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ORIGINAL ARTICLE

# Phylogeography of the pademelons (Marsupialia: Macropodidae: *Thylogale*) in New Guinea reflects both geological and climatic events during the Plio-Pleistocene

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## ABSTRACT

**Aim** Alternative hypotheses concerning genetic structuring of the widespread endemic New Guinean forest pademelons (*Thylogale*) based on current taxonomy and zoogeography (northern, southern and montane species groupings) and preliminary genetic findings (western and eastern regional groupings) are investigated using mitochondrial sequence data. We examine the relationship between the observed phylogeographical structure and known or inferred geological and historical environmental change during the late Tertiary and Quaternary.

**Location** New Guinea and associated islands.

**Methods** We used primarily museum specimen collections to sample representatives from *Thylogale* populations across New Guinea and three associated islands. Mitochondrial cytochrome *b* and control region sequence data were used to construct phylogenies and estimate the timing of population divergence.

**Results** Phylogenetic analyses indicated subdivision of pademelons into 'eastern' and 'western' regional clades. This was largely due to the genetic distinctiveness of north-eastern and eastern peninsula populations, as the 'western' clade included samples from the northern, southern and central regions of New Guinea. Two tested island groups were closely related to populations north of the Central Cordillera; low genetic differentiation of pademelon populations between north-eastern New Guinea and islands of the Bismarck Archipelago is consistent with late Pleistocene human-mediated translocations, while the Aru Islands population showed divergence consistent with cessation of gene flow in the mid Pleistocene. There was relatively limited genetic divergence between currently geographically isolated populations in subalpine and nearby mid-montane or lowland regions.

**Main conclusions** Phylogeographical structuring does not conform to zoogeographical expectations of a north/south division across the cordillera, nor to current species designations, for this generalist forest species complex. Instead, the observed genetic structuring of *Thylogale* populations has probably been influenced by geological changes and Pleistocene climatic changes, in particular the recent uplift of the north-eastern Huon Peninsula and the lowering of tree lines during glacial periods. Low sea levels during glacial maxima also allowed gene flow between the continental Aru Island group and New Guinea. More work is needed, particularly multi-taxon comparative studies, to further develop and test phylogeographical hypotheses in New Guinea.



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**Keywords**

**Biogeography, dispersal, glacial cycles, mammals, New Guinea, phylogeography, Pleistocene, Pliocene, *Thylogale*.**

## INTRODUCTION

New Guinea is a geologically young, topographically complex island with habitats ranging from lowland monsoonal savannas to subalpine grasslands and glaciated montane plateaus. This diversity of habitats has resulted in the radiation of a distinct endemic fauna, with strong biogeographical connections to Australia in the south and to Southeast Asia in the west. Significant gaps exist in our knowledge of the ecology, distribution and evolution of much of the New Guinean fauna, and the formation of the New Guinean landscape. However, our growing understanding of the influence of plate tectonics and historical climate change on habitats in this region is now allowing a much more informed analysis of emerging patterns of higher- and lower-level faunal diversity (e.g. Michaux, 1994; de Boer & Duffels, 1996; Polhemus & Polhemus, 1998; McGuigan *et al.*, 2000; Heads, 2001, 2002a,b; Osborne & Christidis, 2002; Rawlings & Donnellan, 2003; Westerman *et al.*, 2006).

Present-day New Guinea (Fig. 1) is a geological composite consisting of a stable southern lowlands region, a fold belt forming the Central Cordillera and a northern mobile belt (Pigram & Davies, 1987). Collision between the Australian and Pacific tectonic plates initiated formation of the New Guinean landmass, with uplift of the Central Cordillera to more than 4000 m a.s.l. and the accretion of numerous terranes to its north (Hall, 2002; Cloos *et al.*, 2005). The timing of formation of the present landscape is still uncertain, but it is generally thought that uplift of the central mountains above sea level has been occurring since at least the Miocene (Hall, 2002; Cloos *et al.*, 2005). Emergence above sea level of the many terranes north and north-east of the cordillera may have occurred as early as the Late Miocene in western New Guinea (Cooper & Taylor, 1987), but as recently as the Plio-Pleistocene in eastern New Guinea (Abbott *et al.*, 1994). The southern lowlands have remained a geologically stable region of the Australian plate during the Tertiary, and therefore the south-eastern TransFly savanna has zoogeographical affinities to the northern

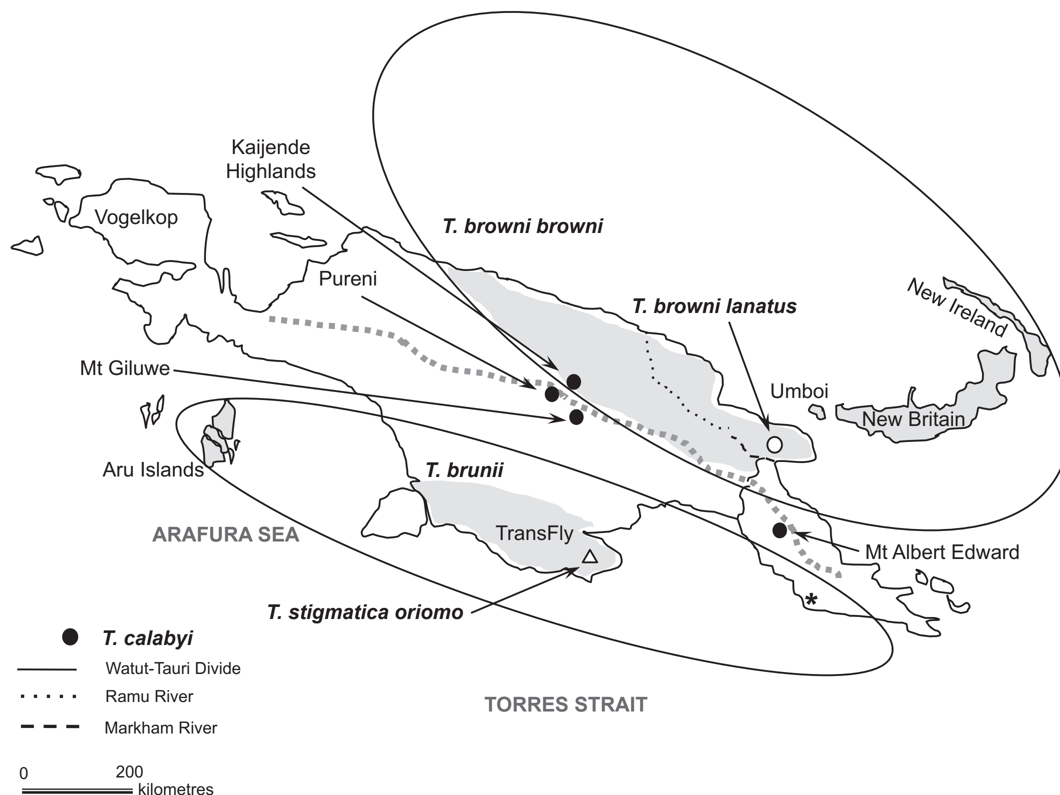


**Figure 1** Map of New Guinea and surrounding islands showing distributions of *Thylogale* species [adapted from Flannery (1995) with more recently surveyed locations for *T. calabyi* (Helgen, 2009) added]. The asterisk indicates the location for the recently extinct population of *T. brunii*. The Central Cordillera of New Guinea is represented by a grey dotted line.


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monsoonal tropics of Australia (Flannery, 1995). In northern New Guinea, isolated mountain ranges form disjunct biogeographical units (e.g. Flannery, 1995; Beehler, 2007) and the lowlands differ from those of the south, lacking the open savanna habitats and strong seasonality of rainfall that occur in that region. The Central Cordillera has extreme elevational variation in climate and habitat, as well as zoogeographical subdivisions from east to west (Beehler, 2007; Helgen, 2007).

Under present climatic conditions, a subalpine zone of grass- and shrublands exists between 3000 and 4000 m a.s.l., with montane broadleaf forests extending from this grassland interface down to 1000 m a.s.l. (Hope, 1976; Flannery, 1995). Closed forests also extend to the coast in the north and south, except where mixed savanna woodlands occur in large areas of the southern lowlands (Paijmans, 1976). Cyclical global cooling during the Pleistocene resulted in extensive glaciation of the highest regions of the Central Cordillera and some outlying ranges (Löffler, 1970, 1971; Hope & Peterson, 1976). Tree lines during glacial maxima are estimated to have lowered by at least 1200 to 1500 m, to approximately 2200 m a.s.l. (Flenley, 1979, 1984; Hope *et al.*, 1983), while mid-montane and lowland forests may have experienced more changes related to floristic composition than to extent and structure (Hope & Tulip, 1994; Hope *et al.*, 2004). Nevertheless, southern areas of savanna probably increased, with tropical lowland forest contracting further inland towards the southern foothills of the Central Cordillera (Nix & Kalma, 1972; van der Kaars, 1991). Lower sea levels during much of the Pleistocene resulted in long intervals of terrestrial connectivity with Australia (Voris, 2000); a plain extended from the Aru Islands in the western extreme of the Arafura Shelf to the Torres Strait in the east at between 75 and 120 m below present levels. The land-bridge environment is likely to have been a continuation of southern savanna habitats, open woodlands and watercourses supporting bordering gallery forests (Nix & Kalma, 1972; Rowe, 2007).

The impact of these vegetational changes on species distributions and current patterns of genetic variation within New Guinea is still poorly understood. Phylogeographical and phylogenetic studies to date indicate that the development of patterns within and among species depends on ecological associations, elevational range and the antiquity of the species within New Guinea (e.g. Norman *et al.*, 2002; Wüster *et al.*, 2005). A pattern of genetic divergence between northern and southern forms has been noted in a number of low to mid-montane taxa (McGuigan *et al.*, 2000; Dumbacher & Fleischer, 2001; Rawlings & Donnellan, 2003; Zwiers *et al.*, 2008) consistent with zoogeographical division across the cordillera (Flannery, 1995; Aplin, 1998). In general, divergence between these forms appears to have occurred during the Pliocene, implying an allopatric process following central montane orogenesis. A study of the montane logrunner (*Orthonyx* spp.), however, found evidence for an east–west pattern of genetic structure (Joseph *et al.*, 2001), consistent with the apparent trend for montane avian species replacement from east to west following the central mountain range (Beehler, 2007). Murphy *et al.* (2007) also found an east–west division in the lowland palm cockatoos (*Probosciger aterrimus*), although this was largely due to late Pleistocene divergence of populations in the distinct biogeographical region of the Vogelkop in north-western West Papua. In contrast, Wüster *et al.* (2005) found identical mitochondrial DNA haplotypes for lowland populations of taipan (*Oxyuranus scutellatus*) from Merauke in south-eastern West Papua, Central Province in the eastern peninsula of Papua New Guinea, and northern Australia.

Due to the limited range of taxa and regions studied, and a still incomplete geological history, general biogeographical hypotheses for New Guinea have not yet been clearly developed. In this study we examined the evolutionary history of the forest edge-dwelling New Guinean pademelons (Marsupialia: Macropodidae: *Thylogale*), which are medium-sized (4.5–7 kg) wallabies. This is the most comprehensive phylogeographical study of a mammal with a broad geographical and elevational distribution conducted in New Guinea to date. A taxonomic revision of the *Thylogale* endemic to New Guinea (Flannery, 1992) discussed three geographically isolated groups concordant with the broad northern, southern and alpine cordilleran zoogeographical regions (Fig. 1). These groups were distinguished by significant differences in cranial and dental measurements and non-metric differences in skull and tooth morphology, as well as variation in dorsal and ventral fur coloration, hip, tail and neck markings, and internal ear and tail fur length. Three species were recognized: *Thylogale brunii* (dusky pademelon), a lowland species found where monsoonal forest and grassland adjoin in the Aru Islands and south of the central New Guinean Cordillera (including the Port Moresby region where it is now considered extinct); *Thylogale calabyi* (Calaby's pademelon), with extant populations known from only four subalpine regions of the Central Cordillera (Flannery, 1992; Helgen, 2009) where it inhabits the forest/grassland interface above approximately 2800 m a.s.l.; and *Thylogale browni* (New Guinea pademelon). *Thylogale browni* was further divided into *Thylogale browni browni*, a lowland to mid-montane subspecies found predominantly north of the Central Cordillera and on some islands of the Bismarck Archipelago, and a montane subspecies, *Thylogale browni lanatus*, limited to the subalpine grassland/forest interface in the Saruwaged and Cromwell ranges on the north-eastern Huon Peninsula. While these subspecies have more recently been considered as full species, *Thylogale browni* and *Thylogale lanatus* (Groves, 2005), Flannery (1992) considered them to be only weakly differentiated based on morphometric comparisons and variation in pelage length and texture. In this study we base our nomenclature on the taxonomic revision of Flannery (1992). A fourth species, *Thylogale stigmatica*, is shared with Australia: the New Guinean subspecies, *Thylogale stigmatica oriomo*, occurs only in the TransFly region of the southern lowlands (Flannery, 1995) and is not considered in this study.

An earlier phylogenetic study of the complete *Thylogale* genus using partial mitochondrial 12S rRNA and cytochrome *b* genes, and a nuclear omega-globin intron (Macqueen *et al.*, 2010) inferred east–west genetic structuring of pademelon




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populations in New Guinea, rather than north–south structuring of populations across the cordillera as expected from current species designations and zoogeographical regions. Here, we expand our sampling of the New Guinean taxa and use the more rapidly evolving mitochondrial control region in addition to cytochrome *b* data to further investigate the genetic structuring of endemic *Thylogale* species. We relate the observed phylogeographical structure and estimates of the timing of population divergence to known or inferred geological and habitat change in New Guinea during the Plio-Pleistocene.

## MATERIALS AND METHODS

### Sampling and laboratory protocols

Forty-eight individuals, of which 18 were included in a previous phylogenetic study (Macqueen *et al.*, 2010), were sampled from regions across New Guinea and two associated island groups (the Aru Islands and New Britain and Umboi Islands in the Bismarck Archipelago). Samples were obtained from representatives of all *Thylogale* species (Flannery, 1992; Groves, 2005). Location and collection details for all samples are listed in Appendix S1 of the Supporting Information. As only eight of the samples used in this study were from specimens examined by Flannery (1992), species designations for all samples were based on the specimen collection location and species distributions and elevational ranges described in Flannery (1992, 1995).

Tissue for DNA extraction was taken primarily from preserved museum specimens (bone fragments, whole teeth and dried muscle or cartilage) (*T. browni*, *T. brunii*, *T. calabyi*), but also from frozen tissue (*T. browni*, *T. calabyi*) and fresh tissue (*T. brunii*). DNA extractions from fresh or frozen tissue were carried out as described in Macqueen *et al.* (2009). DNA extractions from museum specimens were conducted at the Australian Centre for Ancient DNA in a building separate from the post-PCR laboratory. All sample extractions and polymerase chain reaction (PCR) amplifications included negative controls to test for contamination. DNA extractions were carried out using Qiagen DNeasy Blood and Tissue Kits (Qiagen, Doncaster, Australia) following initial sample preparation. Bone and tooth samples were first ground to a fine powder and the powder or soft tissue fragments were then incubated at room temperature for 24 h in 1–1.8 mL 0.5 M ethylenediaminetetraacetic acid (EDTA). All samples were incubated in 380 μL of the supplied Qiagen buffer with 20 μL proteinase K (20 mg $mL^{-1}$) at 55 °C for a further 24 h. DNA extraction followed the manufacturer's instructions except for the addition of 2 μL carrier RNA with the supplied AL buffer.

Reactions for PCR amplification were performed using the primer combinations listed in Appendix S2. Sequences were obtained from fresh or frozen tissue samples using previously published primers (Fumagalli *et al.*, 1997; Bulazel *et al.*, 2007; Macqueen *et al.*, 2010). Due to the poor quality of the DNA in museum specimens, internal primer pairs specific to the New Guinean *Thylogale* control region (CR) and cytochrome *b* (cyt *b*) gene were designed for amplification of short DNA fragments (fewer than 200 base pairs). PCR reactions and cycle conditions for amplification of the mitochondrial CR from tissue samples are described in Macqueen *et al.* (2009) and in Macqueen *et al.* (2010) for amplification of the cyt *b* gene. PCR reactions for amplification of both mitochondrial regions from museum specimen samples were carried out in 25 μL volumes using 0.5 U Platinum Taq High Fidelity DNA Polymerase (Invitrogen, Mulgrave, Australia), 0.4 μM forward and reverse primer, 25 μg rabbit serum albumin and 2 μL of the DNA template. PCR cycle conditions varied depending on the DNA fragment and species (conditions are described in Appendix S2). All PCR products were purified using ExoSAP-IT (USB, Cleveland, OH, USA). Primers used in PCR amplification were also used in sequencing reactions with the BigDye Terminator v3.1 (Applied Biosystems, Melbourne, Australia) sequencing kit. Products were sequenced in both forward and reverse directions on an Applied Biosystems/Hitachi 3130xl Genetic Analyzer.

### Sequence alignment and phylogenetic analysis

Sequences were edited and assembled manually in MEGA v. 4.0 (Tamura *et al.*, 2007) and haplotypes were aligned using Clustal W (Thompson *et al.*, 1994). Cyt *b* sequences were translated to amino acids to verify the absence of premature stop codons and CR sequence alignments were refined by eye, with removal of a short ambiguous region. Following alignment, the full datasets were trimmed to remove sections of sequence only amplified from tissue samples. Due to the fact that some sequences had remaining missing data, the number of different haplotypes was determined manually in MEGA. Numbers of polymorphic sites, overall nucleotide diversity and net pairwise genetic distances between clades identified in the phylogenetic analysis (see Results) were estimated in MEGA using the Tamura–Nei (TrN) distance measure (Tamura & Nei, 1993). Standard errors for pairwise distances were assessed with 1000 bootstrap replicates.

Phylogenetic trees were estimated using a Bayesian approach in MrBayes v. 3.1.2 (Ronquist & Huelsenbeck, 2003) and the maximum parsimony (MP) approach in PAUP* v. 4.0b10 (Swofford, 2002). Gene regions were analysed both separately and, as none of the well-supported clades inferred from individual gene trees were conflicting, also as a combined dataset. The combined dataset only included haplotypes from individuals sequenced for both gene regions. Two outgroup sequences from the species complex *T. stigmatica* (*Thylogale stigmatica wilcoxi* and *Thylogale stigmatica stigmatica*) were included in all analyses, as this complex was found to be the sister group to the New Guinean complex (Macqueen *et al.*, 2010). MP analyses were conducted using a heuristic search with the tree bisection–reconnection branch-swapping algorithm and 100 random addition sequence replicates. Support for nodes was assessed using 1000 bootstrap resamplings of the


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data (Felsenstein, 1985) with 10 random addition sequence replicates each.

Phylogenetic analyses in MrBayes were conducted using models for patterns of DNA substitution selected in jModelTest v. 1.1 (Guindon & Gascuel, 2003; Posada, 2008). Models for the three coding positions of the cyt *b* dataset and the single partition CR dataset were selected using the Akaike information criterion corrected for small sample size ($AIC_c$) as recommended by Posada & Buckley (2004). Six independent analyses were conducted for the individual and concatenated datasets with $10^7$ generations per run and a sampling frequency of 1000. All runs started with a randomly chosen tree, and four Markov chains were employed to improve Markov chain Monte Carlo (MCMC) sampling using the default heating scheme. Analyses were conducted using the Computational Biology Service Unit (CBSU) at Cornell University through the web-based interface (http://cbsuapps.tc.cornell.edu/mrbayes.aspx). Adequacy of chain mixing was assessed by examining estimated sample size (ESS) values in Tracer v. 1.4.1 (Rambaut & Drummond, 2007) and chain swap acceptance rates. Convergence for each run was assessed primarily by examining plots of log-likelihood scores in Tracer. Two convergence diagnostics, potential scale reduction factors (Gelman & Rubin, 1992) and the average standard deviation of split frequencies, were also used to determine if the number of generations for each run was sufficient. An adequate relative burn-in fraction was determined for each run and this fraction of the sampled generations was discarded. Combined posterior probabilities from the six runs were then used to estimate a 50% majority-rule consensus tree.

### Timing of population divergence

We estimated divergence times for major phylogenetic clades (time to most recent common ancestor, TMRCA) for both the cyt *b* and CR datasets using the Bayesian approach implemented in beast v. 1.5.2 (Drummond & Rambaut, 2007). Analysis of the cyt *b* dataset was conducted using the SRD06 model. This model reduces the number of parameters to be estimated and is thought to more adequately model nucleotide evolution for protein-coding data (e.g. Shapiro *et al.*, 2006). The model of evolution for the CR dataset was selected in jModelTest as described above. Both datasets were analysed with a relaxed clock model, assuming uncorrelated substitution rates for each lineage drawn from a lognormal distribution. As adequate fossil or palaeoecological calibration dates for nodes were not available, we used standard estimates of mammalian nucleotide substitution rates to set an initial value for the parameter *ucld.mean* (mean of the branch rates) with a uniform prior for each dataset. For the CR, we used a substitution rate of 0.05 per lineage $Myr^{-1}$ with a lower boundary of 0.010 and an upper boundary of 0.10 based on the range of previously published rate estimates for this genetic region (Brown *et al.*, 1979; Vigilant *et al.*, 1989; Stewart & Baker, 1994; Birungi & Arctander, 2000; Van Tuinen *et al.*, 2008). For the cyt *b* gene we used a substitution rate of 0.01 per lineage $Myr^{-1}$ (Brown *et al.*, 1979; Pesole *et al.*, 1999) with a lower boundary of 0.005 and an upper boundary of 0.015. Remaining parameters were set to default priors. Four independent analyses were run for each dataset; each analysis started with a random tree and was run for $3 \times 10^7$ generations with sampling every 3000 generations. Adequacy of chain mixing and MCMC chain convergence were assessed by the examination of ESS values and plots of the posterior probabilities for each parameter in Tracer. Log files for each run were then combined using LogCombiner v. 1.5.3 (Drummond & Rambaut, 2007) to produce summary estimates of all parameters.

## RESULTS

### Sequence variation and phylogenetic analysis

Partial CR and partial cyt *b* sequences were amplified from 46 *Thylogale* individuals. CR sequence could not be amplified from two samples, C16 and A10 (*T. browni browni*), or for a single DNA fragment from UP3564 (*T. brunii*), and cyt *b* sequences were not amplified from samples THBR2 and THBR5 (*T. brunii*). No premature stop codons or heterozygous sites were observed in any cyt *b* sequences. The use of species-specific primers (e.g. den Tex *et al.*, 2010) and the higher levels of divergence observed among mitochondrial DNA (mtDNA) haplotypes (see results below) relative to those among nuclear intron haplotypes amplified from New Guinean pademelons in a previous study (Macqueen *et al.*, 2010) limits the possibility that nuclear copies of mitochondrial genes were amplified in this study. Following concatenation of the sequence fragments obtained from museum specimens and trimming of missing data, 581 aligned sites for the CR and 396 base pairs of the cyt *b* gene were obtained. The combined cyt *b* and CR dataset consisted of 977 aligned sites. Nucleotide compositions and sequence variation are shown in Appendix S2. All sequences used in this study have been deposited in GenBank under accession numbers JF514560–JF514607, JF694009–JF694036, HQ284004, HQ284007 and HQ283980–HQ283997.

Overall nucleotide diversity was high (CR, 0.115 ± SE 0.009; cyt *b*, 0.040 ± SE 0.006). Haplotype diversity was not estimated for each geographical region due to the wide spread of sampled locations, but the final CR and cyt *b* datasets included 34 and 21 haplotypes, respectively. Only one haplotype was shared between sampled locations; all *T. browni lanatus* individuals in the Huon Peninsula region shared an identical cyt *b* haplotype, and this haplotype was also shared with mid-montane individuals of the subspecies *T. browni browni*.

Bayesian phylogenies for the independent and partitioned mtDNA analyses were inferred using models selected in jModelTest (Appendix S2). ESS values (CR, > 2000; cyt *b*, > 600; combined, > 1500), potential scale reduction factor (PSRF) values (1.00 for all parameters), average standard deviation of split frequencies (CR, < 0.003; cyt *b*, < 0.004; combined, < 0.003), and trace plots of posterior likelihood




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estimates indicated adequate chain mixing and convergence for all runs and all datasets with a default burn-in of 10%. Independent gene phylogenies and the combined phylogeny (50% majority-rule consensus trees) are shown in Fig. 2. Maximum parsimony analyses of individual and combined datasets produced phylogenies with similar clades and topologies to those produced using the Bayesian approach. The MP phylogeny estimated using combined cyt *b* and control region data is shown in Appendix S3.

Gene trees inferred from the CR and cyt *b* data and the combined dataset produced clades that were generally consistent with geographical regions (Fig. 2a–c). Well-supported clades (posterior probability 1.0, bootstrap support 100%) estimated using the combined dataset included Mount Giluwe and TransFly, Northern New Guinea, Northwest New Guinea, Aru Islands, Cromwell Range and Central Province. The *T. browni browni* haplotype from Haia, on the southern edge of the cordillera, was included in a clade with the remaining northern New Guinea haplotypes. A close relationship was inferred between the New Britain and Umboi Island haplotypes and those from the nearby 'mainland' Wanuma haplotypes. The single haplotypes from Mount Albert Edward and Varirata (Central Province), and from Amanab and the Foja Mountains (Northwest New Guinea), were resolved as divergent phylogroups. A single northern New Guinea haplotype from Maprik (haplotype TB19, *T. browni browni*), not successfully amplified for the CR, was in an unresolved position. The phylogeny based on the combined dataset produced well-supported 'Eastern' and 'Western' clades (Figs 2c & 3). The Eastern clade included individuals from Central Province, the Huon Peninsula and Northeast New Guinea. The Western clade was made up of three main clades including Northwest New Guinea, Northern New Guinea/Aru Islands and TransFly/Mount Giluwe.

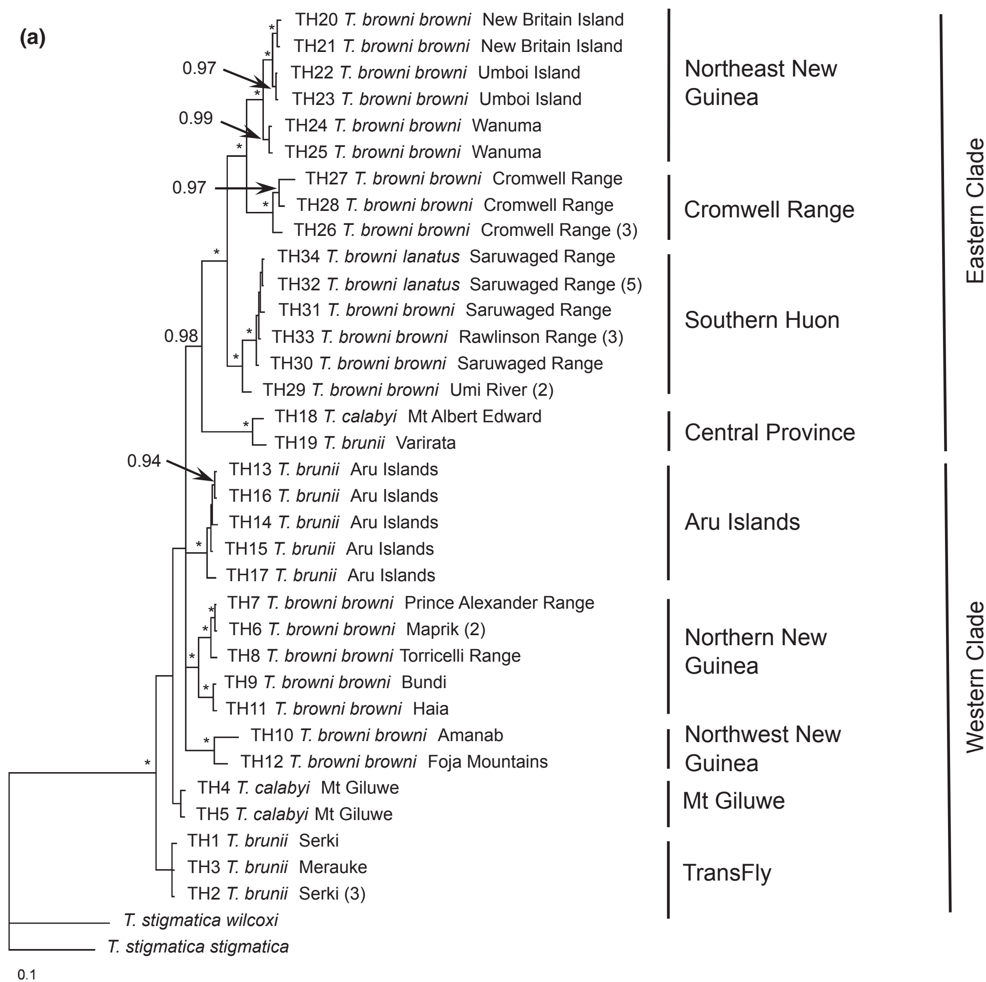


**Figure 2** Bayesian 50% majority-rule consensus tree estimated for New Guinean *Thylogale* from (a) control region data, $n = 48$, (b) cytochrome *b* data, $n = 48$, and (c) combined cytochrome *b* and control region data, $n = 36$. Major clades mentioned in the text are labelled and numbers after the species name and location indicate the number of individuals sharing the same haplotype. Asterisks indicate a posterior probability value of 1.00, and only posterior probability values greater than 0.9 are shown. Letters A to G at nodes in panel (c) correspond to time to most recent common ancestor (TMRCA) estimates listed in Table 2. Coloured symbols correspond to locations shown in Fig. 3.


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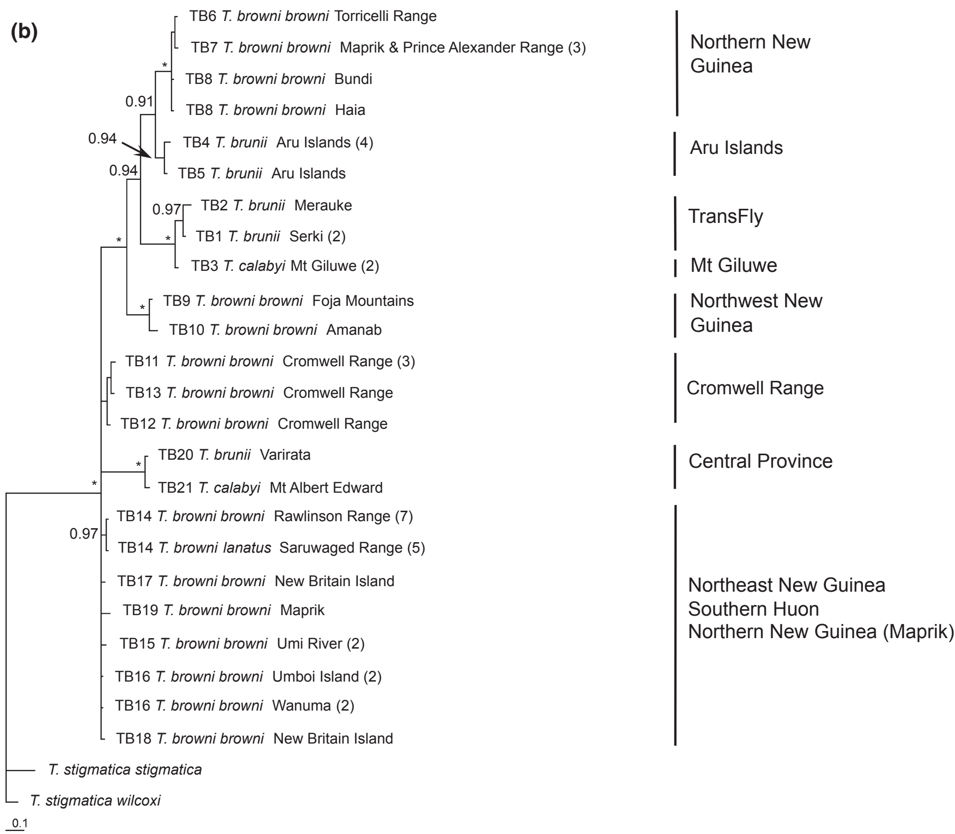


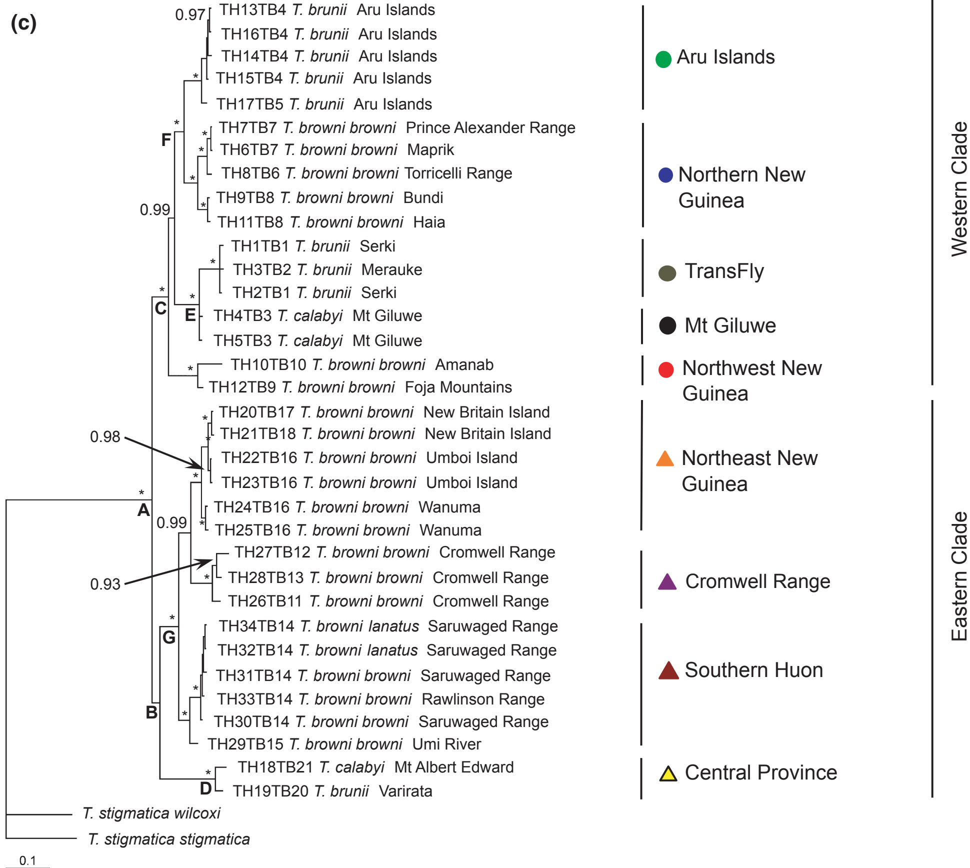


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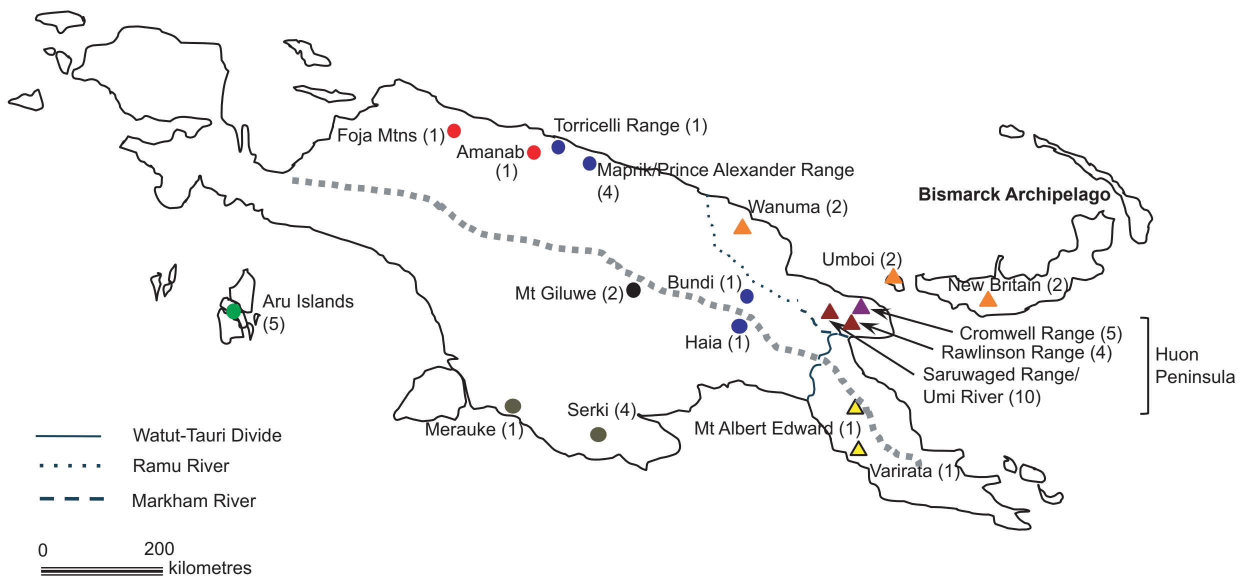


**Figure 3** Map of New Guinea showing sample locations for *Thylogale* populations with the number of samples obtained from each location given in parentheses. The Central Cordillera is represented by a grey dotted line and coloured symbols correspond to subclades in Fig. 2(c).

Net pairwise genetic distances between phylogenetic clades, and genetic distances within clades, are shown in Table 1. Genetic distances between subalpine *T. calabyi* populations and lowland *T. brunii* populations (Mount Albert Edward/Varirata and Mount Giluwe/TransFly) were relatively low (CR 4.4–5.3%; cyt *b* 0.5–0.9%). Generally, there were lower pairwise distances among the Huon Peninsula (Southern Huon and Cromwell Range) and Northeast New Guinea sites than between these and all other regions. CR data indicated that Northern New Guinea was relatively more divergent from the Huon Peninsula and Northeast New Guinea sites than from Northwest New Guinea, and that the Cromwell Range and Northeast New Guinea sites appeared generally divergent from all sites. In contrast, the cyt *b* data indicated the Central Province region was the most highly divergent site, although showing least divergence from Northeast New Guinea and sites on the Huon Peninsula. CR pairwise distances indicated that the population from the Aru Islands was least genetically divergent from the Northwestern, Northern, TransFly and Mount Giluwe sites, although cyt *b* pairwise distances showed relatively high divergence from the Northwest and TransFly sites.

## Timing of population divergence

Estimates of TMRCA for the major phylogroups (nodes labelled in Fig. 2c) are shown in Table 2. ESS values for each run were considered adequate (CR, > 1700; cyt *b*, > 2000) and trace plots of posterior probabilities for each run indicated chain convergence. Values for the *ucld.stdev* parameter for combined runs of the CR and cyt *b* data were 0.34 [95% highest posterior density (HPD) interval is 0–0.64] and 0.84 (95% HPD interval 0.22–1.49), respectively, indicating minor rate heterogeneity among branches in the phylogenies. There was relatively good agreement between datasets despite the lack of common calibration points based on fossil or palaeoecological data. The most disparate estimate was for the TMRCA for all sampled New Guinean *Thylogale* populations (node A), estimated from cyt *b* data as 4.51 Ma, and from CR data as 2.51 Ma. However, 95% HPD intervals were large and overlapping (cyt *b*, 1.865–8.311; CR, 0.987–5.790). Estimates for node C (TMRCA for west and north/central New Guinea) were also relatively different, with a cyt *b* estimate of 3.370 Ma and CR estimate of 1.646 Ma. Again, HPD intervals were overlapping (cyt *b*, 1.348–6.217; CR, 0.611–3.783). The more recent TMRCA estimates (nodes D–G) were similar between datasets and indicated divergence of populations during the Pleistocene.

# DISCUSSION

## Phylogenetic relationships among New Guinean *Thylogale*

Phylogenetic structuring of *Thylogale* populations was congruent with broad geographical regions in New Guinea. The most recently described subalpine *T. calabyi* populations showed limited divergence from lowland *T. brunii* populations. Differentiation of the subalpine and mid-montane *T. browni* populations on the Huon Peninsula was also limited; all individuals in the Rawlinson (*T. browni browni*) and Saruwaged Range (*T. browni lanatus*) populations shared an identical cyt *b* haplotype. The close relationship inferred from morphological analyses of individuals from northern New Guinea and nearby islands of the Bismarck Archipelago (Flannery, 1992) is, however, consistent with our data. The Northeast New Guinea clade included individuals from Wanuma, Umboi and New Britain islands, with intra-clade


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**Table 1** Net pairwise genetic distances between phylogenetic clades (± SE) of endemic *Thylogale* in New Guinea estimated using the Tamura–Nei (TrN) distance measure for the mitochondrial control region (below diagonal, $n = 46$) and cytochrome *b* (in italics above diagonal, $n = 46$) data. Average genetic distances within regions shown in bold (distances for cytochrome *b* in italics).

| Clade | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | TransFly | **0.007** (**0.002**)<br>***0.005*** (***0.003***) | *0.064* (*0.013*) | *0.057* (*0.011*) | *0.059* (*0.013*) | *0.052* (*0.012*) | *0.059* (*0.013*) | *0.056* (*0.013*) | *0.087* (*0.016*) | *0.009* (*0.005*) |
| 2 | Aru Islands | 0.078 (0.012) | **0.017** (**0.004**)<br>***0.003*** (***0.002***) | *0.019* (*0.006*) | *0.053* (*0.012*) | *0.054* (*0.012*) | *0.058* (*0.013*) | *0.055* (*0.012*) | *0.077* (*0.015*) | *0.053* (*0.012*) |
| 3 | Northern New Guinea | 0.082 (0.012) | 0.068 (0.011) | **0.039** (**0.007**)<br>***0.020*** (***0.004***) | *0.041* (*0.009*) | *0.046* (*0.010*) | *0.045* (*0.010*) | *0.045* (*0.010*) | *0.061* (*0.013*) | *0.046* (*0.010*) |
| 4 | Northeast New Guinea | 0.124 (0.016) | 0.132 (0.017) | 0.131 (0.018) | **0.028** (**0.005**)<br>***0.002*** (***0.001***) | *0.042* (*0.011*) | *0.004* (*0.003*) | *0.007* (*0.004*) | *0.040* (*0.011*) | *0.051* (*0.012*) |
| 5 | Northwest New Guinea | 0.075 (0.012) | 0.068 (0.012) | 0.066 (0.012) | 0.109 (0.016) | **0.067** (**0.011**)<br>***0.013*** (***0.006***) | *0.044* (*0.011*) | *0.043* (*0.011*) | *0.062* (*0.014*) | *0.049* (*0.011*) |
| 6 | Southern Huon | 0.111 (0.014) | 0.115 (0.015) | 0.104 (0.015) | 0.087 (0.013) | 0.103 (0.016) | **0.016** (**0.003**)<br>***0.002*** (***0.001***) | *0.012* (*0.005*) | *0.045* (*0.011*) | *0.050* (*0.012*) |
| 7 | Cromwell Range | 0.126 (0.015) | 0.132 (0.016) | 0.137 (0.017) | 0.071 (0.011) | 0.129 (0.017) | 0.106 (0.014) | **0.033** (**0.006**)<br>***0.004*** (***0.002***) | *0.045* (*0.011*) | *0.056* (*0.013*) |
| 8 | Central Province | 0.103 (0.016) | 0.119 (0.018) | 0.090 (0.015) | 0.131 (0.018) | 0.121 (0.019) | 0.116 (0.017) | 0.130 (0.019) | **0.053** (**0.012**)<br>***0.005*** (***0.004***) | *0.083* (*0.016*) |
| 9 | Mt Giluwe | 0.044 (0.009) | 0.070 (0.012) | 0.070 (0.012) | 0.118 (0.016) | 0.067 (0.012) | 0.101 (0.014) | 0.111 (0.015) | 0.093 (0.016) | **0.014** (**0.005**)<br>***0.000*** (***0.000***) |


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**Table 2** Time to most recent common ancestor (TMRCA; millions of years ago, Ma) and 95% highest posterior density (HPD) interval for New Guinean *Thylogale* clades. Estimates were inferred using the relaxed clock model in BEAST based on standard mammalian substitution rates for the mitochondrial control region ($n$ = 46) and cytochrome $b$ gene ($n$ = 46). Letters correspond to nodes labelled in Fig. 2(c).

| | Cytochrome *b* | | Control region | |
|---|---|---|---|---|
| Clade | TMRCA (Ma) | 95% HPD interval | TMRCA (Ma) | 95% HPD interval |
| A | 4.511 | 1.865–8.311 | 2.510 | 0.987–5.790 |
| B | 3.604 | 1.130–7.068 | 2.352 | 0.908–5.449 |
| C | 3.370 | 1.348–6.217 | 1.646 | 0.611–3.783 |
| D | 0.546 | 0.013–1.482 | 0.506 | 0.116–1.224 |
| E | 1.014 | 0.187–2.157 | 0.812 | 0.236–1.905 |
| F | 1.600 | 0.479–3.158 | 1.437 | 0.489–3.322 |
| G | 1.672 | 0.414–3.569 | 1.668 | 0.638–3.852 |

genetic distances of approximately 2.8% and 0.2% for CR and cyt *b* data, respectively. Islands of the Bismarck Archipelago (i.e. New Britain and Umboi) have never been connected by land to New Guinea, but form a separate volcanic arc system (Hall, 2002). It is likely that the island populations originated from human-mediated introductions in the late Pleistocene to Holocene, as previously inferred from archaeological and ethnozoological evidence (Heinsohn, 2001, 2005; Spriggs *et al.*, 2005).

Genetic divergence among populations based on CR data was generally higher than that found among populations and subspecies of Australian macropods (e.g. Pope *et al.*, 1996; Clegg *et al.*, 1998; Le Page *et al.*, 2000; Browning *et al.*, 2001; Macqueen *et al.*, 2009), but was similar to estimates of interspecific divergence in the rock wallaby (*Petrogale*) complex (Eldridge *et al.*, 2001) and the bettongs (*Bettongia*) (Pope *et al.*, 2000). However, genetic distances in our study are biased because we sequenced only the highly polymorphic fragments of the CR and cyt *b* gene in museum specimens. Significant structuring and high sequence divergence among populations and subspecies has been found for a number of New Guinean marsupials (e.g. Bowyer *et al.*, 2003; Westerman *et al.*, 2006; Malekian *et al.*, 2010), reflecting the strong influence of topographic and ecological complexity on gene flow. Nevertheless, while this study and our previous phylogenetic analysis of the genus have highlighted significant variation among populations, specific and subspecific status of the endemic New Guinean *Thylogale* cannot be resolved without further, more comprehensive, sampling for morphological analyses and genetic analyses using nuclear DNA.

## Phylogeographical structure and timing of population divergence

Phylogenetic analyses using both CR and cyt *b* data support the hypothesis of a division of New Guinean populations into eastern and western phylogroups, with the possible exception of a single individual from Maprik in the north whose phylogenetic relationship based on cyt *b* data could not be resolved. Divergence between the two major New Guinean clades was estimated to have occurred in the mid to late Pliocene following colonization of New Guinea in the late Miocene to early Pliocene (Macqueen *et al.*, 2010). Although average estimates for the divergence of these two clades ranged from 2.5 to 4.5 Ma, they are consistent with those from 12S rRNA data (*c.* 3.3 Ma) using a sequence divergence rate of 0.58% and with those from cyt *b* data (*c.* 3.8 Ma) using the same substitution rate as in this study (Macqueen *et al.*, 2010). This relatively deep phylogenetic split is concordant with the location of the Ramu-Markham and Watut-Tauri valleys, separating the biogeographical regions of the eastern Papuan Peninsula (including Central Province) and north-eastern New Guinea (including the Huon Peninsula) from the central cordilleran and northern ranges (Flannery, 1995; Heads, 2001).

### *The Eastern clade*

The timing of divergence between the Central Province and north-eastern populations is broadly consistent with what is known of the geological and historical climate of the region. The Finisterre terrane emerged above sea level around 1.3 Ma to form the landscape of the present peninsula (Abbott *et al.*, 1994). The Ramu–Markham rivers now mark the collision suture between this terrane and older terranes previously accreted to the Australian plate. It is likely that subsequent fragmentation of forest habitat in these valleys during glacial periods limited opportunities for dispersal of forest species from surrounding regions. Previous faunal surveys have noted that the mountain ranges of the Huon Peninsula support only one endemic mammal (Aplin, 1998) and lack some of the common species found elsewhere in New Guinea (van Deusen, 1966; Flannery, 1995). Given their adaptation to forest edge and disturbed habitats, it is not surprising that pademelons were successful migrants to the region. Dispersal from the south-east, rather than from the north-west, may be a result of the continued submergence of much of the northern Ramu and Sepik valleys during the Pleistocene (Chappell, 1993). For example, the individual from Bundi grouped with the Northern New Guinea clade rather than with the Northeast and Huon populations, which are geographically much closer but north of the Ramu Valley. The Ramu and Markham valleys are currently dominated by grasslands, and are likely also to present a strong current barrier to the dispersal of forest species. Nevertheless, the


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limited divergence between the Huon Peninsula haplotypes and a single Northern cyt *b* haplotype from Maprik may indicate recent dispersal into the northern region, or retention of an ancestral haplotype.

Limited genetic divergence between subalpine and lower montane populations on the Huon Peninsula is consistent with recent development of the landscape and the evolution of habitats during a period of dramatic climatic instability. Rapid orogenesis (Abbott *et al.*, 1997) and glacial cycles of 40,000 to 100,000 years' duration (Pisias & Moore, 1981; Schmieder *et al.*, 2000) would have delayed the development of stable habitat associations, resulting in repeated shuffling of the distributions of colonizing species. Evidence of significant glaciation on the Saruwaged Range during the late Pleistocene (Löffler, 1971) suggests that downward shifts in the newly evolved subalpine and upper montane habitats must have occurred. Significant morphological differentiation but limited mtDNA genetic divergence between populations of *T. browni lanatus* and *T. browni browni* may, therefore, reflect incomplete lineage sorting due to the recent development and colonization of subalpine habitats or mtDNA introgression due to admixture of previously geographically isolated populations during the climate-driven elevational shifts in habitat of the Last Glacial Maximum (LGM). It is notable, however, that individuals sampled from the Cromwell Range formed a distinct clade, indicating that at least this highland population may have been isolated from lowland and adjacent Saruwaged and Rawlinson Range populations during the mid to late Pleistocene. Thus, the effects of recent mountain-building on the development of species diversity in the Huon Peninsula are likely to be complex and this region provides a natural laboratory for studying the evolution of morphological and ecological adaptation in species along an elevational gradient. In addition, further genetic studies of regional endemics will provide an opportunity to test evolutionary hypotheses of species diversification in montane habitats.

### *The Central Cordillera*

It is clear that the geographically distant montane populations sampled for this study have not shared a recent common ancestor. During Pleistocene glacial maxima, it has been hypothesized that subalpine habitat was greatly increased in extent, with mixed shrublands and grasslands extending to at least 1000 m below the present tree line (Hope & Hope, 1976; Hope *et al.*, 2004). Given the limited genetic divergence observed between subalpine *T. calabyi* populations and lowland *T. brunii* populations, our data support a scenario of vertical connectivity between pademelon populations during the Pleistocene, rather than increased gene flow along the Central Cordillera. Mount Albert Edward is in the biogeographical zone formed by accretion of the East Papuan composite terrane to the margin of the Australian craton in the mid Miocene and this zone is bounded to the west by the Watut and Tauri valleys (Pigram & Davies, 1987; Heads, 2001). Disjunctions across this Watut–Tauri Divide have been noted for many central New Guinean montane taxa (e.g. Heads, 2001) and Flannery (1995) also noted a high degree of endemism among some mammalian genera to the east of this region. While it is possible that historical connectivity between more proximate central cordilleran pademelon populations may have been facilitated by the expansion of subalpine grasslands during glacial maxima, the apparent lack of connectivity between Mount Albert Edward and the central (Mount Giluwe) montane region might be explained by the persistence of significant areas of closed forest in valleys of the cordillera (e.g. Denham, 2007). Large expanses of dense forest could limit the dispersal of pademelons, particularly subalpine populations, which are associated primarily with forest edge habitats.

There is archaeological evidence for admixture of lowland and montane taxa in West Papua during the late Pleistocene (Pasveer & Aplin, 1998). Remains of mammalian species found at approximately 600 m below their modern distributional limits in West Papua may suggest a historically lower elevational range for montane taxa due to cooling during the LGM. There is also palynological evidence for the invasion of lower montane forest elements into mid-montane regions of the Central Cordillera in Papua New Guinea (Flenley, 1979). These findings are consistent with the persistence of closed forest habitats in lower to mid montane areas of New Guinea throughout the Pleistocene (Hope *et al.*, 2004), but changing faunal and floral assemblages driven by global climatic cycles. Thus, while it is possible that incomplete lineage sorting due to recent colonization of disjunct subalpine habitats may account for the limited genetic divergence between *T. calabyi* and nearby lowland *T. brunii* populations, gene flow between these currently geographically isolated populations as recently as the LGM is also possible. Distinguishing between these scenarios in both the central cordilleran and north-eastern Huon regions will require additional nuclear genetic markers and larger sample sizes than could be obtained for this study. However, further genetic studies of montane taxa with broad elevational distributions in New Guinea will provide valuable comparisons with other geologically recent alpine regions, such as the South American Andes (e.g. Hughes & Eastwood, 2006) and New Zealand Southern Alps (e.g. Buckley & Simon, 2007; Pirie *et al.*, 2010).

### *The Western clade*

Relationships among pademelons in central and western New Guinea may have been more strongly influenced by habitat changes during the Pleistocene than by palaeogeological events. Combined mtDNA data suggest that within the Western clade, the divergence of the southern and northern populations occurred during the Pleistocene, following a split from the north-western population in the late Pliocene/early Pleistocene. The CR data were unable to resolve relationships among the Aru Islands, Northern and Northwestern groups, and provided poor support for the placement of the Mount Giluwe population. Nevertheless, the general pattern of genetic


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differentiation among populations suggests that the Aru population is more closely related to the northern New Guinea populations than to the southern lowland populations. While there is a possibility that these island pademelons, like those of the Bismarck Archipelago, have derived from human-mediated introduction, the extent of genetic differentiation between the Aru and mainland populations is more suggestive of mid-Pleistocene levels of isolation.

The fauna of the Aru Islands is generally thought to be allied with that of the south-eastern New Guinean lowlands due to a shared geological and biogeographical history (Flannery, 1995; O'Connor *et al.*, 2002). The observed phylogeographical structuring in this study, however, may be explained by extrapolation from present habitat preferences of *Thylogale*; within the lowland savannas, pademelons are patchily distributed and confined to areas supporting dense forests. Throughout glacial maxima, the river-dissected landscape of the Aru region probably supported rain forest habitat (Hope & Aplin, 2005), and dispersal between populations in this region and mainland New Guinea would have occurred along forested watercourses, which drained westward from the central mountain ranges (Voris, 2000). Intriguingly, both *T. brunii* and *T. stigmatica* have been found in late Pleistocene fossil deposits on the islands (Aplin & Pasveer, 2005). This may indicate more substantial forest connectivity with the TransFly region, where *T. stigmatica oriomo* is presently found, or an historically much wider distribution for that species encompassing lowland western New Guinea and/or regions on the exposed western Arafura shelf.

Gene flow through valleys across the Central Cordillera in western New Guinea may explain the relatively limited genetic divergence between the pademelon populations of the Aru Islands and the northern region. Levels of genetic divergence between *Thylogale* in subalpine and lowland habitats, and between individuals from the Northern clade and Haia in eastern New Guinea, suggest dispersal across the cordillera. In the Baliem Valley and Mount Jaya region of West Papua, Holocene fossil evidence for two subalpine *Thylogale* species (*Thylogale christenseni* and *Thylogale* sp.) has been found at and above 3000 m a.s.l. (Hope, 1981; Hope *et al.*, 1993). Therefore, it is plausible that during glacial periods lowered tree lines and an increased grassland/shrub mosaic in the Baliem Valley (e.g. Hope & Hope, 1976) also provided a route for dispersal of pademelons across the cordillera. Given that pademelons appear to have tracked the rising tree line to the current subalpine regions of Mount Albert Edward, Mount Giluwe and the Saruwaged Range in central and eastern New Guinea, a similar scenario could have occurred in areas such as the Baliem Valley.

Within the Northern New Guinea clade there is evidence of population structuring, but relatively low genetic divergence. By the mid Pleistocene, all northern terranes were emergent as isolated coastal ranges, with lowland connectivity to the Central Cordillera (Pigram & Davies, 1987; Hall, 2002). Changes in the distributions of lower to mid-montane forests during this period may have been highly site-specific (e.g. Hope *et al.*, 1983; Haberle, 1998), while the total extent of the forests was little changed (Hope & Tulip, 1994; Hope *et al.*, 2004). Gene flow among pademelon populations may have been limited more by lowland swamps, open areas of the Sepik floodplain and the Sepik River itself, than by the effects of forest fragmentation in the Pleistocene. The northern coastal ranges represent distinct biogeographical units for many montane species (Flannery, 1995; Beehler, 2007) and possibly also for more generalist forest species, particularly in the north-west, as indicated by the structuring of populations in this study. However, as our sampling was limited and relatively broad scale in this region, the relationship among north-western and northern populations is difficult to resolve.

## CONCLUSIONS

This study is one of the most comprehensive phylogeographical analyses of a New Guinean mammal conducted to date, and illustrates the importance of extensive geographical sampling for the detection of intra- and inter-specific genetic variation in this region. Although topography and associated habitat variation have clearly influenced the structuring of genetic diversity within the species complex, the observed phylogeographical structuring does not conform to zoogeographical expectations of southern lowland, northern lowland/mid-montane and subalpine divisions among *Thylogale* populations. The results are consistent with a generally broad ecological and elevational tolerance within the species, but also with the restriction of populations to dense forest/forest edge habitats. Finer-scale studies focusing on taxa distributed both within and across biogeographical regions in New Guinea are needed to test whether the phylogeographical patterns observed in this study are common to other species. For example, the pattern of division between eastern and western clades of *Thylogale* and between the north-eastern Huon Peninsula and Central Province *Thylogale* populations could be tested using genetic data from genera of other forest macropods such as *Dendrolagus*, *Dorcopsis* and *Dorcopsulus*. More comprehensive phylogeographical studies of the New Guinean flora and fauna will also provide comparisons for regions with geomorphological parallels to New Guinea. Much of the phylogeographical structuring and speciation in the upper montane regions of New Zealand has been shown to reflect recent trans-oceanic dispersal into the alpine zone (e.g. Tay *et al.*, 2010) and/or dispersal from lowland populations (e.g. Buckley & Simon, 2007). Evolution of the Andean alpine flora, with rapid speciation during the Pleistocene (e.g. Hughes & Eastwood, 2006) also reflects recent orogenesis. Hence, the origins and diversification of the New Guinean montane biota are likely to be complex, dependent on colonization from tropical lowland populations, long-distance dispersal and, in some regions of the cordillera, more ancient vicariant origins on emergent terranes (e.g. Heads, 2002a).

The current taxonomy of New Guinean *Thylogale* is inconsistent with the mitochondrial genetic data presented


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here and in Macqueen *et al.* (2010), and strong genetic structuring indicates that conservation of this species complex at a regional level is warranted. The patchy distribution of pademelons also implies that contemporary connectivity between populations is limited, and while the persistence of populations may be aided by a capacity to exploit disturbed forest habitats, isolated populations are clearly highly vulnerable to hunting pressure. This is evidenced by the recent extinction of the Port Moresby and Mount Wilhelm populations (Flannery, 1992), and the extinction of subalpine populations during the Holocene (Hope *et al.*, 1993). In addition, although *T. calabyi* populations show relatively limited divergence from lowland *Thylogale*, surveys indicate that these subalpine forest-edge populations are now effectively isolated from the lowlands by dense mid-montane forests. It can be anticipated that rapid population growth and the increasing loss of forest habitat in New Guinea (e.g. Shearman & Bryan, 2011) will have an impact on the local persistence of pademelon populations.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Appendix S1** Taxa used in this study including collection and catalogue numbers.
**Appendix S2** Details of the primers and PCR conditions used for mitochondrial DNA amplification, and mitochondrial DNA sequence characteristics and models used for Bayesian analyses.
**Appendix S3** Maximum parsimony tree generated using mitochondrial DNA cytochrome *b* and control region data.

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## BIOSKETCH

**Peggy Macqueen** is a PhD candidate at The University of Queensland in Brisbane, Australia. Her research has investigated the phylogenetic relationships among pademelon species across Australia and New Guinea, as well as the phylogeography of pademelons within these countries.

Author contributions: P.M. coordinated collection of the samples, performed the laboratory and genetic analyses and led the writing of this paper. A.W.G. and J.M.S. assisted with laboratory and genetic analyses and with writing of the paper. J.J.A. assisted with the laboratory work on museum specimens.

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