Phylogenetic relationships of the cuscuses (Diprotodontia: Phalangeridae) of Island
Southeast Asia and Melanesia based on the mitochondrial *ND2* gene

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ABSTRACT

The species-level systematics of the marsupial family Phalangeridae, particularly *Phalanger*, are poorly understood, due partly to the family's wide distribution across Australia, New Guinea, eastern Indonesia, and surrounding islands. In order to refine the species-level systematics of Phalangeridae, and improve our understanding of their evolution, we generated 36 mitochondrial *ND2* DNA sequences from multiple species and sample localities. We combined our new data with available sequences and produced the most comprehensive molecular phylogeny for Phalangeridae to date. Our analyses (1) strongly support the monophyly of the three phalangerid subfamilies (Trichosurinae, Ailuropinae, Phalangerinae); (2) reveal the need to re-examine all specimens currently identified as '*Phalanger orientalis*'; and (3) suggest the elevation of the Solomon Island *P. orientalis* subspecies to species level (*P. breviceps* Thomas, 1888). In addition, samples of *P. orientalis* from Timor formed a clade, consistent with an introduction by humans from a single source population. However, further research on east Indonesian *P. orientalis* populations will be required to test this hypothesis, resolve inconsistencies in divergence time estimates, and locate the source population and taxonomic status of the Timor *P. orientalis*.

Keywords: molecular, New Guinea, *Phalanger orientalis*, Timor, translocation

Introduction

The Phalangeridae are arboreal marsupials from eastern Indonesia, Timor, New Guinea, Melanesia, and Australia (Flannery 1994; Crosby 2002) (Fig. 1). With a total of 29 species in six genera, the Phalangeridae is the most diverse of the extant possum families (Phalangeriformes: Helgen and Jackson 2015). In addition, they have the broadest longitudinal range of any marsupial group and the type species – *Phalanger orientalis* Pallas, 1766 – has the distinction of being the first australidelphian marsupial encountered by Europeans (Calaby 1984; Helgen and Jackson 2015). Phalangeridae is also one of few marsupial families to owe part of its present distribution to purposeful translocation by humans during the late Holocene (Flannery and White 1991; Heinsohn 2010). Despite their early scientific identification, diversity, broad geographic distribution, and fascinating history of human interaction, the evolution and systematics of Phalangeridae remain poorly understood, with a remarkably unstable taxonomy (Ruedas and Morales 2005; Crosby 2007; Helgen and Jackson 2015).

While the superfamily (Phalangerioidea) and family (Phalangeridae) ranks, as originally ratified by Kirsch *et al.* (1997), are now well established (e.g. Beck 2008; Meredith *et al.* 2009; Mitchell *et al.* 2014), the decades-long debate over subfamily groupings has been formalised only recently by Helgen and Jackson (2015). They divided the Phalangeridae into three subfamilies – Trichosurinae (*Wyulda* and *Trichosurus*), Ailuropinae (*Strigocuscus*, and *Ailurops*), and Phalangerinae (*Phalanger* and *Spilocuscus*) – with the Australasian cuscuses (Ailuropinae and Phalangerinae) as the sister group to the Australian scaly- and brush-tailed possums (Trichosurinae) (Table 1). This classification was first suggested by Ruedas and Morales (2005); however, a lack of congruence between morphological relationships and molecular phylogenies, in addition to limited taxonomic coverage in molecular studies (Flannery *et al.* 1987; George 1987; Springer *et al.* 1990, 1995; Hamilton and Springer 1999; Kirsch and Wolman 2001; Osborne and Christidis 2002; Crosby and Norris 2003; Ruedas and Morales 2005; Ratterman *et al.* 2006; Crosby 2007; Meredith *et al.* 2009; Mitchell *et al.* 2014), resulted in a delay of formal recognition (Helgen and Jackson 2015). Helgen and Jackson (2015) also resolved many of the uncertainties surrounding genus-level classification within the family, but highlighted the significant lack of resolution in species-level taxonomy, particularly in *Phalanger*.

Part of the underlying uncertainty regarding classifications of the different *Phalanger* species stems from their scattered island distribution, with numerous populations and taxa isolated by ocean barriers. Isolated, island populations of *Phalanger pelengensis*, *P. ornatus*, *P. gymnotis*, and *P. mimicus* have all been distinguished taxonomically, at times as subspecies or even species. Some of these classifications were later ratified, while others still require further investigation (Helgen and Jackson 2015). For example, studies on the type species of *Phalanger* – *P. orientalis* – reported significant morphological variation among island populations (Flannery 1994). The widespread range of this species has encouraged further taxonomic revisions (Menzies and Pernetta 1986; Norris and Musser 2001; Groves 2005; Helgen and Jackson 2015). However, the six subspecies of *P. orientalis*, proposed by Menzies and Pernetta (1986) based on cranial and dental traits, have received limited scientific attention. Of the four subspecies that have, two (*P. intercastellanus* and *P. mimicus*) were raised to species level (Norris and Musser 2001) while *P. o. breviceps* is the only currently recognised subspecies distinct from *P. o. orientalis* (Flannery 1994 Helgen and Jackson 2015). *Phalanger orientalis vulpecula* was suggested to belong to *P. intercastellanus* (Colgan *et al.* 1993). The Timor (*P. o. timorensis*) and Bougainville (*P. o. kori*) island populations have not been subject to further taxonomic research.

Currently, molecular genetic data for *P. orientalis*-group taxa and populations are too limited to provide useful taxonomic insights (Springer *et al.* 1995; Osborne and Christidis 2002; Amrine-Madsen *et al.* 2003). Furthermore, topotypic *P. orientalis* from Ambon Island have yet to be sampled for any molecular analysis, limiting the phylogenetic validation of sequences currently identified as *P. orientalis*. The vast island distribution of *Phalanger* makes obtaining comprehensive molecular data particularly pertinent to their classification. Similarly, molecular data are sparse for the rest of the Phalangeridae (Helgen and Jackson 2015). Taxon sampling is limited to only a single species per genus or there is incomplete genus-level coverage (e.g. Springer *et al.* 1990; Hamilton and Springer 1999; Kirsch and Wolman 2001; Ruedas and Morales 2005; Ratterman *et al.* 2006). The most inclusive molecular study considered only 12 of the 29 recognised phalangerid species (Osborne and Christidis 2002).

Uncertainties surrounding species- and genus-level classifications within the Phalangeridae have led to multiple reidentifications of vouchered specimens. Not only does this have implications for previous studies using those specimens but has also led to a

lack of reliability of some publicly available molecular sequences, as the corresponding metadata are not always updated in online databases. For example, the '*P. orientalis*' specimen (AMS M18526), from which Springer *et al.* (1995) sequenced the *12S rRNA* gene, was reclassified as *P. intercastellanus* following re-evaluation of the *P. orientalis* species group by Norris and Musser (2001) (see also Ruedas and Morales 2005). GenBank continues to list the species ID of the *12S rRNA* sequence (U33496) as *P. orientalis*. This misattribution has resulted in 'chimeric' taxa, e.g. Kavanagh *et al.* (2004), where U33496 was combined with another *P. orientalis* sequence to expand the intraspecific genetic coverage. To further complicate matters, the specimen from which sequence U33496 was derived has again been revised, to *P. mimicus*, according to the Online Zoological Collections of Australian Museums Database (OZCAM).

Thus, while family and subfamily classifications of the Phalangeridae are now well established, significant work remains at the inter- and intraspecific levels, particularly with respect to differences among island populations (Menzies and Pernetta 1986; Norris and Musser 2001; Groves 2005; Helgen and Jackson 2015; Leary *et al.* 2016a). Here we present the most taxonomically comprehensive molecular phylogeny of the Phalangeridae to date, with a focus on *Phalanger*. We rely on just a single gene (*ND2*) without combining any pre-existing data to remove the risk of multispecies combinations from specimen misidentifications. We present 36 novel *ND2* sequences, including multiple samples and sample localities of the same species. We designed our study to assess which specimen identifications were reliable and which will require further analysis. By including multiple sequences across different localities we also explore the phylogenetic relationships among isolated populations and the different subspecies proposed for *Phalanger*.

Materials and Methods

Species analysed

We included 53 samples from 21 species, of which 18 belong to the Phalangeridae and three are outgroups (Table S1, available as Supplementary material to this paper). Samples were selected to maximise numbers of species, and to include multiple samples per species, where possible, to validate specimen identification (Table S1, Fig. 1).

DNA extraction

DNA was extracted from Timor *P. orientalis* tissue samples using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), following manufacturer's instructions, but with the following modifications: the tissue digestion step (buffer ATL plus Proteinase K) was conducted overnight at 55°C with the addition of dithiothreitol (DTT) (Supplementary material S2.1). DNA from all other tissue samples in the present study was extracted using a Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN, USA)

DNA sequencing

DNA extracted from the Timor *P. orientalis* samples was converted into Illumina sequencing libraries, which were subsequently enriched for mitochondrial DNA (see Supplementary material S2.1). Sequencing reads generated on an Illumina NextSeq 500 were assembled into complete mitochondrial genome sequences for each sample, from which the *ND2* region was isolated for further analysis.

Mitochondrial *ND2* sequences were obtained from all other samples using PCR amplification and Sanger sequenced following the protocols in Supplementary material S2.2.

Alignment and partitions

ND2 sequences were aligned with MUSCLE (Edgar 2004) as implemented in Geneious 11 (<https://www.geneious.com>), resulting in a master alignment of 999 bp for a total of 53 taxa. PartitionFinder 2.1.1 (Lanfear *et al.* 2016) was used to determine an appropriate alignment partitioning scheme and nucleotide substitution model for each partition (Supplementary material S3.1). For the PartitionFinder analysis, we restricted comparisons to models implemented by MrBayes, with the assumption of linked branch lengths, the 'all' search algorithm, and with the Bayesian Information Criterion used for model selection, as suggested by Lanfear *et al.* (2012).

Undated phylogenetic analysis

We performed a Bayesian phylogenetic analysis on our *ND2* dataset using MrBayes 3.2.6 (Ronquist *et al.* 2012) via the CIPRES Science Gateway (Miller *et al.* 2010). The analysis comprised four runs of four chains (one cold, three heated) each, sampling trees every 5000 generations, and run for 10 million generations. The post-burn-in trees were summarised using 50% majority-rule consensus. To evaluate any impact of substitution

saturation at third-codon positions, we also ran our Bayesian analysis with third-codon positions RY-coded (see Phillips and Penny 2003).

We also performed a maximum likelihood (ML) analysis of the *ND2* matrix using RAxML 8.2.10 (Stamatakis 2014), with the same partitions used for our MrBayes analysis. Support for the most likely tree was calculated using 1000 ‘rapid’ bootstrap replicates (see Supplementary material, Fig. S4.1).

Calibrated phylogenetic analysis

To estimate divergence times within Phalangeridae, three internal node calibrations (Phalangerioidea, Burramyidae, and Phalangeridae), in addition to a root calibration (all specified as uniform distributions with ‘hard’ minimum and maximum bounds), were incorporated into a Bayesian analysis using MrBayes (Supplementary material S3.2). We used a single Independent Gamma Rates clock model and implemented a fossilised birth–death tree branching prior that assumed ‘diversity’ sampling (Zhang *et al.* 2016). We chose a sample probability of 0.6 for our tree prior, which is slightly less than the proportion of named phalangerid species in our matrices (0.64) but allows for the existence of a few additional undescribed species. As our molecular analysis includes only modern taxa, the fossilisation prior was fixed as 0. The analysis was then run as described above for the undated dataset. The resulting consensus tree was analysed in FigTree 1.4.3 (FigTree 2016) to extract divergence estimates. As for our undated Bayesian analysis, we also ran the analysis a second time with third-codon positions RY-coded in order to evaluate any possible impacts of substitution saturation on our results. In addition, we calculated the marginal prior on node ages (Fig. S4.4) by running our calibrated analysis without nucleotide data while enforcing the tree topology (but not branch lengths) that we obtained from our initial calibrated analysis.

Results

Undated phylogenetic analysis

Our undated *ND2* analyses of Phalangeridae produced a consensus tree with high support values (BPP > 0.95, Bs >90%) for most clades (Fig. 2). The results of our undated analysis show strong support (BPP = 1, Bs = 98%) for monophyly of the family Phalangeridae and each of its three subfamilies: Ailuropinae, Phalangerinae, Trichosurinae (Table 1). In addition, our results strongly support the position of the subfamily Trichosurinae

(*Trichosurus* and *Wyulda*) as the sister lineage to a clade comprising Ailuropinae and Phalangerinae. Within Phalangerinae, both *Spilocuscus* and *Phalanger* were recovered as monophyletic with strong support (BPP = 1, Bs >95%).

A basal *Phalanger* clade comprising *P. gymnotis* and *P. matanim* receives moderate to low support from our analyses (BPP = 0.86, Bs = 57%), although within this clade the monophyly of both species is strongly supported. Within the rest of *Phalanger*, our results strongly support a clade comprising the Woodlark Island cuscus (*P. lullulae*), *P. mimicus*, *P. intercastellanus*, and three *P. orientalis* samples (BPP = 1, Bs = 100%), which is sister to the remaining *Phalanger* samples.

The silky cuscus (*P. sericeus*) was recovered as a monophyletic species with strong support (BPP = 1, Bs = 100%). In contrast, *P. carmelitae* appears to be polyphyletic: Osborne and Christidis's (2002) sample is well supported as part of a clade including *P. orientalis breviceps* (BPP = 0.95, Bs = 88%). This *P. o. breviceps* clade also includes a single *P. orientalis* sample from New Britain.

The remaining *Phalanger carmelitae* samples and *P. vestitus* form a well supported clade (BPP = 0.99, Bs = 81%) in which the single *P. vestitus* sample from Chimbu Province forms a subclade with the two new *P. carmelitae* samples. This subclade is, in turn, sister to a subclade comprising the rest of the *P. vestitus* samples, both with strong support (BPP = 1, Bs >90%). The remaining *P. orientalis* sequences are recovered as a monophyletic clade with strong support (BPP = 1, Bs = 100%). Almost all the *P. orientalis* samples from West Sepik Province form a strongly supported clade (BPP = 0.99, Bs = 97%), with the exception of a single West Sepik *P. orientalis* sample that is more closely related to the two *P. orientalis* samples from Madang (although with low support; BPP = 0.23, Bs = 24%). All the *P. orientalis* samples from Timor are recovered in a single strongly supported clade (BPP = 1, Bs = 100%) although relationships among the samples within the Timor clade, as well as between the Timor clade and its sister clade (Madang + one West Sepik sample), are poorly resolved.

In the Bayesian analysis, the '*P. o. breviceps*' clade is recovered as sister to a clade comprising both the '*P. vestitus*' and *P. orientalis* clades. However, in the ML analysis this sister relationship is reversed, with the '*P. o. breviceps*' clade instead being sister to the remaining *P. orientalis* clade (Fig. S4.1). In both analyses the branching order of these two clades (i.e. *breviceps* and *vestitus*) and *P. orientalis* receives low support.

Calibrated phylogenetic analysis

The calibrated phylogenetic analysis produced a tree with a topology matching that of the undated analysis (Fig. 3). Similarly, strong support (BPP = 1) was also recovered for the monophyly of all phalangerid subfamilies and genera represented.

By conducting a calibrated phylogenetic analysis on our *ND2* dataset, we produced estimates of the divergence times for the different groups within Phalangeridae (Table 2). Our median node age estimates fall within the range of estimates for comparable clades from recent *Phalanger*-specific studies (Ruedas and Morales 2005; Raterman *et al.* 2006), except for the dates recovered for crown Phalangerinae and *Phalanger* by Ruedas and Morales (2005). Apart from crown Phalangeroidea, all our median divergence estimates are older than the ranges recovered in the phylogeny of Meredith *et al.* (2009). The 95% Highest Posterior Densities (HPD) recovered by our study do, however, overlap with all the comparable estimates shown in Table 2 (including Meredith *et al.* 2009 with the exception of crown Phalangerinae), and the age of deeper clades (i.e. Phalangeroidea and Phalangeridae) are also found to be congruent with those reported by other broader-scale studies (i.e. Beck 2008).

Impacts of RY-coding third codon positions

To test for any influence of substitution saturation at third-codon positions on our phylogenetic and molecular dating results, we RY-coded the third-codon partition of our *ND2* matrix and reran our previous analyses. For the undated analysis (Fig. S4.2), the tree topology generated using the dataset including RY-coded nucleotides was very similar to that obtained from our original analysis. The main difference was that when third-codon positions were RY-coded the *Ailurops*+*Strigocuscus* clade was recovered as sister to the *Trichosurus*+*Wyulda* clade, while the '*P. breviceps*' and '*P. vestitus*' clades swapped positions on the tree (concordant with the ML results). In the calibrated analysis of the dataset including RY-coded nucleotides (Fig. S4.3), the *P. gymnotis*+*P. matanim* clade was recovered as sister to the *P. lullulae*+*P. mimicus*+*P. intercastellanus* clade, while the '*P. breviceps*'+'*P. carmelitae*' clade also shares a sister relationship with *P. orientalis*, displacing the '*P. vestitus*' clade. The divergence date estimates recovered in both analyses are very similar (substantially overlapping 95% HPDs), suggesting that saturation in third-codon position has had little impact on this portion of the study.

Discussion

Classifications and divergence times within Phalangeridae

Overall, the relationships among taxa are congruent with recent mitochondrial (i.e. Ruedas and Morales 2005) and nuclear (i.e. Raterman *et al.* 2006; Meredith *et al.* 2009) molecular phylogenetic studies. Accepted families, subfamilies, and genera (as per Helgen and Jackson 2015) are each recovered with strong support.

We do not find any support for the inclusion of *Strigocuscus* within Trichosurinae, as previously suggested by morphological studies (Flannery *et al.* 1987; Crosby 2002). Instead, *Strigocuscus celebensis* appears to be sister taxon to *Ailurops ursinus* within the subfamily Ailuropinae. While our undated Bayesian analyses of the dataset including RY-coded third-codon positions did recover a sister relationship between Ailuropinae and Trichosurinae, support for this relationship is very low (BPP = 0.47) (Fig. S4.2) and not replicated in any of our other analyses. However, the lack of mitochondrial sequences for *St. sangirensis* (the only other species recognised in the genus: Helgen and Jackson 2015) as well as *A. melanotis* and *A. furvus* means the relationships of the other species within Ailuropinae remain uncertain. The lack of additional sequences for this subfamily also limits our interpretations of divergence date estimates.

Our time-calibrated phylogeny suggests that dispersal of the common ancestor of Ailuropinae and Phalangerinae (the cuscuses) out of Australia occurred during the late Oligocene to early Miocene, corresponding with the emergence of the New Guinea Bird's Head, in turn resulting from the collision of the Australian and Pacific plates (Hall 2009). The Early Miocene collision of the Sula Spur and the North Sulawesi volcanic arc, which resulted in mountainous regions over 1000 m on palaeo-Sulawesi for the first time (Nugraha and Hall 2018), also supports our estimate for an early Miocene dispersal of the ancestral Ailuropinae. The subsequent diversification events within the Phalangerinae, following its divergence from Ailuropinae, likely result from the various geological and climatic changes experienced by the New Guinea and east Indonesian region during the Miocene (Zachos *et al.* 2001; Hall 2009).

On classifications within Phalanger

Our study focuses largely on relationships within *Phalanger*, particularly the current identifications of different '*P. orientalis*' specimens. In accordance with current classifications, we recover *P. gymnotis*, *P. matanim*, and *P. sericeus* as monophyletic in our MT-ND2 phylogeny with good support. However, no other *Phalanger* species

included in our study is recovered as monophyletic. *P. orientalis* is recovered as highly polyphyletic. Our phylogeny recovers three distinct ‘*orientalis*-morphotype’ clades: the *P. mimicus*–*P. intercastellanus* clade, *P. o. breviceps* clade, and the West Sepik–Madang–Timor clade. We recover the Timor cuscus population as monophyletic, supporting all previous studies that suggest it constitutes a single species belonging to the *P. orientalis* species group (Menzies and Pernetta 1986; Helgen and Jackson 2015). For clarity, we assume that the *P. orientalis* specimens from Timor are ‘true’ members of the *P. orientalis* species, and only the New Guinea *P. orientalis* samples which are recovered in monophyly with the Timor clade belong in *P. orientalis*. Verification of this assumption, however, will require sequencing of, and comparison with, cuscus samples taken from the *P. orientalis* type locality. All *P. orientalis* samples not recovered as monophyletic to the West Sepik–Madang–Timor clade are consequently considered here as misidentifications.

The New Britain ‘*P. orientalis*’ sample forms a clade with the *P. o. breviceps* samples, strongly suggesting that this is a misidentified *P. o. breviceps* sample (Figs 1, 2). The reidentification of the New Britain ‘*P. orientalis*’ sample as *P. o. breviceps* is supported by Thomas’ (1888, 1895) original classification of the New Britain populations with the Solomon Island populations, and more recently by Helgen and Jackson’s (2015) proposed species distributions. As the *P. o. breviceps* clade is separated from the *P. orientalis* clade and appears sister to the *P. carmelitae* from Oro Province (Fig. 2), we suggest that this subspecies be returned to its full species classification: *P. breviceps* Thomas, 1888 (Thomas 1895). This revision supports Thomas’s (1895) taxonomy, although it is contrary to Menzies and Pernetta (1986) and currently accepted classifications (Helgen and Jackson 2015). The location of the *P. breviceps* clade in our main phylogenies, polyphyletic with respect to the ‘true’ *P. orientalis* clade, further supports this (Figs 2, 3).

We recovered the pre-existing *P. carmelitae* sequence from the study by Osborne and Christidis (2002) as a basal member of the *P. breviceps* clade and polyphyletic with respect to our other *P. carmelitae* samples (Fig. 2). The provenance of the Osborne and Christidis (2002) sample, in the north-eastern Oro Province (Figs 1, 2), is within the range of the type locality for the species (Thomas 1898), supporting its identification as *P. carmelitae*. A north-easterly location suggests that it likely represents a descendant of the ‘mainland’ (New Guinean) source for the island *P. breviceps* populations.

The early divergence estimates of this Oro *P. carmelitae*+*P. breviceps* clade further supports the elevation to species rank of *P. breviceps* (Fig. 3). Additional research into the various known and probable *P. breviceps* populations throughout the Bismarck Archipelago and the Solomon Islands will be required to further clarify the evolutionary history of this clade. In particular, the archaeological record of '*P. orientalis*' in the region suggests human introductions of *P. breviceps* (or its ancestor) to New Ireland as early as ~22.5–23.6 thousand years ago (Leavesley *et al.* 2002; Leavesley and Chappell 2004; Summerhayes 2007) and Buka Island (Solomons) at ~8.5–9 thousand years ago (Wickler 2001; Helgen and Jackson 2015). Improved identifications of the archaeological material, possibly with the use of ancient DNA, along with direct dating of the specimens would also play an important role in improving our understanding of this species-group and its dispersal history.

While samples such as the New Britain '*P. orientalis*' sequence in this study can be confidently revised to *P. breviceps*, other specimens, such as those placed with the *P. lullulae*+*P. mimicus*+*P. intercastellanus* group, cannot be revised any further than '*P. orientalis*' due to the lack of monophyly of the species recovered within this clade. When Norris and Musser (2001) first identified *P. mimicus* as a species separate from *P. orientalis* and *P. intercastellanus*, they also identified distinct geographic ranges for each species. When these geographic ranges are compared with our sample locations (Fig. 1, 2) (Norris and Musser 2001, fig. 1) the '*P. orientalis*' sequence from Morobe Province falls within the geographic range of *P. intercastellanus*, while all the closely related Southern Highland Province sequences (including the '*P. intercastellanus*' sequence) are within the range of *P. mimicus*. Further analysis of the samples used in this study, in addition to those reviewed by Norris and Musser (2001), will be required to resolve the relationships within this clade and the identifications of these samples.

Phalanger vestitus is known currently from four separate regions in New Guinea, and it has been proposed that the taxonomy of these separate populations requires investigation (Helgen and Jackson 2015; Leary *et al.* 2016b). However, we only included sequences from the central-eastern New Guinean population (Fig. 1, Table S1) (Leary *et al.* 2016b). These *P. vestitus* sequences are recovered in a clade with '*P. carmelitae*' (with the exception of the previously published sequence from Oro Province), a relationship that is supported by an allozyme study (Colgan *et al.* 1993). However, as the Oro province *P. carmelitae* originates from the type locality of the species, it seems more likely that these

two new '*P. carmelitae*' are in fact misidentified *P. vestitus*. Our *P. vestitus*+*P. carmelitae* clade can be further split into two sister clades: a '*P. carmelitae*'+'*P. vestitus*' clade and a *P. vestitus* clade (Fig. 2). A single *P. vestitus* sample from Chimbu Province is recovered with the '*P. carmelitae*' sequences. Based on the BPP support for its position in the phylogeny and the Pliocene divergence estimate (Fig. 3), this clade may represent a different species or subspecies within the *P. vestitus* complex (see Helgen and Jackson 2015). A thorough examination of the other three *P. vestitus* populations in addition to *P. carmelitae* samples from western and eastern Papua New Guinea will be required to further understand the relationships and classification of these two species.

The origin of Phalanger orientalis on Timor

We recover the *P. orientalis* sequences from Timor as a single monophyletic group, supporting biological and archaeological conclusions that only a single species inhabits the island (Fig. 2) (Glover 1986; Flannery 1994; Heinsohn 2001, 2005; O'Connor 2015). Our analysis also supports the hypothesis that this population shares a close relationship with the modern New Guinean *P. orientalis*. While our analysis suggests a close relationship to *P. orientalis* populations from central New Guinea, populations further west in New Guinea (and closer to Timor) were not sampled. Menzies and Pernetta (1986) proposed a separate subspecies for Timor, *P. o. timorensis*, which is somewhat supported by our phylogeny. Further genetic research, however, on both the Ambon type population and cuscus populations from western New Guinea, will be required to determine whether the Timor clade merits recognition as a distinct subspecies.

Our estimates for the timing of the divergence of the Timor *P. orientalis* from its sister clade in New Guinea range from 820 to 570 thousand years ago (Table 2, Fig. 3). Whether or not this divergence happened on Timor is not revealed by our analyses. While divergence dates for other taxa recovered from our analysis agree with previous molecular dating studies (Table 2), a date of 0.82 million years ago for the establishment of a Timor cuscus population runs contrary to both the fossil and archaeological records, the latter of which suggest an introduction date of ~3 thousand years ago (Glover 1986; O'Connor 2015).

Two scenarios could account for this disparity if our estimated divergence times are correct. First, this could indicate natural dispersal into Timor and subsequent omission from all palaeontological and archaeological sites until ~3000 years ago. Such a scenario

would be consistent with the fact that the earliest records for humans in the region are significantly younger than our divergence estimate (Summerhayes *et al.* 2010; Clarkson *et al.* 2017; Hawkins *et al.* 2017) and may be accounted for archaeologically by a change of hunting technologies 3000 years ago. However, we consider this scenario substantially less likely than the alternative. This is principally due to the extensive archaeological records and the more moderate palaeontological records that have failed to recover a single phalangerid fossil, despite thousands of similarly sized, and likely ecologically convergent, giant rodent fossils (Aplin and Helgen 2010; Louys *et al.* 2017). The alternative scenario would require people to have translocated multiple *P. orientalis* from an (as yet) unsampled source population whose ancestors diverged from the eastern New Guinea population ~0.82 million years ago. As with the classification difficulties discussed above, further work to identify potential source populations and the relationships of *P. orientalis* on neighbouring islands (i.e. western New Guinea, Ambon, Seram, Babar, Wetar, assuming that biological surveys for other Lesser Sunda and Moluccan islands are accurate) will require additional geographical sampling.

Several methodological factors may have influenced our age estimates for the divergences among the Timor *P. orientalis* individuals, possibly causing these dates to be overestimated: saturation at third-codon positions, tree model mis-specification, or time-dependency of molecular rates. We excluded the possibility that saturation impacted our results by rerunning our analyses with third-codon positions RY-coded (see Phillips and Penny 2003), which resulted in largely similar age estimates (Fig. S4.3). However, our posterior distributions for the age of divergences among Timor *P. orientalis* individuals were substantially younger than the marginal prior distributions for these nodes (95% HPDs did not overlap: Fig. S4.4), suggesting that our specification and parameterisation of the birth–death tree prior may have resulted in overestimation of more shallow nodes. Finally, as our study was calibrated by constraining the age of nodes deep in the phylogeny, more shallow divergences may have been overestimated due to the time-dependency of rates previously reported for mitochondrial data (see Ho *et al.* 2005). Unfortunately, these latter two possibilities are challenging to assess or circumvent without serially sampled data (e.g. ancient DNA from dated specimens), which is not currently available for this taxon. Thus, while we can be reasonably confident in our species (and higher-level) divergence estimates, for several reasons there remains the possibility that our divergence estimates within the Timor *P. orientalis* clade are significantly older than

the ‘true’ dates. Further research into the palaeontological and archaeological record of these species and the incorporation of nuclear data into future phylogenetic analyses will improve our understanding of the evolution of these taxa.

Conclusion

Despite being among the first australidelphian marsupials encountered by Europeans, most known cuscus species in the Phalangeridae have received a surprising lack of attention in modern genetic research. The most comprehensive molecular phylogeny, until now, included only 12 of the 29 species recognised. Furthermore, despite being the type species for the genus, *Phalanger orientalis* has received minimal genetic attention, and suffers from classification and specimen identification problems. Here we presented a comprehensive *ND2* gene sequence molecular phylogeny for the Phalangeridae, with a particular focus on identifications of *P. orientalis* samples and the inclusion of the *P. orientalis* populations on Timor.

Our phylogenetic results and divergence date estimates are congruent with previous molecular analyses and palaeobiogeography. *Phalanger orientalis*, *P. mimicus*, *P. intercastellanus*, *P. vestitus*, and *P. carmelitae* are identified as species that require further investigation, both for sampling populations in the field but also a greater investigation of specimens held in museum collections, particularly *P. orientalis*. While our analyses are based on a single locus (*ND2*), and thus in some cases may not reflect the true underlying species-tree (e.g. Nilsson *et al.* 2018; Wright *et al.* 2018), they are sufficient to identify sample misidentifications and test some phylogenetic hypotheses. We recommend that future studies of phalangerid taxa with problematic identifications begin with *ND2* sequencing and comparison against our matrix before undertaking more comprehensive, accurate, and costly analyses (e.g. nuclear genome sequencing). A similar screening approach may be appropriate for other marsupial taxa in New Guinea and surrounding islands, which have a similarly convoluted history of collections and classifications.

Our study revealed that the Solomon Island cuscus, previously considered a subspecies of *P. orientalis*, forms a polyphyletic clade with respect to other *P. orientalis* samples and should be considered a distinct species, *P. breviceps*, with a sister relationship to *P. carmelitae* and an ancestral source population likely located in New Guinea’s Oro Province. Some support is recovered for Menzies and Pernetta’s (1986) classification of a distinct subspecies – *P. orientalis timorensis* – for the Timor cuscus; however, further

research is required to confirm this. Our results, combined with fossil and archaeological evidence, suggest the introduction of *P. orientalis* to Timor by humans from an original source population of *P. orientalis* on New Guinea. Future sampling of *P. orientalis* populations in Indonesian Papua (west New Guinea) and the Ambon type population are required to pinpoint the immediate source population of the Timor cuscus and its subspecific status. Finally, our study highlights the need to thoroughly evaluate the Phalangeridae across its various geographic ranges on New Guinea and throughout the islands of Melanesia and Indonesia. This will have significant impacts not only for our understanding of cuscus evolution and biogeography but also for the conservation of these species.

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Supplementary Information

Additional information on taxa and samples (S1), methods of DNA extraction and sequencing (S2), sequence partitions, nucleotide substitution models, and node calibrations (S3), and additional model test results (S4) are in the supplementary material. For NEXUS files of the undated and calibrated matrices, RY-coded and prior-only matrices, see the supplementary NEXUS file. Raw tree files produced from all analyses are also provided in nexus format in the corresponding file. All new *ND2* sequences are available on GenBank under the accession numbers listed in Table S1; see <https://www.ncbi.nlm.nih.gov/genbank/>.

Conflicts of Interest

The authors declare there are no known conflicts of interest.

References

- Amrine-Madsen, H., Scally, M., Westerman, M., Stanhope, M. J., Krajewski, C., and Springer, M. S. (2003). Nuclear gene sequences provide evidence for the monophyly of australidelphian marsupials. *Molecular Phylogenetics and Evolution* **28**, 186–196.
- Aplin, K. P., and Helgen, K. M. (2010). Quaternary murid rodents of Timor Part I: new material of *Coryphomys buehleri* Schaub, 1937, and description of a second species of the genus. *Bulletin of the American Museum of Natural History* **341**, 1–80.
- Beck, R. M. D. (2008). A dated phylogeny of marsupials using a molecular supermatrix and multiple fossil constraints. *Journal of Mammalogy* **89**, 175–89.
- Calaby, J. H. (1984). Foreword. In ‘Possums and Gliders’. (Eds A. P. Smith and I. D. Hume.) Pp. iii–iv. (Surrey Beatty & Sons with Australian Mammal Society: Sydney)
- Clarkson, C., Jacobs, Z., Marwick, B., Fullagar, R., Wallis, L., Smith, M., Roberts, R. G., Hayes, E., Lowe, K., Carah, X., Florin, S. A., McNeil, J., Cox, D., Arnold, L. J., Hua, Q., Huntley, J., Brand, H. E. A., Manne, T., Fairbairn, A., Shulmeister, J., Lyle, L., Salinas, M., Page, M., Connell, K., Park, G., Norman, K., Murphy, T., and Pardoe, C. (2017). Human occupation of northern Australia by 65,000 years ago. *Nature* **547**, 306–310.
- Colgan, D. J., Flannery, T. F., Trimble, J., and Aplin, K. P. 1993. Electrophoretic and Morphological Analysis of the Systematics of the *Phalanger orientalis* (Marsupialia) Species Complex in Papua New Guinea and the Solomon Islands. *Australian Journal of Zoology*, **41**, 355–378.
- Crosby, K. (2002). Studies in the diversity and evolution of phalangeroid possums (Marsupialia; Phalangerida; Phalangerioidea). PhD Thesis, University of New South Wales, Sydney.
- Crosby, K. (2007). Re-diagnosis of the fossil species assigned to *Strigocuscus* (Marsupialia, Phalangeridae), with description of a new genus and three new species. *Alcheringa: An Australasian Journal of Palaeontology* **31**, 33–58.
- Crosby, K., and Norris, C. (2003). Periotic Morphology in the Trichosurin Possums *Strigocuscus celebensis* and *Wyulda squamicaudata* (Diprotodontia, Phalangeridae) and a Revised Diagnosis of the Tribe Trichosurini. *American Museum Novitates* **3414**, 1–14.

514 Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high
515 throughput. *Nucleic Acids Research* **32**, 1792–1797.

516 FigTree. (2016). FigTree v1.4.3. Available at <http://tree.bio.ed.ac.uk/software/figtree/>
517 [accessed January 2018].

518 Flannery, T. (1994). ‘Possums of the World: A Monograph of the Phalangerioidea.’
519 (Australian Museum/Geo Productions: Sydney.)

520 Flannery, T. F., and White, J. P. (1991). Animal translocation. *National Geographic*
521 *Research and Exploration* **7**, 96–113.

522 Flannery, T., Archer, M., and Maynes, G. (1987). The phylogenetic relationships of living
523 phalangerids (Phalangerioidea: Marsupialia) with a suggested new taxonomy. In ‘Possums
524 and Opossums: Studies in evolution, V.II’. (Ed. M. Archer.) Pp. 477–506. (Surrey Beatty
525 & Sons and the Royal Zoological Society of New South Wales: Sydney.)

526 George, G. G. (1987). Characterisation of the living species of cuscus (Marsupialia:
527 Phalangeridae). In ‘Possums and Opossums: Studies in evolution, V.II’. (Ed. M. Archer.)
528 Pp. 507–526. (Surrey Beatty & Sons and the Royal Zoological Society of New South
529 Wales: Sydney.)

530 Glover, I. (1986). ‘Archaeology in Eastern Timor, 1966–67.’ *Terra Australis* **11**, 1–241.
531 (Australian National University: Canberra.)

532 Groves, C. P. (2005). Order Diprotodontia. In ‘Mammal Species of the World: A
533 Taxonomic and Geographic Reference. 3rd Edition’. (Eds. D. E. Wilson and D. M. Reeder)
534 Pp. 43–70. (The Johns Hopkins University Press: Baltimore, MD.)

535 Hall, R. (2009). Southeast Asia's changing palaeogeography. *Blumea-Biodiversity,*
536 *Evolution and Biogeography of Plants* **54**, 148–161.

537 Hamilton, A. T., and Springer, M. S. (1999). DNA sequence evidence for placement of the
538 ground cuscus, *Phalanger gymnotis*, in the tribe Phalangerini (Marsupialia:
539 Phalangeridae). *Journal of Mammalian Evolution* **6**, 1–17.

540 Hawkins, S., O'Connor, S., Maloney, T. R., Litster, M., Kealy, S., Fenner, J. N., Aplin, K.,
541 Boulanger, C., Brockwell, S., Willan, R., Piotto, E., and Louys, J. (2017). Oldest human
542 occupation of Wallacea at Laili Cave, Timor-Leste, shows broad-spectrum foraging
543 responses to late Pleistocene environments. *Quaternary Science Reviews* **171**, 58–72.

- Heinsohn, T. E. (2001). Human influences on vertebrate zoogeography: animal translocation and biological invasions across and to the east of Wallace's Line. In 'Faunal and Floral Migrations and Evolution in SE Asia-Australasia'. (Eds I. Metcalfe, J. M. B. Smith, M. Morwood and I. Davidson.) Pp. 153–170. (A.A. Balkema: Lisse.)
- Heinsohn, T. E. (2005). Den sites and habitats utilised by the northern common cuscus *Phalanger orientalis* (Marsupialia: Phalangeridae) in East Timor. *Australian Mammalogy* **27**, 99–101.
- Heinsohn, T.E. (2010). Marsupials as introduced species: Long-term anthropogenic expansion of the marsupial frontier and its implications for zoogeographic interpretation. In 'Altered Ecologies: Fire, climate and human influence on terrestrial landscapes. Terra Australia Vol. 32'. (Eds S. Haberle, J. Stevenson, and M. Prebble) pp.133-176. (ANU E Press: Canberra.)
- Helgen, K. M., and Jackson, S. M. (2015). Family Phalangeridae (Cuscuses, Brush-tailed Possums and Scaly-tailed Possum). In 'Handbook of The Mammals of the World, Vol. 5: Monotremes and Marsupials'. (Eds D. E. Wilson and R. A. Mittermeier.) Pp. 456–497. (Lynx Edicions: Barcelona.)
- Ho, S.Y., Phillips, M.J., Cooper, A., and Drummond, A.J. (2005). Time dependency of molecular rate estimates and systematic overestimation of recent divergence times. *Molecular Biology and Evolution* **22**, 1561–1568.
- Kavanagh, J. R., Burk-Herrick, A., Westerman, M., and Springer, M. S. (2004). Relationships among families of Diprotodontia (Marsupialia) and the phylogenetic position of the autapomorphic honey possum (*Tarsipes rostratus*). *Journal of Mammalian Evolution* **11**, 207–22.
- Kirsch, J. A. W., and Wolman, M. A. (2001). Molecular relationships of the bear cuscus, *Ailurops ursinus* (Marsupialia: Phalangeridae). *Australian Mammalogy* **23**, 23–30.
- Kirsch, J. A., Springer, M. S., and Lapointe, F. J. (1997). DNA-hybridisation studies of marsupials and their implications for metatherian classification. *Australian Journal of Zoology* **45**(3), 211-280. doi:10.1071/ZO96030

572 Lanfear, R., Calcott, B., Ho, S. Y., and Guindon, S. (2012). PartitionFinder: combined
573 selection of partitioning schemes and substitution models for phylogenetic analyses.
574 *Molecular Biology and Evolution* **29**, 1695–1701.

575 Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., and Calcott, B. (2016).
576 PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular
577 and morphological phylogenetic analyses. *Molecular Biology and Evolution* **34**, 772–773.

578 Leary, T., Singadan, R., Menzies, J., Helgen, K., Wright, D., Allison, A., Hamilton, S.,
579 Salas, L., and Dickman, C. (2016a). *Phalanger orientalis*. The IUCN Red List of
580 Threatened Species 2016:e.T16847A21951519.

581 Leary, T., Singadan, R., Menzies, J., Helgen, K., Wright, D., Allison, A., Aplin, K., Salas,
582 L., and Dickman, C. (2016b). *Phalanger vestitus*. The IUCN Red List of Threatened
583 Species 2016:e.T16850A21950723.

584 Leavesley, M. G., Bird, M. I., Fifield, L. K., Hausladen, P. A., Santos, G. M., and Di Tada,
585 M. L. (2002). Buang Merabak: early evidence for human occupation in the Bismarck
586 Archipelago, Papua New Guinea. *Australian Archaeology* **54**(1), 55–57.
587 doi:10.1080/03122417.2002.11682070

588 Leavesley, M. G. and Chappell, J. (2004). Buang Merabak: additional early radiocarbon
589 evidence of the colonisation of the Bismarck Archipelago, Papua New Guinea. *Antiquity*
590 **78**(301).

591 Louys, J., Kealy, S., O'Connor, S., Price, G. J., Hawkins, S., Aplin, K., Rizal, Y., Zaim, J.,
592 Tanudirjo, D. A., Santoso, W. D., and Hidayah, A. R. (2017). Differential preservation of
593 vertebrates in Southeast Asian caves. *International Journal of Speleology* **46**, 379–408.

594 Menzies, J. I., and Pernetta, J. C. (1986). A taxonomic revision of cuscuses allied to
595 *Phalanger orientalis* (Marsupialia: Phalangeridae). *Journal of Zoology (London)* **1**, 551–
596 618.

597 Meredith, R. W., Westerman, M., and Springer, M. S. (2009). A phylogeny of
598 Diprotodontia (Marsupialia) based on sequences for five nuclear genes. *Molecular*
599 *Phylogenetics and Evolution* **51**, 554–571.

600 Miller, M. A., Pfeiffer, W., and Schwartz, T. (2010). Creating the CIPRES Science
601 Gateway for inference of large phylogenetic trees. In ‘Proceedings of the Gateway

602 Computing Environments Workshop (GCE), New Orleans, LA, 14-14 Nov. 2010', Pp. 1–
603 8.

604 Mitchell, K. J., Pratt, R. C., Watson, L. N., Gibb, G. C., Llamas, B., Kasper, M., Edson, J.,
605 Hopwood, B., Male, D., Armstrong, K. N., Meyer, M., Hofreiter, M., Austin, J.,
606 Donnellan, S. C., Lee, M. S. Y., Phillips, M. J., and Cooper, A. (2014). Molecular
607 phylogeny, biogeography, and habitat preference evolution of marsupials. *Molecular*
608 *Biology and Evolution* **31**, 2322–2330.

609 Nilsson, M.A., Zheng, Y., Kumar, V., Phillips, M.J., and Janke, A. (2017). Speciation
610 generates mosaic genomes in kangaroos. *Genome Biology and Evolution* **10**, 33–44.

611 Norris, C. A., and Musser, G. G. (2001). Systematic revision within *Phalanger orientalis*
612 complex (Diprotodontia, Phalangeridae): a third species of lowland gray cuscus from New
613 Guinea and Australia. *American Museum Novitates* **3356**, 1–20.

614 Nugraha, A. M. S. and Hall, R. (2018). Late Cenozoic palaeogeography of Sulawesi,
615 Indonesia. *Palaeogeography, Palaeoclimatology, Palaeoecology* **490**, 191-209. doi:
616 10.1016/j.palaeo.2017.10.033

617 O'Connor, S. (2015). Rethinking the Neolithic in Island Southeast Asia, with particular
618 reference to the archaeology of Timor-Leste and Sulawesi. *Archipel* **90**, 15–47.

619 Osborne, M. J., and Christidis, L. (2002). Molecular relationships of the cuscuses,
620 brushtail and scaly-tailed possums (Phalangerinae). *Australian Journal of Zoology* **50**,
621 135–149.

622 Phillips, M.J., and Penny, D. (2003). The root of the mammalian tree inferred from whole
623 mitochondrial genomes. *Molecular Phylogenetics and Evolution* **28**, 171–185.

624 Raterman, D., Meredith, R. W., Ruedas, L. A., and Springer, M. S. (2006). Phylogenetic
625 relationships of the cuscuses and brushtail possums (Marsupialia: Phalangeridae) using the
626 nuclear gene BRCA1. *Australian Journal of Zoology* **54**, 353–361.

627 Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget,
628 B., Liu, L., Suchard, M. A., and Huelsenbeck, J. P. (2012). MrBayes 3.2: efficient
629 Bayesian phylogenetic inference and model choice across a large model space. *Systematic*
630 *Biology* **61**, 539–542.

- Ruedas, L. A., and Morales, J. C. (2005). Evolutionary relationships among genera of
Phalangeridae (Metatheria: Diprotodontia) inferred from mitochondrial DNA. *Journal of*
Mammalogy **86**, 353–365.
- Springer, M. S., Hollar, L. J., and Burk, A. (1995). Compensatory substitutions and the
evolution of the mitochondrial 12S rRNA gene in mammals. *Molecular Biology and*
Evolution **12**, 1138–1150.
- Springer, M. S., Kirsch, J. A., Aplin, K., and Flannery, T. (1990). DNA hybridization,
cladistics, and the phylogeny of phalangerid marsupials. *Journal of Molecular Evolution*
30, 298–311.
- Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-
analysis of large phylogenies. *Bioinformatics* **30**, 1312–1313.
- Summerhayes, G. R. (2007). Island Melanesian pasts: a view from archaeology. In ‘Genes,
Language, & Culture History in the Southwest Pacific’. (Ed. Friedlaender, J.S.) pp.10-35.
(Oxford University Press: New York.)
- Summerhayes, G. R., Leavesley, M., Fairbairn, A., Mandui, H., Field, J., Ford, A., and
Fullagar, R. (2010). Human adaptation and plant use in highland New Guinea 49,000 to
44,000 years ago. *Science* **330**, 78–81.
- Thomas, O. (1888). ‘Catalogue of the Marsupialia and Monotremata in the collection of
the British Museum.’ (British Museum (Natural History): London.)
- Thomas, O. (1895). On some mammals collected by Mr. Albert Meek on Fergusson
Island, D’Entrecasteaux Group. *Novitates Zoologicae* **2**, 163–165.
- Thomas, O. (1898). Descriptions of two new cuscuses (*Phalanger*) obtained by Dr. Loria
in British New Guinea. *Annali del Museo Civico di Storia Naturale di Genova* **2^a**(19(39)),
5–8.
- Wickler, S. (2001). ‘The prehistory of Buka: a stepping stone island in the northern
Solomons. Terra Australis Vol. 16’. (Department of Archaeology and Natural History and
Centre for Archaeological Research, Australian National University: Canberra.)
- Wright, J.L., Wasef, S., Heupink, T.H., Westaway, M.C., Rasmussen, S., Pardoe, C.,
Fourmile, G.G., Young, M., Johnson, T., Slade, J., and Kennedy, R. (2018). Ancient

660 nuclear genomes enable repatriation of Indigenous human remains. *Science Advances* **4**,
661 eaau5064.

662 Zachos, J., Pagani, M., Sloan, L., Thomas, E., and Billups, K. (2001). Trends, rhythms,
663 and aberrations in global climate 65 Ma to present. *Science* **292**, 686–693.

664 Zhang, C., Stadler, T., Klopstein, S., Heath, T. A., and Ronquist, F. (2016). Total-
665 evidence dating under the fossilized birth–death process. *Systematic Biology* **65**, 228–249.

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Tables

Table 1: Currently accepted classifications of the Phalangeridae following Helgen and Jackson (2015).

Order Diprotodontia Owen, 1866
Family Phalangeridae Thomas, 1888
Subfamily Trichosurinae Flynn, 1911
<i>Trichosurus</i> Lesson, 1828
<i>Wyulda</i> Alexander, 1918
Subfamily Ailuropinae Flannery, Archer and Maynes, 1987
<i>Ailurops</i> Wagler, 1830
<i>Strigocuscus</i> Gray, 1862
Subfamily Phalangerinae Thomas, 1888
<i>Phalanger</i> Storr, 1780
<i>Spilocuscus</i> Gray, 1862

Table 2: Divergence dates (in millions of years, to 2 d.p.) for select nodes from the calibrated phylogenetic analysis compared with most recent estimates from molecular phylogenies of Phalangeridae.^a

Clade	95% HPD ^b (Ma)	Median Age (Ma)	Ruedas and Morales (2005) ^a	Rateman <i>et al.</i> (2006) ^{*a}	Meredith <i>et al.</i> (2009) ^a
Burramyidae – Phalangeridae split (= crown Phalangerioidea)	51.18 – 31.14	40.84	n/a	n/a	49.4 – 36.6
Trichosurinae (<i>Trichosurus</i> + <i>Wyulda</i>) – Ailuropinae+Phalangerinae split (= crown Phalangeridae)	35.54 – 18.57	26.74	27.3 – 24.7	36.2 – 17.55	21.7 – 13.6
Ailuropinae (<i>Ailurops</i> + <i>Strigocuscus</i>) – Phalangerinae (<i>Phalanger</i> + <i>Spilocuscus</i>) split	30.39 – 15.55	22.49	23.2 – 21.1	32.47 – 13.95	17.9 – 10.7
Crown Phalangerinae	24.42 – 11.93	17.72	16.1 – 14.6	19.43 – 6.00	11.6 – 6.2**
<i>Phalanger</i>	19.12 – 9.16	13.94	13.8 – 12.5	16.18 – 4.07	10.5 – 5.4***
<i>Phalanger orientalis</i>	4.07 – 1.13	2.37	n/a	n/a	n/a
<i>Phalanger orientalis</i> Timor samples	1.5 – 0.35	0.82	n/a	n/a	n/a

^bHPD = Highest posterior density. *date ranges are the compound ranges of both their Multidivtime and BEAST estimates. **Analysis included *Strigocuscus pelengensis*, which is absent from our study. ***Following George's (1987) classification of *P. pelengensis* in *Phalanger*, as supported by Meredith *et al.* (2009).

Figure Captions

Fig. 1: Maps showing the distribution of the Phalangeridae and sampling localities for the sequences generated in this study. A) Map of Australasia with distribution of the species from the Phalangeridae shaded in grey. Insets B and C are indicated. B) Island of Timor with collecting sites indicated by red triangles. C) Papua New Guinea and the Solomon Islands with the provinces and islands sampled highlighted and labelled (see Table S1 for location codes).

Fig. 2: Undated *ND2* phylogeny of the Phalangeridae. Nodes are coloured according to mean Bayesian posterior probabilities (BPP): black ≥ 0.91 , orange 0.80-0.91, red ≤ 0.80 . Bootstrap values (Bs) > 50 are shown on the branches. Scale bar represents 0.06 substitutions per site. Phalangerinae samples are highlighted according to sampling localities, with colours corresponding to Fig. 1C and key in top left.

Fig. 3: Time-calibrated phylogeny of the Phalangeridae. Bayesian posterior probabilities (BPP) are indicated by coloured nodes as per Fig. 2. Branch lengths are proportional to time and correspond to the scale at the base, in millions of years before present. Bars at nodes represent 95% Highest Posterior Densities (HPD) of divergence time estimates.