Tree-kangaroos *Dendrolagus* in Australia: are *D. lumholtzi* and *D. bennettianus* sister taxa?

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ABSTRACT

Tree-kangaroos *Dendrolagus sp.* are a poorly known group of folivorous arboreal macropodid marsupials endemic to the rainforests of north-eastern Australia and New Guinea. Over the last century there has been little agreement on the relationship between the two Australian species, D. *lumholtzi* and *D. bennettianus*. In light of this uncertainty, we undertook a phylogenetics study based on 430 bp of sequence from the mitochondrial DNA gene cytochrome *b* (cyt*b*). Samples were collected from *D. lumholtzi* (n = 45) and *D. bennettianus* (n = 3), as well as representative of six New Guinean *Dendrolagus* species. Results from distance and parsimony analyses strongly support a sister relationship between the two Australian species. However, *D. lumholtzi* and *D. bennettanius* show considerable sequence divergence (5.3%), suggesting that their speciation predates the mid-late Pleistocene. Although inter-relationships amongst New Guinean *Dendrolagus* were not fully resolved by our preliminary analysis, a number of distinct lineages are apparent. *D. goodfellowi, D. matschiei* and *D. spadix* form a monophyletic group of closely related taxa, as do the two sampled subspecies of *D. dorianus*. However, unexpectedly large divergences were obtained both between these subspecies (7.4%) and within one of them (*D. d. stellerum,* 7.8%). This suggests that *D. dorianus* may actually consist of a complex of related species, but more extensive research is required.

Keywords: *Dendrolagus,* phylogeny, cytochrome b, DNA sequencing, SSCP.

Introduction

Tree-kangaroos *Dendrolagus* sp. are a group of folivorous arboreal macropodid marsupials found in the rainforests of north-eastern Australia (2 species) and New Guinea (8 species) (Flannery *et al.* 1996). Due to their scarcity, cryptic nature and preferred habitat, relatively little is known of any *Dendrolagus* species (Flannery *et al.* 1996). However, comparatively more is known of the two Australian species, Lumholtz's Tree-kangaroo *D. lumholtzi* and Bennett's Tree-kangaroo *D. bennettianus* (Procter-Gray 1984, 1985; Martin 1992, 1995; Newell 1999a, b, c; Bowyer *et al.* 2002). The ten recognised *Dendrolagus* species are currently divided into 17 taxa (Table 1) and, with the exception of the two Australian taxa, all are considered 'threatened' or 'insufficiently known' (Flannery *et al.* 1996). In New Guinea, new *Dendrolagus* taxa continue to be discovered with four species/subspecies having been described since 1990 (Flannery *et al.* 1996).

Over the last century there has been little agreement on the relationship between the two Australian (*D. lumholtzi, D. bennettianus*) and New Guinean *Dendrolagus* species. An early attempt to understand interspecies relationships within *Dendrolagus* was conducted by Rothschild and Dollman (1936) and was based on the position of the hair whorl and coat colour. Rothschild and Dollman identified 3 distinct groups within *Dendrolagus*, placing *D. lumholtzi* and *D. bennettianus* together in their third group with the New Guinea species *D. inustus* and *D. ursinus*. Tate (1948) also recognised three groups, although their composition differed from that of Rothschild and Dollman. In Tate's classification *D. lumholtzi* was placed alone in Group 3, while *D. bennettianus* was placed in Group 1 as a race of the New Guinean *D. dorianus*. Groves (1982) conducted a comprehensive morphological study of *Dendrolagus* and concluded that two groups existed within the genus; a group of relatively primitive taxa (*D. lumholtzi* and the

Table 1 Current taxonomy and classification of *Dendrolagus* (Flannery *et al.* 1996).

Long-footed group		*D. bennettianus*
(plesiomorphic)		*D. lumholtzi*
		D. inustus (2 subspecies)
Short-footed group		*D. ursinus*
(apomorphic)	Goodfellow's complex	*D. goodfellowi* (3 subspecies)
		D. matschiei
		D. spadix
	Doria's complex	*D. dorianus* (4 subspecies)
		D. scottae (2? subspecies)
		D. mbasio

Table 2 Collection localities of *Dendrolagus* specimens used in this study. WSP, West Sepik Province; SHP, Southern Highlands Province; PNG, Papua New Guinea. Numbers beginning with "M " refer to specimens from the Australian Museum Sydney. Number beginning with "S " refer to tissue/DNA samples held at Macquarie University.

Specimen ID	Taxon	Locality
M32253, S1629	*D. bennettianus*	Daintree, Qld, Australia
M23242	*D. bennettianus*	Shiptons Flat, Qld, Australia
M17374, M17746	*D. dorianus notatus*	Mt Sisa, Bobole Village, SHP, PNG
M17153,	*D. dorianus notatus*	Waro, SHP, PNG
M30720, M30750, M30753	*D. dorianus stellarum*	Tempagapura area, Irian Jaya, Indonesia
M16699	*D. dorianus stellarum*	Sol River Valley, Telefomin, WSP, PNG
S1606	*D. goodfellowi buergersi*	Taronga Zoo, Sydney, NSW, Australia
M17149	*D. inustus finschi*	Pual River, near Vanimo, WSP, PNG
M24426	*D. inustus finschi*	Torricelli Mountains, WSP, PNG
M32217, S1438, S1439, S1450-1487, S1575-1577	*D. lumholtzi*	Atherton Tablelands, Qld, Australia
S1621	*D. matschiei*	National Zoo, Washington DC, USA
M30751	*D. mbasio*	Tempagapura area, Irian Jaya, Indonesia
M17212	*D. spadix*	Fogamaiyu, SHP, PNG

Table 3. Average sequence divergence (2KP) (%) amongst cyt*b* haplotypes within (on diagonal; where n > 1) and between (below diagonal) *Dendrolagus* taxa. Taxon labels are abbreviations of the specific or subspecific names.

	D. lum	D. ben	D. goo	D. spa	D. mat	D. d. ste	D. d. not	D. inu
D. lum	0.3							
D. ben	5.3	0.0						
D. goo	11.8	12.0	-					
D. spa	9.8	11.5	6.4	-				
D. mat	7.5	8.9	5.4	4.7	-			
D. d. ste	9.8	11.9	12.1	9.8	8.2	7.8		
D. d. not	8.7	11.2	11.7	9.7	8.4	7.4	0.0	
D. inu	12.7	13.5	13.4	12.5	9.9	13.2	13.3	0.2
D. mba	10.8	12.2	11.0	10.7	9.4	11.2	11.2	13.6

New Guinean *D. inustus*) and a group of more derived taxa. The relationship of *D. bennettianus* was regarded as equivocal, but Groves concluded the affinities of this taxon probably lay with the more derived *D. ursinus*. In a further morphological study, Flannery *et al.* (1995), also divided *Dendrolagus* into 2 groups; a plesiomorphic (and probably paraphyletic) long-footed group (*D. lumholtzi*, *D. bennettianus*, *D. inustus*) and an apomorphic, monophyletic short-footed group (Table 1). These groupings were maintained in Flannery *et al.* (1996), although in this classification the two Australian species *D. lumholtzi* and *D. bennettianus* were placed as sister taxa.

The proposed sister relationship between *D. lumholtzi* and *D. bennettianus* has significance for our understanding of the role of Pleistocene climatic fluctuations in promoting speciation and diversity in tropical rainforests (reviewed in Moritz *et al.* 2000). Within the Australian Wet Tropics the current distribution of *D. lumholtzi* and *D. bennettianus* largely coincides with the proposed location of the two main rainforest refugia that existed at the height of the last glacial maximum ~18,000 YBP (Winter 1997). However, the role of these refugia in promoting speciation within Australian *Dendrolagus* remains uncertain (Winter 1997) and is clearly dependent on whether or not *D. lumholtzi* and *D. bennettianus* are infact sister taxa.

In light of the uncertainty surrounding the relationship of *D. lumholtzi* and *D. bennettianus* and relationships within *Dendrolagus* generally, we proposed to undertake a molecular phylogenetics study using the mitochondrial DNA gene cytochrome *b* (cyt*b*). Cyt*b* has been widely used in phylogenetic studies and appears most helpful at resolving relationships within and between related species (Taylor *et al.* 1994; Krajewski *et al.* 1997; Sinclair and Westerman 1997; Taylor *et al.* 1999; Osborne *et al.* 2000). It should therefore be useful at clarifying relationships within *Dendrolagus* which appears to have radiated within the last seven million years (Kirsch *et al.* 1997; Campeau-Peloquin *et al.* 2001).

Materials and methods

Sample collection and DNA extraction

Frozen and alcohol preserved tissue (skin, skeletal muscle, liver, kidney) was obtained from 61 specimens of *Dendrolagus*, including representatives of eight species. For the two Australian species, samples were collected from 45 specimens of *D. lumholtzi* from 19 localities on the Atherton Tablelands, Qld (Bowyer *et al.* 2002) and three specimens of *D. bennettianus* from two localities north of the Daintree River, Qld (Table 2). For the New Guinean species, samples were obtained from seven specimens of *D. dorianus* (four localities), two specimens of *D. inustus* (two localities) and single examplars of *D. goodfellowi*, *D. matschiei*, *D. mbasio* and *D. spadix* (Table 2). Total cellular DNA was extracted from the tissue samples using the high salt method of Sunnucks and Hales (1996).

PCR-SSCP (polymerase chain reaction - single stranded conformation polymorphism) analysis of cyt*b*

Cyt*b* variation in *Dendrolagus* was assessed using PCR-SSCP (Girman 1996; Sunnucks *et al.* 2000) followed by automated sequencing of representative haplotypes. A ~500 bp fragment from the 5' end of the cyt*b* gene was amplified using the primers H15149 and L14724 (Irwin *et al.* 1991). SSCP methods were performed as described in Sunnucks *et al.* (2000).

Sequencing of cyt*b*

Approximately 450 bp of cyt*b* sequence was obtained for 1-4 representatives of each identified haplotype. PCR was performed in a total volume of 25 µl using the primers H15149 and L14724 (Irwin *et al.* 1991). The product was cut from a 2% agarose gel and the DNA was extracted using the BRESA-CLEAN nucleic acid purification kit (Bresatec) according to manufacturers instructions. 100 - 200 ηg of purified DNA was sequenced via automated sequencing. All samples was sequenced using both forward and reverse primers; H15149 and L14724 respectively.

Phylogenetic analysis of cyt*b* sequence

The PILEUP option in Web ANGIS was used to align the sequences and where possible ambiguities were resolved and the sequences adjusted by eye. Phylogenetic analysis was then conducted using PAUP* (Swofford 2000) on an aligned block of 430 bp of cytb sequence. Firstly, the relationship of *Dendrolagus* haplotypes was determined by maximum parsimony (Felsenstein 1983) analysis utilizing the Branch and Bound search option, with all substitutions weighted equally, regardless of type. Statistical confidence of the branching points was assessed by 500 bootstrap replications (Felsenstein 1985). Secondly, distance trees were constructed by neighbour-joining (Saitou and Nei 1987) from pairwise genetic distances estimated using the sequence evolution assumptions of Kimura (1980) and Tamura and Nei (1993). The level of resolution in the distance trees was assessed by 500 bootstrap replicates (Felsenstein 1985). For both analyses, sequence from two species of *Thylogale* were used as outgroup taxa.

Results

PCR-SSCP analysis revealed 13 distinct cyt*b* haplotypes amongst the 61 examined *Dendrolagus* specimens (Fig. 1). No additional haplotype were detected through sequencing 1-4 representatives of each identified haplotype. Single unique haplotypes were detected in *D. bennettianus* (n = 3), as well as *D. goodfellowi*, *D. matschiei*, *D. mbasio* and *D spadix* (all n = 1). Two unique haplotypes were detected in *D. inustus* (n = 2), while three unique haplotypes were detected within *D. lumholtzi* (n = 45) and *D. dorianus* (n = 7).

Sequence divergence between species averaged 10.3% (range 4.7 – 13.6). The two Australian species, *D. lumholtzi* and *D. bennettianus*, differed by 5.3% (Table 3). Intraspecific sequence divergence averaged 1.6%, but ranged from 0.0 (*D. bennettianus*) to 7.5% (*D. dorianus*). The average divergence between *D. d. stellerum* and *D. d.*

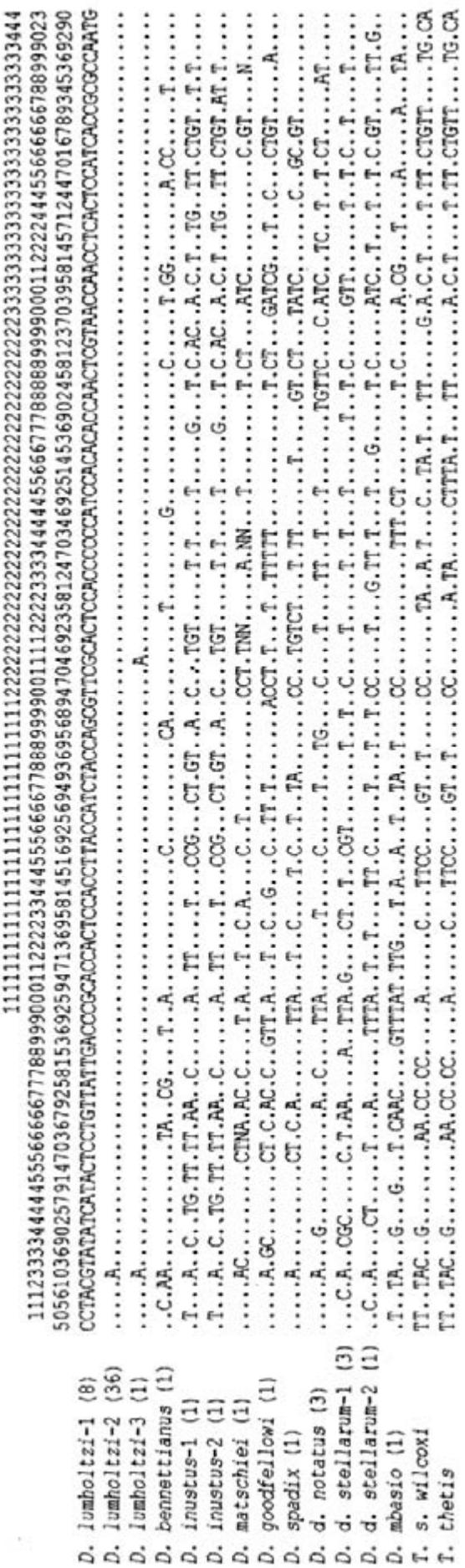

Figure 1 Sequence variation among 13 partial cyt*b* sequences found in eight species of *Dendrolagus*. Only variable sites are shown, with their positions indicated above. Complete sequences are available from GenBank (Accession numbers AY226564-76). The numbers of individuals with each haplotype are shown in parentheses. Letters indicate nucleotide substitutions; . indicates identity with the consensus sequence. Two species of *Thylogale* were used as outgroup taxa.

notatus (7.4%) was greater than that detected between some pairs of recognised species (e.g., *D. lumholtzi* and *D. bennettianus,* 5.3%). Considerable divergence was also present within *D. dorianus stellerum* where two haplotypes differing by 7.8% were detected. One haplotype was found in three individuals from Tempagapura (Irian Jaya) while the second was unique to a single animal from Telefomin (Papua New Guinea). In contrast, the three sampled individuals of *D. d. notatus* (from two localities) all shared a single haplotype. Additionally, the haplotypic variants detected within *D. lumholtzi* and *D. inustus* only differed by 0.3% and 0.2% respectively (Table 3).

Amongst *Dendrolagus*, 116 variable sites were present within the 430 bp of cyt*b* sequence (Fig. 1), 80 of which were phylogenetically informative. Including the outgroup sequences (*Thylogale*), 125 variable sites were present, with 93 being phylogenetically informative. Both the neighbor-joining and maximum parsimony analysis produced phylogenetic trees of identical topology (Fig. 2). Haplotypes from the two Australian species (*D. lumholtzi*, *D. bennettanius*) formed a strongly supported (95-100% bootstrap) monophyletic group (Fig. 2). Within this Australian lineage the three *D. lumholtzi* haplotypes were monophyletic (99-100% bootstrap) and formed a sister group to the *D. bennettanius* haplotype. The two *D. inustus* haplotypes also formed a strongly supported (100% bootstrap) monophyletic group and appeared basal to all other *Dendrolagus* (Fig. 2). Amongst the haplotypes from the remaining New Guinean taxa, three lineages were apparent; a well supported (87-93% bootstrap) group comprising haplotypes from *D. goodfellowi*, *D. matchiei* and *D spadix*; a second less well supported (56-87% bootstrap) group comprising haplotypes from *D. dorianus* and lastly the ungrouped *D. mbasio* haplotype (Fig. 2). The relationship amongst these lineages and between the Australian and New Guinean haplotypes could not be resolved.

Discussion

Both the distance and parsimony analyses of the partial cyt*b* sequence strongly support a sister relationship between haplotypes from *D. lumholtzi* and *D. bennettanius* (Fig. 2). This is consistent with Flannery *et al.*'s (1996) proposed phylogenetic arrangement within *Dendrolagus* based on morphology. However, while Flannery *et al.* (1996) placed *D. lumholtzi* and *D. bennettanius* basal to all other *Dendrolagus*, our data suggest that *D. inustus* is basal (Fig. 2), although the bootstrap support for this arrangement was weak (< 65%). Within *Dendrolagus*, Flannery *et al.* (1996) regarded *D. inustus* along with *D.*

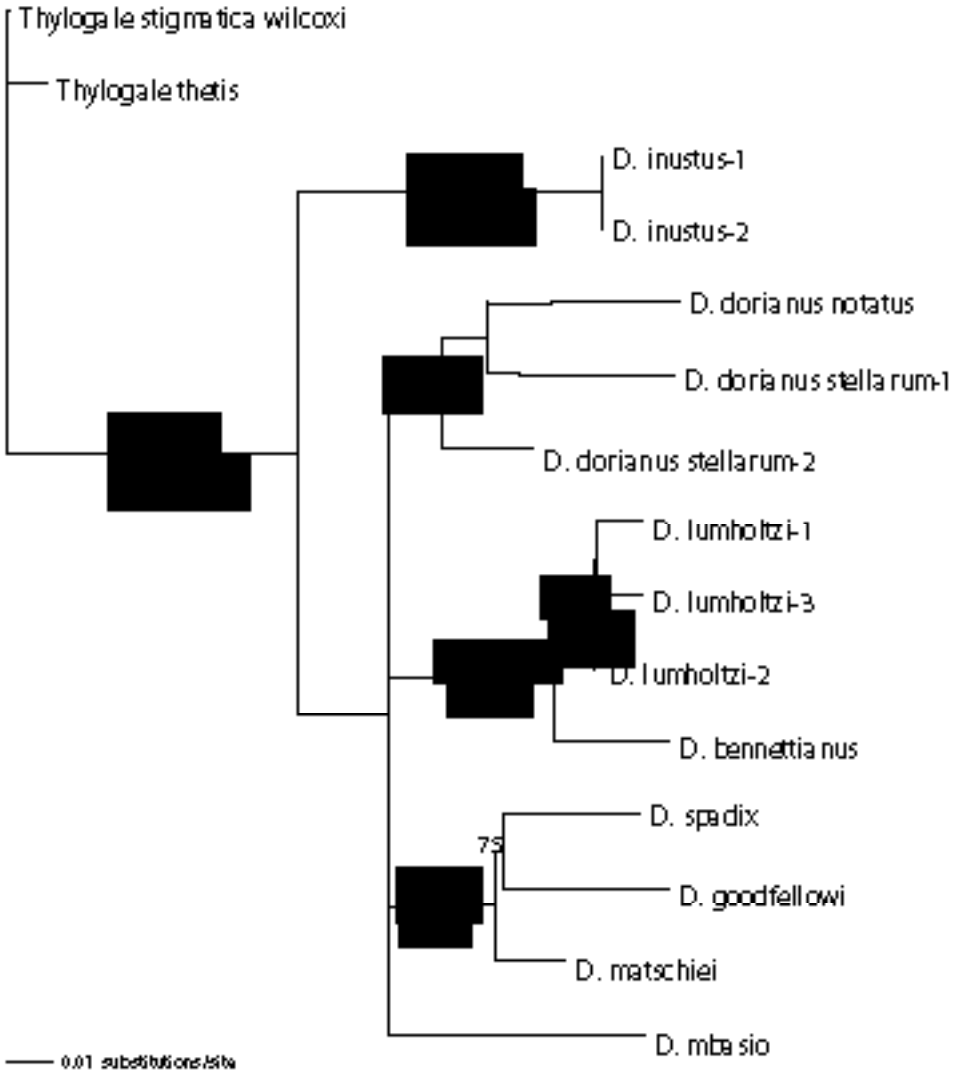

Figure 2 Phylogenetic relationships amongst 13 cyt*b* haplotypes from eight *Dendrolagus* species following neighbour-joining (NJ) and maximum parsimony (MP) analysis of 430 bp of sequence. Haplotype designations are as in Fig. 1. Numbers on branches indicate bootstrap support (% of 500 pseudoreplicates); above, NJ analysis; below, MP analysis. Only bootstrap value > 65% are shown. The tree was rooted using *Thylogale* as an outgroup.

lumholtzi and *D. bennettanius* as members of the same, relatively plesiomorphic, long-footed group (Table 1). Although a number of other distinct mtDNA lineages are apparent within New Guinean *Dendrolagus*, our data could not fully resolve relationships amongst them and the Australian species (Fig. 2).

Although our data do suggest that *D. lumholtzi* and *D. bennettanius* are sister species, it seems unlikely that the most recent glacial maximum (~18, 000 YBP) was influential in their speciation. As well as pronounced morphological differences (Flannery *et al.* 1996), *D. lumholtzi* and *D. bennettanius* show considerable sequence divergence in both cyt*b* (5.3%) (this study) and the mtDNA control region (17.3% (Bowyer *et al.* 2002). In mammals, cyt*b* is widely assumed to evolve at ~2% per million years (Brown *et al.* 1979; Krajewski *et al.* 1997; Avise *et al.* 1998; Moritz *et al.* 2000; Osborne *et al.* 2000) so the divergence of *D. lumholtzi* and *D. bennettanius* almost certainly predates the mid-late Pleistocene. However, Pleistocene climatic fluctuations do appear to have significantly influenced the distribution of both species (Winter 1997), as well as the genetic diversity of at least *D. lumholtzi* (Bowyer *et al.* 2002). Similarly, Moritz *et al.* (2000) reported that most divergences between sister species of tropical forest vertebrates, appeared to predate the Pleistocene. However, Pleistocene rainforest contractions appear to have profoundly affected the distribution and abundance of genetic diversity in many endemic tropical forest taxa (Joseph and Moritz 1993, 1994; Joseph *et al.* 1995; Cunningham and Moritz 1998; Schneider *et al.* 1998; Schneider and Moritz 1999; Schneider *et al.* 1999; Pope *et al.* 2000).

Although inter-relationships amongst New Guinean *Dendrolagus* were not fully resolved by our analysis, a number of distinct lineages were present. The Goodfellow's complex (Table 1) of Flannery *et al.* (1996) is supported by our data, with *D. goodfellowi*, *D. matschiei* and *D. spadix* forming a monophyletic group of closely related taxa (Fig. 2, Table 3). However, our data were limited to a single specimen of each taxon. Support for the Doria's complex

(Table 1) of Flannery *et al.* (1996) was more equivocal with the relationship between *D. dorianus* and the highly divergent *D. mbasio* (Table 3) being unresolved (Fig. 2). However, representatives from two of the four *D. dorianus* subspecies (*D. d. stellerum* and *D. d. notatus*) did form a monophyletic group (Fig. 2), although unexpectedly large divergences were obtained both between the subspecies and within *D. d. stellerum* (Table 3). The degree of genetic differentiation within *D. dorianus* warrants further investigation since the average sequence divergence between *D. d. stellerum* and *D. d. notatus* exceeds that found between *D. lumholtzi* and *D. bennettanius* (5.3%) and amongst the three species of the Goodfellow's complex (5.5%). More extensive sampling throughout the range of all four subspecies of *D. dorianus* is clearly required, but it seems possible that *D. dorianus* comprises a complex of related species. At least *D. d. dorianus* and *D. d. notatus* are well differentiated morphologically and do not appear to intergrade where parapatric in eastern Papua New Guinea (Kawei 1989).

Finally it is important to note a number of limitations in the current study. Firstly, cyt*b* represents only a single genetic marker and only part of the gene has been analysed. Future studies should ideally include a greater portion of the mtDNA genome as well as sequences from nuclear genes. Secondly, not all *Dendrolagus* taxa have been sampled and sample sizes for most taxa were unavoidably small. To fully resolve relationships within the genus a much larger study is required that more thoroughly examines the degree of genetic divergence, at multiple loci, both within and between the currently recognised species. As most taxa of interest inhabit remote and relatively inaccessible areas of New Guinea obtaining an appropriate number and geographic spread of samples will be a formidable challenge.

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