. uva*) noting the possibility of a third (a red slender loris *L. tardigradus* from Rakwana). Using mostly genetic data, Gamage *et al.* (2019) did not find enough support for the recognition of *L. t. nycticeboides* or *L. l. uva*.

Gamage *et al.* (2017) published their taxonomic views on the grey and red slender lorises of Sri Lanka based on skull morphology, facemasks and pelage characteristics (grey slender lorises from India were not included in their study). The findings were significant for the evaluation of the conservation status and potential management implications of the two species and their putative subspecies in Sri Lanka, six being deemed valid by Gamage *et al.* (2017). Gamage *et al.* (2017) made reference to an upcoming or preliminary molecular study that would corroborate (some of) their findings, including how certain taxa were related to each other. The molecular study was published in 2019. It was based on partial cytochrome *c* oxidase subunit 1 (*co1*) sequences (Gamage *et al.* 2019), and the sequences were made public (in GenBank). In both of the papers, the different taxa are labelled as geographic groups, viz. groups 1 to 7 (apart from group 3, Gamage *et al.* [2017] gave each group a subspecific name) (see Table 1). Gamage *et al.* (2019) found that their subsequent molecular phylogenetic analyses provided strong support for just one of these groups as a subspecies: *L. t. parvus* [group 1]. Group 3 was highlighted as a possible new subspecies. Weak ("not strong") or no support was found for the other subspecies (Gamage *et al.* 2019). While Gamage *et al.* (2019) make reference to their earlier study, there were no conclusions on the validity of the names listed and proposed in Gamage *et al*. (2017), and there was no discussion on conservation and/or management implications of their findings.

Here we take the opportunity to examine some of the taxonomic intricacies of Gamage *et al*. (2017, 2019) to present an alternative molecular phylogeny of the slender lorises of Sri Lanka, and to discuss the conservation implications. There are some issues that we note with the Gamage *et al.* (2019) study: there is no mention of the sequence editing or alignment method that they used; as far as we are aware RAxML (Stamatakis 2014) is unable to estimate neighbor-joining or maximum-parsimony trees; there is no mention of which software was used for maximum-likelihood analyses; there are no Bayesian inference results presented; references associated with phylogenetic programs are incorrect; there is no information provided about how each analysis was run, for example, number of bootstraps or how the data was partitioned. Most importantly, the analyses of Gamage *et al.* (2019) are purportedly based on 604 bp of *co1* data but only a maximum of 291 bp for each sample is deposited in GenBank. It is for these reasons that we decided to reanalyze and reinterpret the data generated by Gamage *et al.* (2019).

Table 1. Selected overview of the taxonomic history of slender lorises in India and Sri Lanka

	Osman Hill (1953)	**Groves (1998)**	**Nekaris (2013)**	**Gamage *et al.* (2017)**	**Gamage *et al.* (2019)**
	Morphology	Morphology	Morphology & behavior	Morphology	Genetics
Sri Lanka	*L. t. tardigradus*	*L. t. tardigradus*	*L. t. tardigradus*	*L. t. tardigradus*	*L. t. tardigradus*
	L. t. nycticeboides	*L. t. nycticeboides*	*L. t. nycticeboides*	*L. t. nycticeboides*	
				L. t. parvus	*L. t. parvus*
				L. t. ssp. 'Rakwana'	
	L. t. nordicus	*L. l. nordicus*	*L. l. nordicus*	*L. l. nordicus*	*L. l. nordicus*
	L. t. grandis	*L. l. grandis*	*L. l. grandis*	*L. l. grandis*	*L. l. grandis*
				L. l. uva	
India	*L. t. lydekkerianus*	*L. l. lydekkerianus*	*L. l. lydekkerianus*	Not assessed	Not assessed
	L. t. malabaricus	*L. l. malabaricus*	*L. l. malabaricus*	Not assessed	Not assessed

Table 2. Samples of slender lorises used in this study, indicating sequence length and accession numbers. Subspecies names are taken from Gamage *et al.* (2019). bp = base pairs.

GenBank accession number, taxon	Length (bp)	Comment
KX761807 *Loris tardigradus parvus*	291	
KX761808 *Loris tardigradus parvus*	291	
KX761809 *Loris tardigradus parvus*	291	
KX761810 *Loris tardigradus tardigradus*	291	
KX761811 *Loris tardigradus tardigradus*	291	
KX761812 *Loris tardigradus tardigradus*	291	
KX761813 *Loris tardigradus tardigradus*	291	
KX761814 *Loris tardigradus tardigradus*	291	
KX761815 *Loris tardigradus tardigradus*	291	
KX761816 *Loris tardigradus nycticeboides*	291	
KX761817 *Loris tardigradus nycticeboides*	291	
KX761818 *Loris tardigradus tardigradus*	291	*L. lydekkerianus* in GenBank
KX761819 *Loris tardigradus tardigradus*	291	*L. lydekkerianus* in GenBank
KX761820 *Loris lydekkerianus grandis*	291	
KX761821 *Loris lydekkerianus grandis*	291	
KX761822 *Loris lydekkerianus uva*	291	
KX761823 *Loris lydekkerianus uva*	291	
KX761824 *Loris lydekkerianus nordicus*	291	
KX761825 *Loris lydekkerianus nordicus*	231	*L. tardigradus* in GenBank
KX761826 *Loris lydekkerianus nordicus*	231	*L. tardigradus* in GenBank
KX761827 *Loris lydekkerianus nordicus*	291	
NC_012763 *Loris tardigradus*	16,776	
KJ543732 *Loris lydekkerianus*	603	
KC757402 *Loris lydekkerianus*	16,791	
KJ543733 *Loris tardigradus*	606	
KX397281 *Nycticebus pygmaeus*	16,856	

Methods

Approach and data management

Gamage *et al.* (2019) reported four errors or inconsistencies in the naming of samples, their species identity and GenBank accession numbers. According to Table 1 in Gamage *et al.* (2019), KX761818 and KX761819 are *L. t. tardigradus* but in GenBank these are listed as *L. lydekkerianus*. Conversely, KX761825 and KX761826 are listed as *L. l. nordicus* in Gamage *et al.* (2019) but in GenBank these are labelled as *L. tardigradus* (Table 2). In our phylogenetic analyses, we keep their labels as indicated in Gamage *et al*. (2019) as this allows us to assign them to "subspecies."

The molecular phylogeny presented by Gamage *et al*. (2019) is based on a partial region of the co1 gene; in primates this gene is 1,541 base pairs [bp] long and the proximate 658 bp is widely used as a marker for DNA barcoding (Hebert *et al.* 2003). In Gamage *et al*. (2017), this is referred to as "the CO1 region", while in Gamage *et al.* (2019) this is repeatedly referred to as a 604 bp gene. It is unclear what the 604 bp region of *co1* that Gamage *et al.* (2019) used is, because the sequences that they deposited in GenBank are 231–291 bp and no sequence alignments have been deposited online. In the absence of longer sequences our analysis is thus based on the Gamage *et al.* (2019) GenBank sequences (Table 2).

Sequence alignment and phylogenetic analyses

Sequences generated by Gamage et al. (2019) were downloaded from GenBank, along with other sequences of *Loris* and one *Nycticebus pygmaeus*, which was used as an outgroup (Table 2). Sequences were aligned using MUSCLE (Edgar 2004) in Geneious Prime. The dataset was trimmed to 291 bp to match the alignment of Gamage *et al.* (2019)'s sequences. Due to some missing data, there is only a maximum of 192 bp of overlapping sequence data among all samples.

Four different phylogenetic reconstruction approaches were used following, as best as possible, Gamage *et al*. (2019)'s approach. However, the approach of Gamage *et al.* (2019) is not detailed and there are numerous inaccuracies (as explained in the Introduction of this article) so we reconstructed phylogenetic relationships using: (1) MEGA X (Kumar *et al.* 2018) to reconstruct neighbor-joining (NJ) and maximum-parsimony (MP) trees, (2) RAxML v.8.2.12 (Stamatakis 2014) to reconstruct maximum-likelihood (ML) trees, and (3) MrBayes v.3.2.7 (Ronquist *et al*. 2012) to reconstruct Bayesian inference (BI) trees. Due to the short sequences, we opted against partitioning the alignment by codon. RAxML and MrBayes were implemented in the CIPRES Science Gateway (Miller *et al*. 2010). NJ trees were estimated using p-distances with pairwise deletion and run for 500 bootstraps. MP trees were estimated using the SPR method with partial deletion and run for 500 bootstraps. ML trees were estimated with 500 bootstraps using the GTRCAT model; the final tree was evaluated under the GTRGAMMA model.

For the BI analyses, the best-fit model of sequence evolution was estimated for each alignment using PartitionFinder v.2.1.1 (Lanfear *et al.* 2016). BI analyses were run with one cold and three heated chains for 10^6 generations and sampled every 10,000 generations. The first 10% of trees were discarded as burn-in. Convergence was assessed by looking for good mixing and ESS values >200 in Tracer v.1.7 (Rambaut *et al.* 2018). Tree topology is visualized as a strict consensus.

Results

Some support exists for the two species of *Loris tardigradus* and *L. lydekkerianus* being monophyletic (Fig. 1). Whilst most analyses support the distinction of the two species, internal relationships within these two clades are overwhelmingly lacking.

In the sequences that Gamage *et al.* (2019) deposited in GenBank only 12 positions are variable (Fig. 2). Between the two species there is a mean p-distance of 2.9% sequence divergence. Within each species there is a maximum of 1% sequence divergence (p-distance) observed between any member of the same species. The largest p-distance between any individuals is 3.8%, compared to Gamage *et al.* (2019), who reported 5.6%.

Discussion

Gamage *et al.* (2017) stated that their initial mitochondrial DNA study (presumably the study that forms the basis of the Gamage *et al.* 2019 paper) shows that *L. t. nycticeboides* [group 4, in Gamage *et al*. 2019] is genetically close to *L. t. tardigradus* [group 2]. Based on Gamage *et al*. (2019), however, it appears that *L. t. tardigradus* [group 2] is not monophyletic, with the majority of samples (individuals) forming a poorly resolved clade with *L. t. parvus* [group 1], slender lorises from Rakwana [group 3] and *L. t. nycticeboides* [group 4], and a smaller number of samples being sister to this clade. As such, there is no clear support for a close genetic (sister) relationship between *L. t. nycticeboides* [group 4] and *L. t. tardigradus*, which is supported by our reanalysis. While Gamage *et al.* (2017) indicated that *L. l. uva* [group 6] was genetically very close to *L. l. grandis* [group 5], their most recent paper indicates that *L. l. grandis* [group 5] is in fact a sister taxon to *L. l. nordicus* [group 7] and that these two groups are sister to *L. l. uva* [group 6]. We likewise were not able to demonstrate a close relationship between *L. l. uva* [group 6] and *L. l. grandis* [group 5].

Gamage *et al.* (2019) concluded that "[t]he CO1 region data strongly supported the proposed new subspecies from the wet zone clade, northwestern red slender loris (*Loris tardigradus parvus*)". They reported 2.4% uncorrected pairwise differences between both *L. t. parvus* [group 1] and *L. t. nycticeboides* [group 3], and *L. t. parvus* [group 1] and *L. t. tardigradus* [group 4]. We directly compared the three *L. t. parvus* sequences (KX761807, KX761808, KX761809) with the two *L. t. nycticeboides* sequences (KX761817, KX761818) and

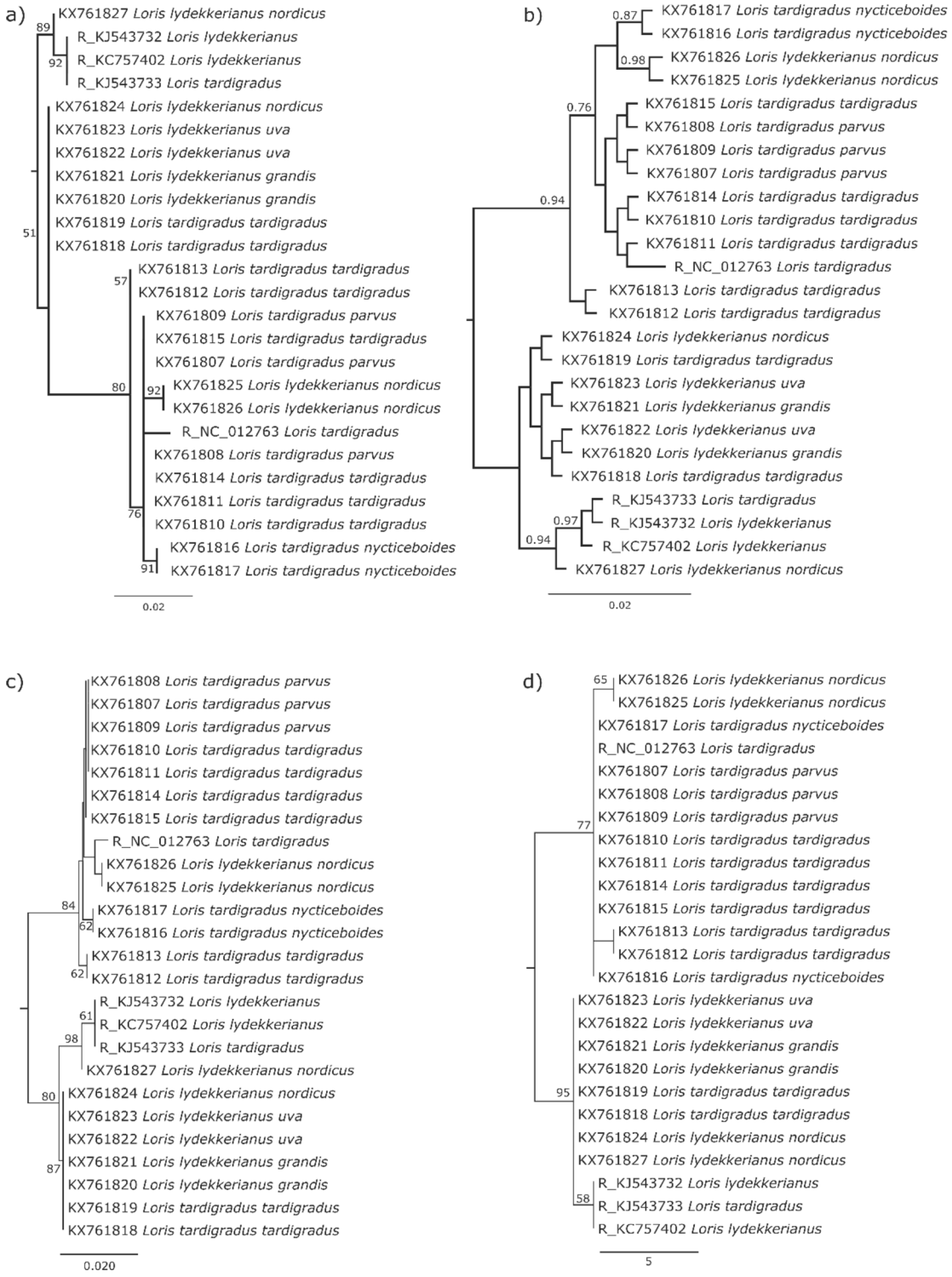

Figure 1. Phylogenetic relationships of *Loris* taxa from sequence data produced in Gamage *et al.* (2019): a) maximum-likelihood tree, b) Bayesian inference tree, c) neighbor-joining tree, d) maximum-parsimony tree. Outgroups have been pruned from all trees. Bootstrap/ Bayesian posterior probability values are shown on branches; only support values >50% are shown.

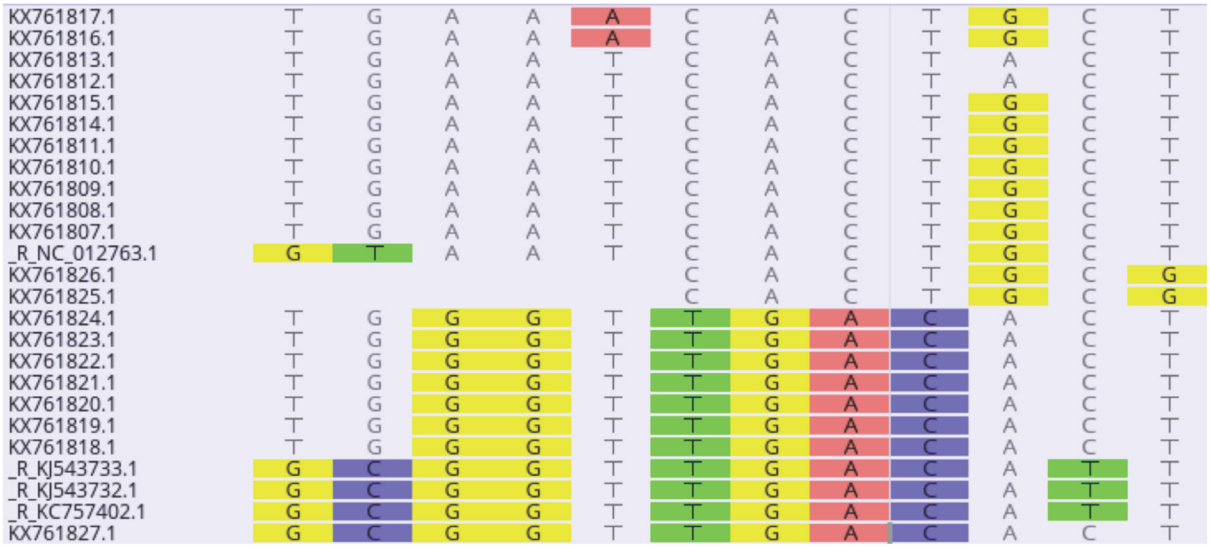

Figure 2. Mutational differences exhibited among all Loris samples used in this study.

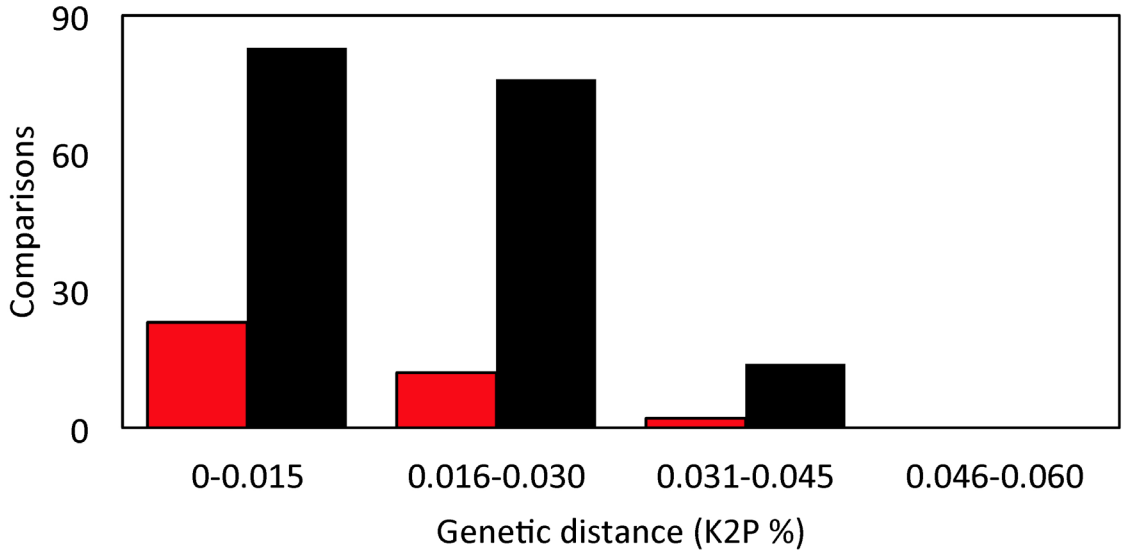

Figure 3. Inter- and intrasubspecific genetic distances for co1 sequences of slender lorises, showing a clear lack of a barcoding gap. Intrasubspecific distances are in red, intersubspecific distances are in black. Comparisons indicate the number of sequences that were compared with each other.

the six *L. t. tardigradus* sequences (KX761810, KX761811, KX761812, KX761813, KX761814, KX761815) and found that *L. t. parvus* and *L. t. nycticeboides* differ in only one site (0.3%), and *L. t. parvus* and four of the *L. t. tardigradus* sequences are identical, and the two others (KX761812, KX761813) differ again at only one site (0.3%).

The proximal 658 bp of the co1 locus has become a popular DNA barcoding marker (Hebert *et al.* 2003; DeSalle and Goldstein 2019) and allows for the molecular identification of primates (Nijman and Aliabadian 2010). Its efficiency declines rapidly as smaller and smaller sections of the sequence ("mini-barcodes") are used. Based on 1,197 *co1* sequences of 179 putative species of primates from GenBank, the shortening of the sequence length from 648 bp to 216 bp reduces the efficiency of unambiguously distinguishing species to 53% (5' end of the Folmer region), 69% (central section) and 54% (3' end) when compared to the full barcode sequence (Nijman and Robbins 2017). Yeo *et al.* (2019) noted that "very short mini-barcodes (<200 bp) perform poorly, especially when they are located near the 5' end of the Folmer region". This underscores the importance of ensuring the whole (or at least a substantial part) of the *co1* gene is sequenced when using it for identification. Many advocates of DNA barcoding have indicated that it is rarely a suitable method for phylogenetic analysis or for describing new species, but that it could flag-up distinct lineages worthy

of further investigation (Hajibabaei *et al.* 2007; DeSalle and Goldstein 2019). This caution of course should sound louder when very short DNA sequences are used.

One of the appealing aspects of DNA barcoding is that in its simplest approach it is easy to understand: the amount of genetic variation within a taxon should be considerably smaller than that between taxa (for example, families, species and even subspecies) (Aliabadian *et al.* 2014). The difference between the within- and between-taxa genetic distance is the DNA barcoding gap. Figure 3 shows the inter- and intrasubspecific distances for the slender lorises; there is no indication of a barcoding gap and the genetic differences between subspecies of slender lorises is as small as are the differences within subspecies.

In conclusion, the molecular data referred to in Gamage *et al.* (2019) does not support the recognition of three subspecies of the grey slender loris nor the recognition of three (or four) subspecies of the red slender loris in Sri Lanka. The phylogenetic relationships between taxa as alluded to in Gamage *et al.* (2017) are not supported by their own subsequent molecular analysis nor indeed ours. While it is quite appropriate to name taxa based on morphological data, as indeed was done by Gamage *et al.* (2017), the corresponding molecular data should corroborate the morphological data. Further analyses should be performed with more information before assigning/reassigning subspecies/species status for nomenclatural stability.

Our current understanding of the taxonomy of the grey slender loris suggests that this is one species (Nekaris 2013), with an extent of occurrence about eight times larger in India than in Sri Lanka. The populations of this species in India are recognized as two different subspecies, *L. l. lydekkerianus* and *L. l. malabaricus*, and it is well possible that these are genetically differentiated from the populations in Sri Lanka; unfortunately, however, no genetic analysis has been conducted on any of the Indian populations. With respect to the conservation of grey slender lorises, it is important to recognize that this requires the concerted efforts of the Indian and Sri Lankan governments, regional bodies and NGOs in both countries. The red slender loris is found only in Sri Lanka; its conservation is thus highly dependent on the efforts and actions of both the authorities and people of Sri Lanka. We furthermore recognize that the management and practical conservation of slender lorises requires the efforts of dedicated individuals working on the ground in both India and Sri Lanka. The availability of data on distribution, morphology and genetics, such as from Gamage *et al.* (2017, 2019), can then provide the basis for evaluation such that sound conservation measures can be planned and executed.

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