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 (Phalangeroidea) 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 *Ailuropus*), 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; Raterman *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; Raterman *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 (Phalangeroidea, 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.
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).

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. matanin 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 volcanic arc 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 climactic 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 ‘not-*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 Providence 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.

**Acknowledgments**

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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.
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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
   Trichosurus Lesson, 1828
   Wyulda Alexander, 1918
Subfamily Ailuropinae Flannery, Archer and Maynes, 1987
   Ailurops Wagler, 1830
   Strigocuscus Gray, 1862
Subfamily Phalangerinae Thomas, 1888
   Phalanger Storr, 1780
   Spilocuscus 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.\(^a\)

<table>
<thead>
<tr>
<th>Clade</th>
<th>95% HPD(^b) (Ma)</th>
<th>Median Age (Ma)</th>
<th>Ruedas and Morales (2005)(^a)</th>
<th>Raterman et al. (2006)**</th>
<th>Meredith et al. (2009)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burramyidae – Phalangeridae split (= crown Phalangeroidae)</td>
<td>51.18 – 31.14</td>
<td>40.84</td>
<td>n/a</td>
<td>n/a</td>
<td>49.4 – 36.6</td>
</tr>
<tr>
<td>Trichosurinae (Trichosurus + Wyulda) – Ailuropinae + Phalangerinae split (= crown Phalangeridae)</td>
<td>35.54 – 18.57</td>
<td>26.74</td>
<td>27.3 – 24.7</td>
<td>36.2 – 17.55</td>
<td>21.7 – 13.6</td>
</tr>
<tr>
<td>Crown Phalangerinae</td>
<td>24.42 – 11.93</td>
<td>17.72</td>
<td>16.1 – 14.6</td>
<td>19.43 – 6.00</td>
<td>11.6 – 6.2**</td>
</tr>
<tr>
<td>Phalanger</td>
<td>19.12 – 9.16</td>
<td>13.94</td>
<td>13.8 – 12.5</td>
<td>16.18 – 4.07</td>
<td>10.5 – 5.4***</td>
</tr>
<tr>
<td>Phalanger orientalis</td>
<td>4.07 – 1.13</td>
<td>2.37</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Phalanger orientalis Timor samples</td>
<td>1.5 – 0.35</td>
<td>0.82</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

\(^b\)HPD = 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.