Phylogeography of Eastern Grey Kangaroos, *Macropus giganteus*, Suggests a Mesic Refugium in Eastern Australia

Brett A. Coghlan[1☯]*, **Anne W. Goldizen**[1☯], **Vicki A. Thomson**[1☯¤], **Jennifer M. Seddon**[2☯]

1 School of Biological Sciences, University of Queensland, St. Lucia, Queensland, Australia, 2 School of Veterinary Science, University of Queensland, Gatton, Queensland, Australia

☯ These authors contributed equally to this work.
¤ Current address: School of Biological Sciences, University of Adelaide, Adelaide, South Australia, Australia
* brett.coghlan@uqconnect.edu.au

Abstract

Phylogeographic studies around the world have identified refugia where fauna were able to persist during unsuitable climatic periods, particularly during times of glaciation. In Australia the effects of Pleistocene climate oscillations on rainforest taxa have been well studied but less is known about the effects on mesic-habitat fauna, such as the eastern grey kangaroo (*Macropus giganteus*). The eastern grey kangaroo is a large mammal that is common and widespread throughout eastern Australia, preferring dry mesic habitat, rather than rainforest. As pollen evidence suggests that the central-eastern part of Australia (southeast Queensland and northern New South Wales) experienced cycles of expansion in mesic habitat with contraction in rainforests, and vice versa during glacial and interglacial periods, respectively, we hypothesise that the distribution of the eastern grey kangaroo was affected by these climate oscillations and may have contracted to mesic habitat refugia. From 375 mitochondrial DNA control region sequences from across the distribution of eastern grey kangaroos we obtained 108 unique haplotypes. Phylogenetic analysis identified two clades in Queensland, one of which is newly identified and restricted to a small coastal region in southern Queensland north of Brisbane, known as the Sunshine Coast. The relatively limited geographic range of this genetically isolated clade suggests the possibility of a mesic habitat refugium forming during rainforest expansion during wetter climate cycles. Other potential, although less likely, reasons for the genetic isolation of the highly distinct clade include geographic barriers, separate northward expansions, and strong local adaptation.

Introduction

During past Pleistocene climatic cycles, many species survived in pockets of preferred habitat known as refugia, allowing them to persist through unfavourable local and regional climatic conditions [1, 2]. Climatic oscillations in Australia are thought to have been less extreme than

Competing Interests: The authors have declared
that no competing interests exist.

those in the northern hemisphere, varying between cool/dry and warm/wet conditions rather
than glacial/non-glacial [3–6]. Warm/wet cycles allowed rainforest habitat in eastern Australia to
expand and conversely cool/dry cycles allowed mesic habitat to expand as rainforests contracted
to refugial areas [7–10]. The boundaries of these rainforest refugia throughout the continent
were constantly changing due to climate fluctuations [11]. In fact, phylogeographic patterns have
been recognised for various rainforest species that support the hypothesis that multiple rainforest
refugia occurred throughout the east coast of Australia during periods of climate fluctuation, al-
lowing certain species to survive and diverge [12–15].

The identification of mesic barriers restricting gene flow among rainforest fragments, result-
ing in rainforest refugia, provides support for the expansion of mesic habitat [16]. The best-
studied mesic-habitat barrier is the Black Mountain Barrier (BMB) in the Wet Tropics rainfor-
est region of northern Queensland (QLD) in north-eastern Australia, which is considered a
habitat barrier rather than purely a geographic (mountain) barrier [14, 17]. Several rainforest
species spanning this region exhibit a north/south separation across the BMB, indicating con-
nectivity early in the Pleistocene but subsequent separation by unsuitable habitat during the
Last Glacial Maximum (LGM) that still persists today [15, 18, 19]. Examples of similar mesic-
habitat barriers in other parts of QLD include the Burdekin Gap south of Townsville and the
Broad Sound Barrier/St. Lawrence Gap, both in central-east QLD (e.g. [20, 21]).

In contrast to these studies of rainforest refugia, there has been little research conducted on
the effects of refugia on mesic, open-woodland species in Australia (but see [22–24]). It is likely
that the mesic habitats, such as dry and open woodlands that were previously barriers to rain-
forest species, may have acted as refugia for mesic-adapted species when tropical and subtropi-
cal rainforests expanded during warm/wet climate cycles. Here we test this hypothesis in
southeast QLD (including the areas surrounding Brisbane and the Sunshine Coast region
north of Brisbane) where there is a modified mosaic of closed rainforest interspersed with drier
habitat along, and to the east (coastal side) of, the Great Dividing Range (GDR; Fig 1) [16, 25,
26]. Estimates of rainforest species distributions indicate that until recently rainforests were
contiguous across such high altitudes as the GDR [16]. In addition, pollen records support re-
peated cycles of expanding/contracting rainforest and mesic forest vegetation in north-eastern
New South Wales (NSW; located directly south of QLD) and southeast QLD during the Pleis-
tocene [8, 9, 27]. Furthermore, rainforest species in the region show genetic patterns that are
consistent with their having had such restricted ranges during the cool, dry period of the LGM
[16]. However, there is little direct evidence for refugia and expansion patterns in mesic-habitat
species in the area.

The eastern grey kangaroo (*Macropus giganteus*) is useful for examining the hypothesis that
mesic-habitat refugia occurred in southeast QLD since it is widespread and common (non-
threatened), prefers mesic habitat, and avoids rainforests [28]. Although the kangaroos' large
size makes them difficult to trap, their abundance facilitates non-invasive sampling from faecal
material. Eastern grey kangaroos range throughout QLD and NSW, as well as other states in
the eastern part of Australia including Victoria (VIC), parts of Tasmania (TAS), and the ex-
treme eastern part of South Australia, and prefer open-woodland habitat that provides ade-
quate grass cover for forage and shade for rest during the heat of the day [28–31]. The eastern
grey kangaroo is a vagile species that does not appear to exhibit genetic isolation-by-distance at
a widespread scale, suggesting contemporary gene flow occurs across the wider species' distri-
bution [23]. A similar species, the red kangaroo (*Macropus rufus*), occupies drier habitat than
the eastern grey kangaroo and exhibits significantly higher diversity across its range and struc-
ture that correlates with topography, suggesting regions of higher altitude may have provided
refuges during past climate cycles [32]. A previous phylogeographic study on eastern grey kanga-
roos, based on the mitochondrial DNA control region (mtDNA *CR*), found clear differentiation

Fig 1. Contemporary temperate rainforest in southeast Queensland. Shaded areas indicate rainforest. Note that rainforest has been cleared in many areas since European colonisation. Cities/towns are represented by stars. Rainforest GIS layer package from Nauman [25] and locator map layer from Commonwealth of Australia (Geoscience Australia) [26]. ACT—Australian Capital Territory; ∧– Great Dividing Range.

doi:10.1371/journal.pone.0128160.g001

between northern individuals in inland QLD and southern individuals in NSW, VIC, and TAS, suggesting a likely northwards migration from a southern origin possibly as environmental amelioration occurred after past climate change events [23], however this study lacked samples from southern and coastal (eastern) QLD. Based on these prior studies, we hypothesise that the distribution of eastern grey kangaroos was affected by the climate oscillations of previous eras. Thus, this paper has two aims. The first is to investigate possible refugial areas by examining the genetic variation in eastern grey kangaroos within the mosaic of mesic and rainforest habitats at a regional scale in southeast QLD to investigate the evidence for mesic refugia, while the second is to investigate species-wide phylogeographic patterns by filling large gaps in existing, yet patchy, range-wide datasets to more accurately determine how southeast QLD kangaroos fit into the wider phylogeographic patterns exhibited by this species.

Materials and Methods

Sample collection

A total of 317 faecal and tissue samples were collected from 39 sites throughout QLD and NSW. Faecal samples were collected fresh and either placed in paper bags, air-dried and stored at -20°C, or placed in sterile containers and frozen at -20°C as soon as feasible [33]. We chose to primarily use non-invasive sampling techniques due to sampling within the semi-urban environment, cost, and time-effectiveness that precluded getting samples from kangaroo harvesters or from trapping a large mammal such as the eastern grey kangaroo. Although we cannot confirm that every faecal sample came from a different individual, when possible we observed an individual kangaroo defecating and collected that sample. Samples from Elanda Point and Sundown National Park were collected from different individual kangaroos, identified as part of other behavioural studies. Our phylogenetic analyses do not rely on haplotype frequency and thus the presence of multiple samples from a single individual will not have affected inferences from phylogenetic analyses. Tissue samples were opportunistically taken as ear clips from road-killed or otherwise deceased kangaroos in QLD only and were stored in 100% ethanol at -20°C. It was not possible to obtain samples from harvested kangaroos, as kangaroos are not harvested in the semi-urban area that was the main focus of our study. In addition to 41 samples collected in 2008 and the 215 collected in 2012 for this study, 58 eastern grey kangaroo mtDNA sequences were obtained from GenBank from a previous phylogeographic study (GenBank accession numbers AF443122.1–AF443172.1, EF555437.1–EF555443.1) [23] and three sequences from western grey kangaroos (*Macropus fuliginosus*; AF443173.1–AF443175.1) were used as an outgroup (Fig 2; S1 File).

Ethics Statement. The collection protocol was approved by the University of Queensland Native/Exotic Wildlife and Marine Animals ethics committee and collection permits WISP10606612 and WITK10606712 were obtained from Queensland's Department of Environment and Resource Management. No permits were required for faecal sample collection in New South Wales. This study did not involve any endangered or protected species.

DNA extraction

DNA was extracted from the majority of faecal samples using the QIAmp DNA Stool Mini Kits (QIAGEN, Chadstone Centre) with a 200 μL elution, and 22 samples were extracted using the ISOLATE Fecal DNA Kit (Bioline, Alexandria) with a 100 μL elution. DNA extractions from tissue samples followed a Proteinase K digestion and a salting-out procedure similar to Nicholls *et al.* [34].
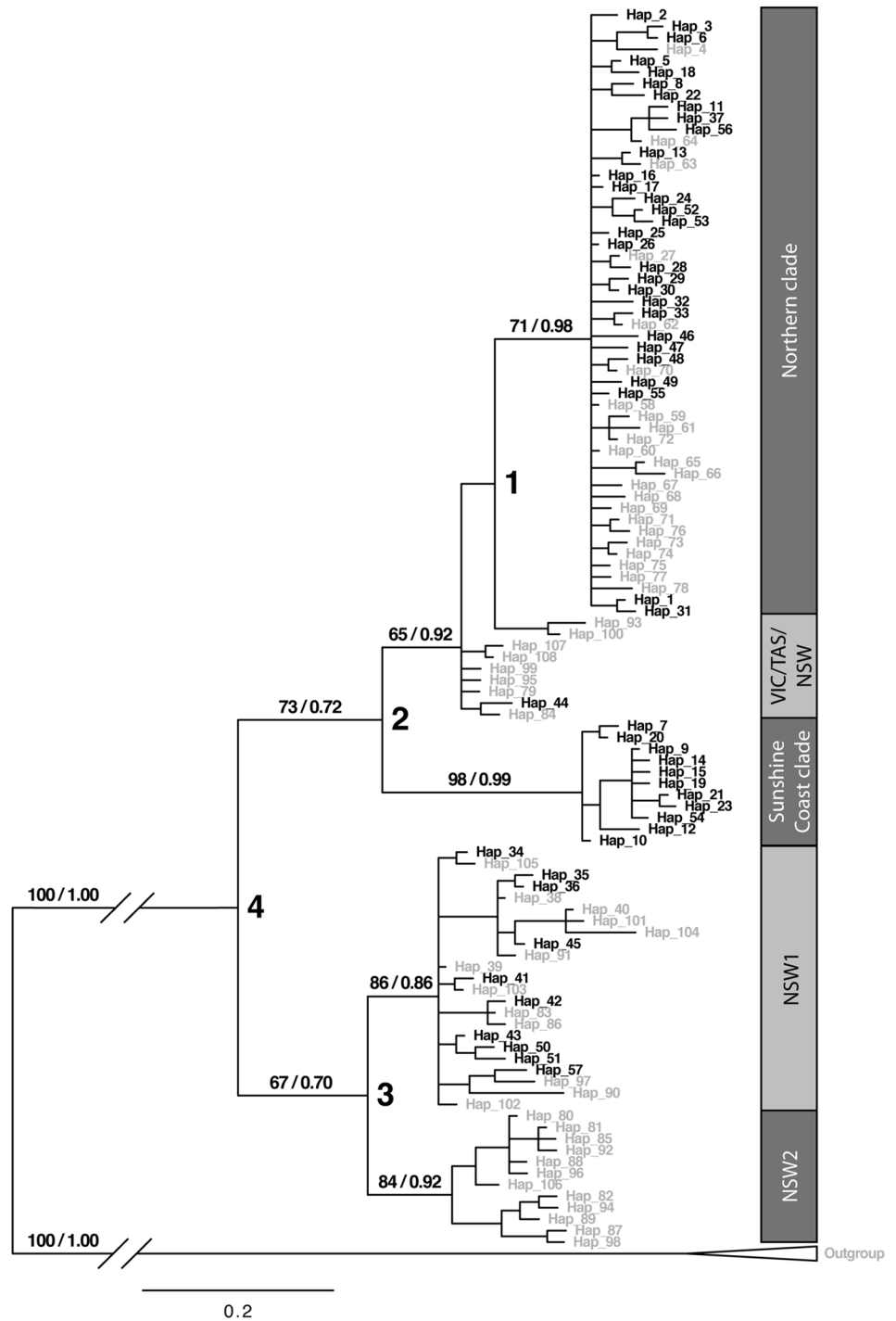
Fig 2. Bayesian inference phylogenetic tree of mtDNA control region for *M. giganteus*. Numbers on branches represent ML bootstrap support/ Bayesian posterior probabilities. Support is only shown on major clades (Bayesian posterior probability > 0.5). Numbers at nodes correspond to splitting events discussed in the text. Haplotypes in black indicate new haplotypes from this study, while haplotypes in grey represent sequences from Zenger *et al*. [23].

mtDNA sequencing

We sequenced 570 bp of the mtDNA *CR* using marsupial-specific primers H16498M and L15999M [35]. We used a 10 μL polymerase chain reaction (PCR) mixture comprised of 1x PCR buffer (Qiagen, Chadstone Centre), 1.5 mM MgCl$_2$, 0.25 μM of each primer, 0.15 mM each dNTP, and 0.5 U HotStar Taq DNA polymerase (Qiagen, Chadstone Centre). PCR reactions were run with the following conditions: initial denaturation at 94°C for 15 minutes, 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute, followed by a final annealing step at 50°C for 5 minutes and a final extension at 72°C for 10 minutes.

PCR products were purified by adding 5 U exonuclease I, *Escherichia coli* (Fermentas, Scoresby) and 0.5 U shrimp alkaline phosphatase (Promega, Hawthorn) and incubating at 37°C for 30 minutes followed by an enzyme inactivation step of 80°C for 15 minutes. Following purification, we performed a cycle sequencing reaction by using a 12 μL reaction mixture with 3.0 μL 5x sequencing buffer, 0.5 μL primer, and 1.0 μL BigDye v3.1 (Applied Biosystems, Mulgrave). Both strands were sequenced using the respective PCR primers. The products were precipitated following the manufacturer's protocol of using EDTA, sodium acetate, and ethanol. Capillary electrophoresis was undertaken by the Animal Genetics Laboratory (AGL) at the University of Queensland, Gatton, on an ABI 3730xl or 3130xl Genetic Analyzer (Applied Biosystems, Mulgrave). We attempted to examine nuclear markers but were unsuccessful due to low microsatellite amplification rates, likely a result of low quality DNA in the faecal samples.

Analyses

Sequences were assembled and checked for errors in ChromasPro (Technelysium, South Brisbane) and aligned using Clustal W in MEGA v5.1 [36, 37]. Nucleotide diversity (π), haplotype diversity (h), Tajima's D [38], and Fu and Li's F^* [39] were calculated in DnaSP v5.10.1 [40] using the 256 sequences obtained in this study and 61 GenBank sequences to form a complete data set (n = 317). Fu's F_S [41] for each clade and respective *P*-values were calculated in Arlequin v3.5.1.2 [42] using 10,000 simulated samples.

A maximum likelihood (ML) tree was constructed in MEGA v5.1 [36] and Bayesian phylogenetic analysis was conducted using MrBayes v3.2.1 [43]. Using jModelTest v2.1.1 [44, 45], the best-fit nucleotide substitution model for phylogenetic analysis was a Tamura-Nei model (TrN) + gamma distribution [46] with gamma shape parameter 3.1020. This model was used in the maximum likelihood analysis while the generalised time-reversible (GTR) + gamma distribution model was used in MrBayes, given that MrBayes does not provide TrN as an available substitution model.

We ran the Bayesian analysis for 5,000,000 generations to obtain a standard deviation of split frequencies less than 0.01 and discarded 25% as burn-in. As the large number of sequences proved computationally intensive, maximum likelihood and Bayesian phylogenetic analyses were only conducted on a smaller set of unique haplotypes, generated in DnaSP. The tree was edited for publication using FigTree v1.4.0 [47]. We also used MrBayes to determine if the ancestry of nodes 2, 3, and 4 from the resulting phylogenetic tree was northern, southern, or of outgroup (western grey kangaroo) origin. Median joining haplotype networks were created for all unique *M. giganteus* haplotypes, plus the Sunshine Coast clade separately using Network v4.6.1.3 (www.fluxus-engineering.com [48, 49]). Arlequin v3.5.1.2 [42] was used to estimate demographic expansion parameters of the Sunshine Coast clade through mismatch distributions with 100 bootstrap replicates.

Results

A total of 256 samples were successfully sequenced (S1 File) and aligned (S2 File) with the 61 sequences from Zenger et al. [23]. Among the full dataset (n = 317), there were 111 unique haplotypes with 141 polymorphic sites, of which 121 were parsimony informative. New haplotypes were uploaded to GenBank (KF316482–KF316538).

Both ML and Bayesian methods of phylogenetic analysis consistently reconstructed five clades, although with differing support levels (Fig 2). Importantly, we identified a strongly supported, highly distinct clade that was restricted to southeast QLD, predominately the area near the Sunshine Coast (hereafter termed the Sunshine Coast clade). The other four clades were less strongly supported but were consistent with those identified in Zenger et al. [23]: 1) a Northern clade with individuals from QLD and northern NSW extending south to Glen Innes (NSW) and Moree (NSW); 2) a clade consisting of individuals from VIC, TAS, and NSW (VIC/TAS/NSW) was identified on the ML tree, however these individuals did not form a clade on the Bayesian tree but rather were sister taxa to the monophyletic Northern clade; 3) and 4) two clades consisting mainly of NSW individuals, with a few haplotypes from southern QLD (Fig 3). Our network analysis identified five groups consistent with the clades identified in our Bayesian inference tree (Fig 4). The geographical distribution of the clades is shown in Fig 3; however, the two NSW clades are represented as one in the figure due to lack of detailed sampling locations available from Zenger et al. [23] leading to an inability to determine exact locations within NSW for a few of the samples. Bayesian posterior probability support in the Bayesian inference tree was strongest for the Sunshine Coast clade (0.99) and the Northern clade (0.96) and lower for the other clades, ranging from 0.82 to 0.89 (Fig 2). The topology of major clades in the ML tree was identical to the Bayesian tree with the ML tree containing some clades with lower bootstrap values, although all clades had bootstrap values greater than 65 (Fig 2). Our analysis to determine the origin of nodes 2, 3, and 4 resulted in the highest probabilities of P(south) = 0.98 for node 2 [P(north) = 0.018; P(outgroup) = 0.0034], P(south) = 0.99 for node 3 [P(north) = 0.00099; P(outgroup) = 0.00034], and P(outgroup) = 0.99 for node 4 [P(north) = 0.00037; P(south) = 0.00056].

The Tajima's D and Fu and Li's F^* statistics for all five major clades were non-significant (Table 1), suggesting population stability. However Fu's F_S values were significant for the Northern clade, the NSW clades combined, each of the NSW 1 and NSW 2 clades separately, as well as the species as a whole (Table 1), indicating recent population expansion for these groups. Haplotype diversity was high ($h > 0.5$) for all clades, with nucleotide diversity also high ($\pi > 0.5\%$) for all clades except the Sunshine Coast clade ($\pi = 0.402\%$; Table 1).

Monophyly of the newly discovered Sunshine Coast clade had high support values in both of the mtDNA CR phylogenetic trees (Fig 2) and this clade was genetically divergent from both the Northern clade (5%) and NSW clades (7%). The Sunshine Coast clade was comprised of eleven haplotypes, with the network showing one common haplotype with four closely related haplotypes and six more distant haplotypes (Fig 5 inset). All haplotypes in this clade except one occurred within a 23,000-km^2 area in south-eastern QLD along and inland from the Pacific coast, between Hervey Bay (to the north), North Lakes (to the south), Kingaroy (to the west), and the Pacific Ocean (to the east; Fig 3; Fig 5). A single individual with a Sunshine Coast haplotype was found in Stanthorpe, QLD (~170 km southwest of Brisbane), while all other individuals from Stanthorpe (n = 6) belonged to the Northern clade.

Discussion

Here we identify a mtDNA clade that was predominantly restricted to the Sunshine Coast region of QLD (plus areas just to the north and west of there) and was genetically divergent from
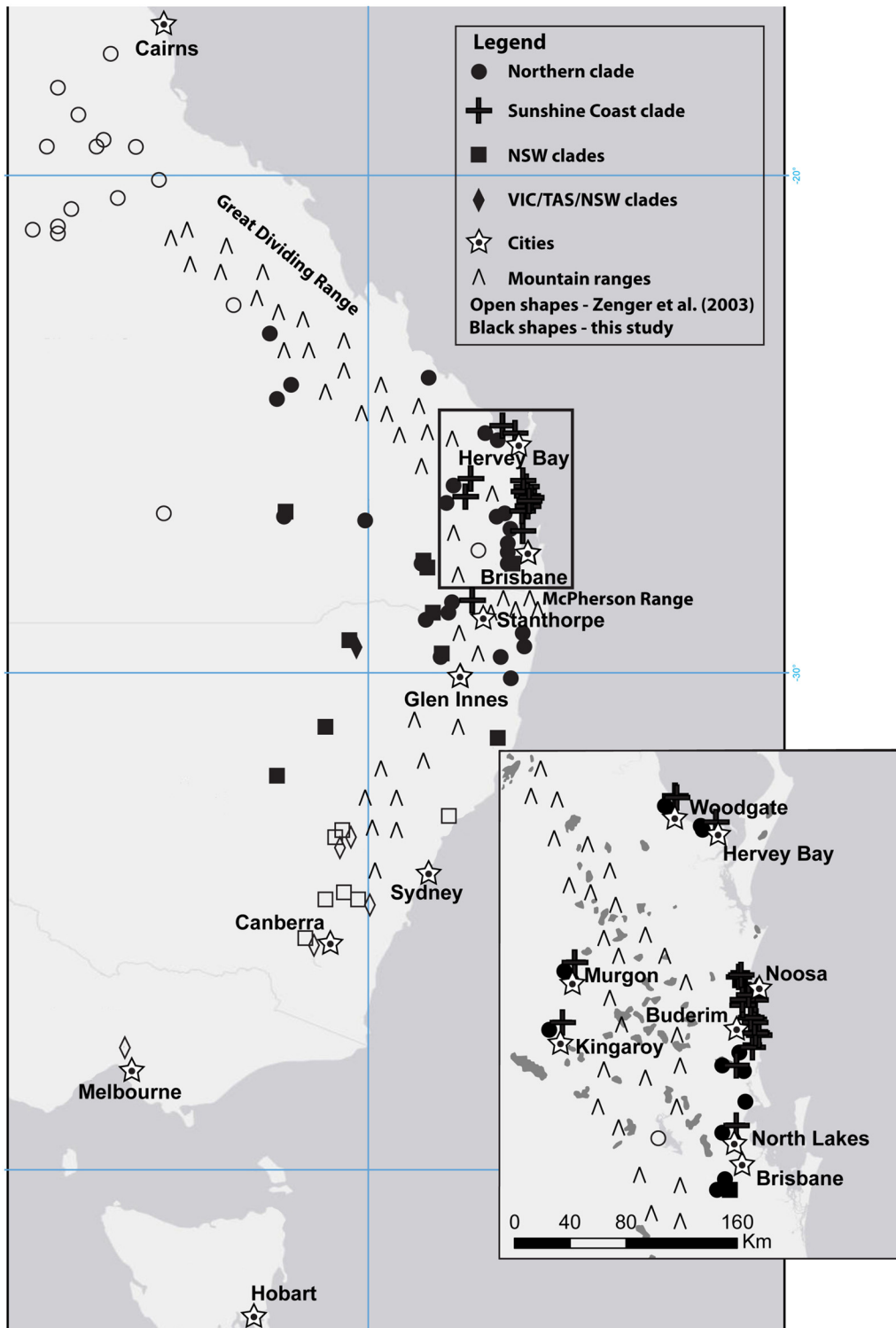
Fig 3. Sampling and haplotype locations. Map showing location and haplotype designations of each clade according to the phylogenetic tree. Circles represent the Northern clade, diamonds represent the VIC/TAS/NSW clade, crosses represent the Sunshine Coast clade, squares represent both NSW 1 and NSW 2 clades, and stars represent cities. The open shapes represent haplotypes from Zenger et al. [23] and solid black coloured shapes represent new haplotypes sampled in this paper. Inset is the Sunshine Coast region; shading in inset indicates contemporary temperate rainforest.
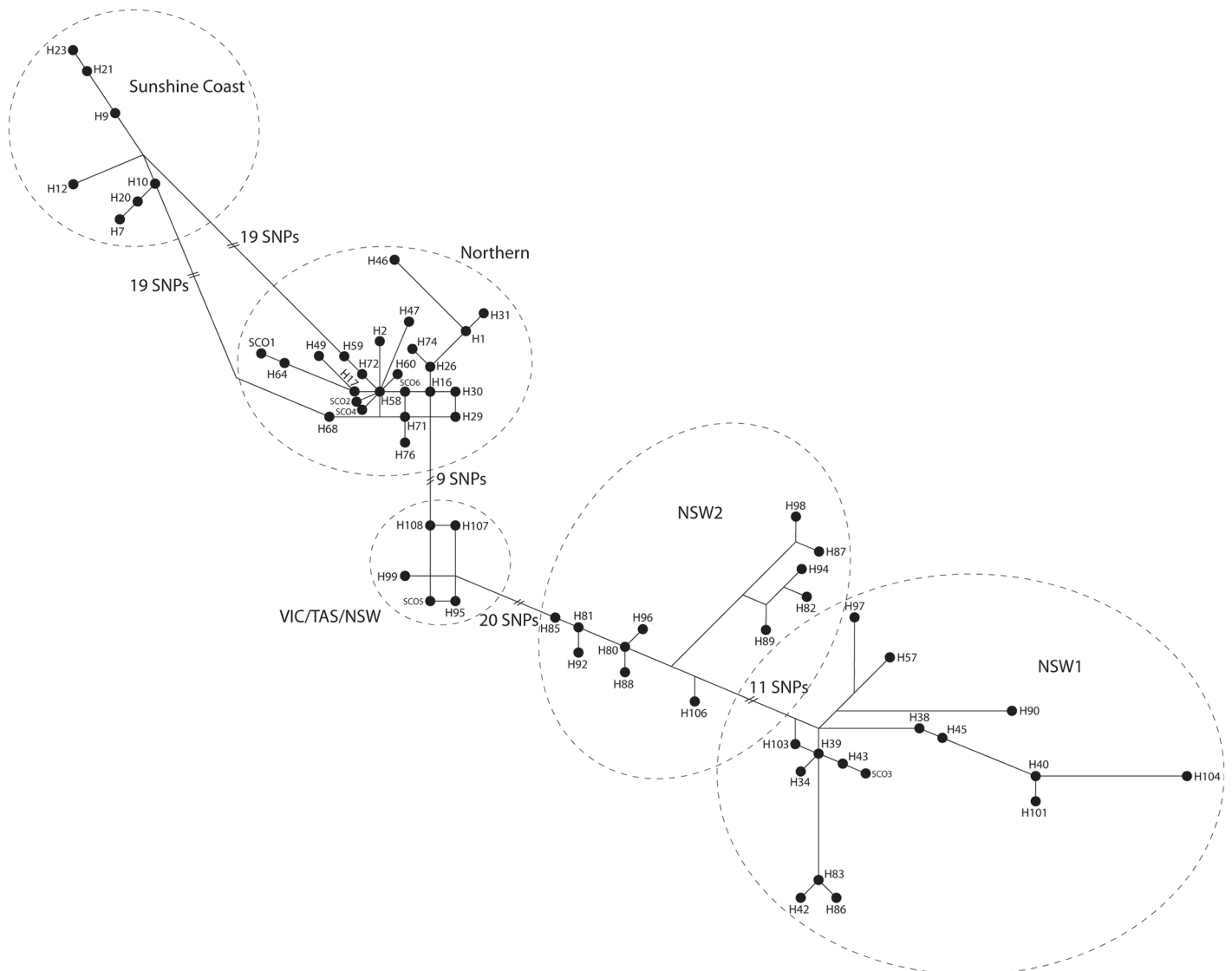
doi:10.1371/journal.pone.0128160.g003

Fig 4. Haplotype network of *M. giganteus*. The haplotype numbers are the same as in the phylogenetic tree and the dashed circles correspond to clades from Fig 2. Nodes labelled "SCO" indicate multiple haplotypes collapsed into a single node by star contraction in Network. SCO1 = H_11, H_37, H_56; SCO2 = H_8, H_13, H_22, H_24, H_27, H_28, H_32, H_52, H_53, H_55, H_63, H_67; SCO3 = H_50, H_51; SCO4 = H_33, H_62, H_69; SCO5 = H_44, H_79, H_84; SCO6 = H_3, H_4, H_5, H_6, H_18, H_65, H_66.

doi:10.1371/journal.pone.0128160.g004

clades in surrounding regions currently connected by suitable habitat. The limited geographic range of the Sunshine Coast clade, together with its clear divergence from other nearby populations, is consistent with a population of eastern grey kangaroos having survived in a mesic refugium for long periods of time. The repeated cycles of alternating expansion and contraction of rainforest and mesic forest vegetation in northern NSW and southeast QLD during the Pleistocene [8, 9, 27] may have been the driving force behind such a refugium. Genetic divergences in two other species with similar habitat preferences to the eastern grey kangaroo, the sugar glider (*Petaurus breviceps*) [50] and the squirrel glider (*Petaurus norfolcensis*) [51], also showed parallels with climatic cycles. These gliders show evidence of range contractions during time periods when climate hindered their ideal habitat, but range expansions when the climate supported their ideal habitat. In addition, koalas (*Phascolarctos cinereus*) show evidence of mtDNA

Table 1. Diversity statistics for clades identified in Bayesian phylogenetic analysis.

Clade	No. of haplotypes	π	h	k	Tajima's D	P-value	Fu and Li's F^*	P-value	Fu's F_S	P-value
Northern	54	0.010	0.960	5.833	-1.223	> 0.10	-2.129	> 0.05	-23.887	0.003[a]
Sunshine Coast	11	0.004	0.644	2.086	-0.672	> 0.10	-0.403	> 0.10	-7.323	0.085
VIC/TAS/NSW	10	0.010	1.000	6.156	-0.608	> 0.10	-0.378	> 0.10	-1.931	0.099
NSW 1 and 2 combined	35	0.018	0.960	10.852	-0.785	> 0.10	-1.420	> 0.10	-24.038	< 0.001[a]
NSW 1	23	0.012	0.940	7.044	-0.930	> 0.10	-1.924	> 0.10	-20.140	< 0.001[a]
NSW 2	12	0.012	1.000	7.682	0.038	> 0.10	0.060	> 0.10	-4.469	0.017[a]
M. giganteus (total)	108	0.041	0.960	20.304	0.351	> 0.10	-0.353	> 0.10	-23.794	< 0.001[a]

Diversity statistics of the supported clades using the complete data set (n = 317). Tajima's D and Fu and Li's F^* were not significant for any clade. Some statistics were not calculated for *M. fuliginosus* due to the low sample size (n = 3). π—nucleotide diversity, h—haplotype diversity k—average number of nucleotide differences within each clade.

[a] Indicates significance (< 0.02) of Fu's F_S.

doi:10.1371/journal.pone.0128160.t001

divisions between coastal and inland populations, consistent with closed rainforests at higher altitude having acted as a wet habitat barrier to the preferred mesic habitat of this species [24].

Although refugial populations characteristically undergo reductions in population size, we found contradictory evidence for a bottleneck or post-bottleneck expansion in the Sunshine Coast clade. When compared to the eastern grey kangaroo species as a whole, this clade exhibits low nucleotide diversity but medium-high haplotype diversity (Table 1), which can be indicative of a bottleneck followed by population growth [52]. Similarly indicative of a bottleneck, the majority of Sunshine Coast individuals exhibited a single haplotype (Hap_9; Fig 5 inset; although it should be noted this may reflect sampling bias), with the area around Noosa (QLD) showing the least amount of incursion from haplotypes that were assigned to other clades (Fig 3). Our high concentration of samples from near Elanda Point and Noosa might explain the apparently high frequency of Hap_9. Equally intensive sampling in other regions of the Sunshine Coast may identify additional common haplotypes. Furthermore, with our non-invasive sampling techniques we cannot say for certain if all samples and sequences came from different individuals although the samples from Elanda Point were all from identified individuals. However, additional statistics such as Tajima's D, Fu and Li's F^*, and Fu's F_S that can test for population bottlenecks were non-significant (Table 1). Hence, it is possible but not conclusive that the Sunshine Coast population has remained at a long-term low effective population size. For the eastern grey kangaroo species as a whole, the high nucleotide and high haplotype diversity suggests a large and stable population [52], as would be expected for a species with its large body size and natural history [53]; the 2011 estimated population of eastern grey kangaroos in Queensland was nearly 10.8 million [54].

An alternative explanation for genetic isolation is an effect of physical or topographical barriers to gene flow. However, there are no obvious physical or topographical barriers preventing gene flow immediately to the north, south, and west of the Sunshine Coast region. At a wider scale, geographic barriers in eastern Australia including the GDR, Brisbane River, and McPherson Range, appear not to have restricted dispersal in eastern grey kangaroos of the Sunshine Coast and Northern clades. Hence, the genetic isolation of the Sunshine Coast clade is more consistent with historical isolation due to unsuitable habitat than with clear geographic barriers.

Haplotypes from the Northern and Sunshine Coast clades currently overlap in regions surrounding the Sunshine Coast (Murgon, Kingaroy, Woodgate, Hervey Bay, Buderim, and North Lakes; Fig 3; Fig 5). The genetic isolation of these clades suggests that this geographic overlap
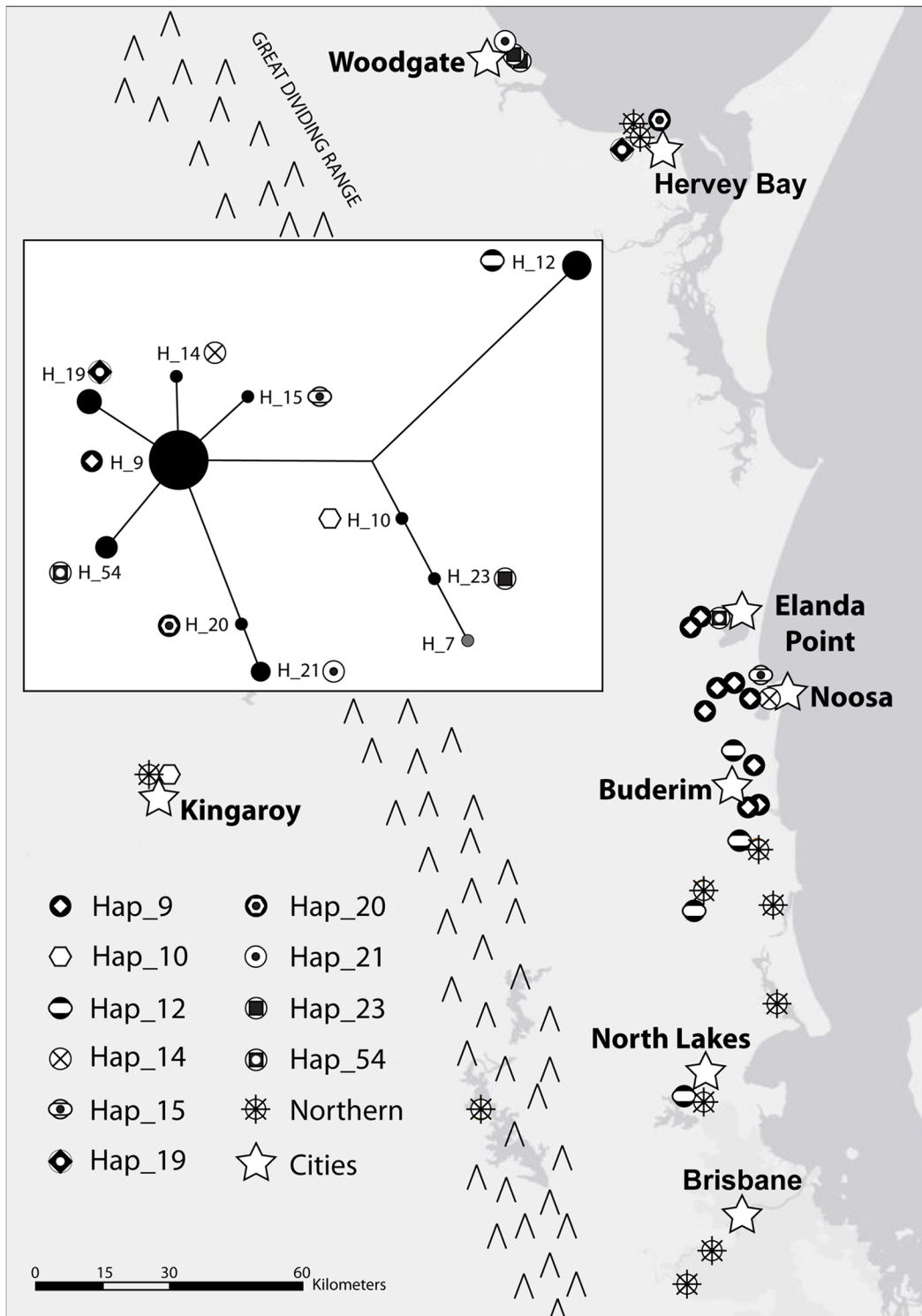
Fig 5. Distribution of haplotypes in the Sunshine Coast region. Map showing the distribution of Sunshine Coast clade haplotypes and Northern clade haplotypes in the Sunshine Coast region. Hap_7 of the Sunshine Coast clade (not pictured) was located ~170 km SW of Brisbane in Stanthorpe, QLD. A single individual with a haplotype from the NSW clade (not labelled) was found at Elanda Point, ~18 km NNW of Noosa. Inset is a haplotype network of the Sunshine Coast clade. Node size corresponds to relative number of individuals with that haplotype and distance between nodes corresponds to number of mutations.

doi:10.1371/journal.pone.0128160.g005

was not present, or at least not as widespread, during the period when the Sunshine Coast region was isolated. There has been significant anthropogenic habitat modification such as urbanisation and forest clearing in coastal areas of eastern Australia, including the Sunshine Coast region, in recent times. This modification will have increased kangaroo-favourable habitat and allowed kangaroos to expand outwards from the Sunshine Coast, thereby increasing contact with individuals from the Northern clade.

Another explanation for the presence of two distinct clades of eastern grey kangaroos in northern Australia is that there may have been two separate incursions of eastern grey kangaroos into QLD from southern Australia. Zenger *et al.* [23] proposed that eastern grey kangaroos in QLD originated from a single northward migration from southern regions (divergence between NSW and QLD clades at node 4 in Fig 2). Our results are compatible with a separate migration into southeast QLD, with the Sunshine Coast the remnant of this migration or expansion event (Sunshine Coast clade divergence at node 2 in Fig 2). The low probability of a northern origin for nodes 2, 3, and 4 supports the southern origin of the species. The high probability of a southern origin for node 2 [P(south) = 0.98] specifically suggests that the individuals in the Sunshine Coat clade were not a recent split from a northern population and that they had migrated from the south. Under this scenario, it is possible that the northern migration suggested by Zenger *et al.* [23] was actually a separate, possibly subsequent, migration northward into QLD (the Northern clade divergence at node 1 in Fig 2), with eastern grey kangaroos at that time successfully colonising a widespread part of eastern QLD. However, we did not identify any haplotypes of the Sunshine Coast clade along a possible expansion route, either inland or along the coast, nor in any potential source population in southern Australia, and so we consider this explanation unlikely. Further sampling required would be required to test it thoroughly.

It is also possible that the Sunshine Coast clade represents a population of kangaroos that underwent strong local adaptation, possibly to soil type. However, our data are insufficient to address this hypothesis and sampling of soils and vegetation as well as more genetic data, particularly from autosomal markers, would be necessary to confirm or rule out local adaption.

In our phylogeographic trees the nodes of southern lineages in NSW appear more basal, providing support for the conclusion of Zenger *et al.* [23] that eastern grey kangaroos originated in southern Australia and migrated northwards. However, our study identified that the Northern clade extends farther south into NSW than previously thought (Fig 3) [23]. The area of mixing between the Northern and NSW 1 clades appears to centre on the QLD/NSW border with individuals from both clades having been sampled in Glen Innes (NSW), Moree (NSW), Sundown National Park (QLD), and Wacol (QLD). The cause of the original separation between the Northern and NSW clades remains unclear [23]. Another finding is the unusual placement of the VIC/TAS/NSW clade as sister to the Northern clade. There are no contemporary records of translocations of kangaroos from the mainland to Tasmania [55] and the nodes of most other haplotypes from the southern part of the eastern grey kangaroos' range not a part of the VIC/TAS/NSW clade are basal to the Northern clade. The origin of this VIC/TAS/NSW clade also remains unclear.

The presence of similar or identical haplotypes in geographically distant sampling locations suggests gene flow has occurred over long distances in the eastern grey kangaroos, a pattern also seen in the previous study [23]. Given that the maximum observed dispersal distance of an eastern grey kangaroo is about 17 km [56], we cannot completely rule out the possibility that a few of the instances of apparent long-distance dispersal events in our data were caused by orphaned kangaroos that were taken into care being released at a distance from their capture location. Possible examples of such cases include one individual sampled from Elanda Point in the Sunshine Coast region that clustered with NSW samples in the phylogenetic analysis, one

individual from Roma, QLD (~430 km WNW of Brisbane) that clustered with a NSW clade, and one individual from Stanthorpe, QLD that clustered with the Sunshine Coast clade. It is always possible though that other individuals with haplotypes from our Sunshine Coast clade occur in other areas but our sampling process simply failed to obtain those individuals due to sampling bias, just as it is possible that our sampling process missed individuals from the Northern clade that may occur in the Sunshine Coast region or in New South Wales and vice versa.

In conclusion, we identified a divergent Sunshine Coast clade of eastern grey kangaroos that most likely resulted from a mesic-habitat refugium in southeast QLD. However confirming the presence of a refugium in this region and determining its extent requires geographically fine-scale studies of other mesic taxa as well as historical vegetation modelling. With regards to the eastern grey kangaroo, generating additional genetic data based on nuclear loci will be necessary to assess whether the Sunshine Coast clade warrants designation as a management or evolutionarily significant unit. Nuclear markers may also establish whether the divergence patterns that we identified are influenced by the species' male-biased dispersal and identify any recent genetic admixture between animals from different mtDNA clades [23, 56].

Supporting Information

S1 File. Information for each sample collected. Sample name, location, haplotype, clade, GenBank accession number, and source for all *M. giganteus* samples included in this study.
(XLSX)

S2 File. Sequence alignment. The alignment of all 108 *M. giganteus* haplotypes included in our analysis as well as information of haplotype frequencies. Invariable sites have been removed from the alignment.
(TXT)

Acknowledgments

Author Contributions

Conceived and designed the experiments: BAC AWG VAT JMS. Performed the experiments: BAC VAT. Analyzed the data: BAC AWG VAT JMS. Contributed reagents/materials/analysis tools: AWG JMS. Wrote the paper: BAC AWG VAT JMS.

References

1. Hewitt GM. The genetic legacy of the Quaternary ice ages. Nature. 2000; 405(6789):907–13. PMID: 10879524

2. Hewitt GM. Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transactions of the Royal Society of London Series B: Biological Sciences. 2004; 359(1442):183–95. doi: 10.1098/rstb.2003.1388 PMID: 15101575

3. Crisp M, Cook L, Steane D. Radiation of the Australian flora: what can comparisons of molecular phylogenies across multiple taxa tell us about the evolution of diversity in present–day communities? Philosophical Transactions of the Royal Society of London Series B: Biological Sciences. 2004; 359(1450):1551–71. doi: 10.1098/rstb.2004.1528 PMID: 15519972

4. Crisp MD, West JG, Linder HP. Biogeography of the terrestrial flora. Flora of Australia. 1. Melbourne, Australia: Australian Biological Resources Study/CSIRO Publishing; 1999. p. 321–67.

5. Kershaw AP, D'Costa DM, McEwen Mason JRC, Wagstaff BE. Palynological evidence for Quaternary vegetation and environments of mainland southeastern Australia. Quaternary Sci Rev. 1991; 10(5):391–404. doi: 10.1016/0277-3791(91)90003-D

6. Sniderman JMK. Early Pleistocene vegetation change in upland south-eastern Australia. J Biogeogr. 2011; 38(8):1456–70. doi: 10.1111/j.1365-2699.2011.02518.x

7. Gallagher SJ, Greenwood DR, Taylor D, Smith AJ, Wallace MW, Holdgate GR. The Pliocene climatic and environmental evolution of southeastern Australia: evidence from the marine and terrestrial realm. Palaeogeography, Palaeoclimatology, Palaeoecology. 2003; 193(3):349–82. doi: 10.1016/s0031-0182(03)00231-1

8. Longmore ME. Quaternary palynological records from perched lake aediments, Fraser Island, Queensland, Australia: rainforest, forest history and climatic control. Aust J Bot. 1997; 45(3):507–26. doi: 10.1071/BT96109

9. Longmore ME, Heijnis H. Aridity in Australia: Pleistocene records of palaeohydrological and palaeoecological change from the perched lake sediments of Fraser Island, Queensland, Australia. Quatern Int. 1999;57–58:35–47. doi: 10.1016/S1040-6182(98)00048-2

10. VanDerWal J, Shoo LP, Williams SE. New approaches to understanding late Quaternary climate fluctuations and refugial dynamics in Australian wet tropical rain forests. J Biogeogr. 2009; 36(2):291–301. doi: 10.1111/j.1365-2699.2008.01993.x

11. Graham CH, VanDerWal J, Phillips SJ, Moritz C, Williams SE. Dynamic refugia and species persistence: tracking spatial shifts in habitat through time. Ecography. 2010; 33(6):1062–9. doi: 10.1111/j.1600-0587.2010.06430.x

12. Byrne M, Yeates DK, Joseph L, Kearney M, Bowler J, Williams MAJ, et al. Birth of a biome: insights into the assembly and maintenance of the Australian arid zone biota. Mol Ecol. 2008; 17(20):4398–417. doi: 10.1111/j.1365-294X.2008.03899.x PMID: 18761619

13. Chapple DG, Chapple SNJ, Thompson MB. Biogeographic barriers in south-eastern Australia drive phylogeographic divergence in the garden skink, Lampropholis guichenoti. J Biogeogr. 2011; 38(9):1761–75. doi: 10.1111/j.1365-2699.2011.02531.x

14. Chapple D, Hoskin C, Chapple S, Thompson M. Phylogeographic divergence in the widespread delicate skink (Lampropholis delicata) corresponds to dry habitat barriers in eastern Australia. BMC Evol Biol. 2011; 11(1):191. doi: 10.1186/1471-2148-11-191

15. Moritz C, Patton JL, Schneider CJ, Smith TB. Diversification of rainforest faunas: an integrated molecular approach. Annu Rev Ecol Syst. 2000; 31(ArticleType: research-article / Full publication date: 2000 / Copyright © 2000 Annual Reviews):533–63. doi: 10.2307/221742

16. McGuigan K, McDonald K, Parris K, Moritz C. Mitochondrial DNA diversity and historical biogeography of a wet forest-restricted frog (Litoria pearsoniana) from mid-east Australia. Mol Ecol. 1998; 7(2):175–86. doi: 10.1046/j.1365-294x.1998.00329.x PMID: 9532760

17. Byrne M. Evidence for multiple refugia at different time scales during Pleistocene climatic oscillations in southern Australia inferred from phylogeography. Quaternary Sci Rev. 2008; 27(27–28):2576–85. doi: 10.1016/j.quascirev.2008.08.032

18. Schneider C, Moritz C. Rainforest refugia and Australia's Wet Tropics. Proceedings of the Royal Society of London B: Biological Sciences. 1999; 266(1415):191–6. doi: 10.1098/rspb.1999.0621

19. Schneider CJ, Cunningham M, Moritz C. Comparative phylogeography and the history of endemic vertebrates in the Wet Tropics rainforests of Australia. Mol Ecol. 1998; 7(4):487–98. doi: 10.1046/j.1365-294x.1998.00334.x

20. Schäuble CS, Moritz C. Comparative phylogeography of two open forest frogs from eastern Australia. Biol J Linn Soc. 2001; 74(2):157–70. doi: 10.1111/j.1095-8312.2001.tb01384.x

21. Rix MG, Harvey MS. Phylogeny and historical biogeography of ancient assassin spiders (Araneae: Archaeidae) in the Australian mesic zone: evidence for Miocene speciation within Tertiary refugia. Mol Phylogenet Evol. 2012; 62(1):375–96. doi: 10.1016/j.ympev.2011.10.009 PMID: 22040763

22. Neaves LE, Zenger KR, Prince RIT, Eldridge MDB, Cooper DW. Landscape discontinuities influence gene flow and genetic structure in a large, vagile Australian mammal, Macropus fuliginosus. Mol Ecol. 2009; 18(16):3363–78. doi: 10.1111/j.1365-294X.2009.04293.x PMID: 19659477

23. Zenger KR, Eldridge MDB, Cooper DW. Intraspecific variation, sex-biased dispersal and phylogeography of the eastern grey kangaroo (Macropus giganteus). Heredity. 2003; 91(2):153–62. PMID: 12886282

24. Lee KE, Seddon JM, Corley SW, Ellis WAH, Johnston SD, Villiers DL, et al. Genetic variation and structuring in the threatened koala populations of Southeast Queensland. Conserv Genet. 2010; 11(6):2091–103. doi: 10.1007/s10592-009-9987-9

25. Nauman R. Queensland Australia temperate rain forest (data basin dataset) 2010 [cited 2013 18 June 2013]. Available from: http://www.arcgis.com/home/item.html?id=6acd8a7f17ea494fb399d94b898e782e.

26. Commonwealth of Australia (Geoscience Australia). States (including coastal waters)—Australian Maritime Boundaries 2006 [cited 2015 27 February 2015]. Available from: http://spatial.ala.org.au/layers/more/cw_state_poly.

27. McGrath R, Boyd WE. Holocene vegetation history of Bundjalung National Park and Bungawalbin Creek, north-eastern New South Wales. Australian Geographer. 1998; 29(2):205–21. doi: 10.1080/00049189808703215

28. Poole WE. Macropus giganteus. Mammalian Species. 1982;(187:):1–8. doi: 10.2307/3504005

29. Davis NE, Coulson G, Forsyth DM. Diets of native and introduced mammalian herbivores in shrub-encroached grassy woodland, south-eastern Australia. Wildl Res. 2008; 35(7):684–94. doi: 10.1071/WR08042

30. Hill GJE. A study of habitat preferences in the grey kangaroo. Australian Wildlife Research. 1981; 8(2):245–54. doi: 10.1071/WR9810245

31. Taylor RJ. The diet of the eastern grey kangaroo and wallaroo in areas of improved and native pasture in the New England tablelands. Australian Wildlife Research. 1983; 10(2):203–11. doi: 10.1071/WR9830203

32. Clegg SM, Hale P, Moritz C. Molecular population genetics of the red kangaroo (Macropus rufus): mtDNA variation. Mol Ecol. 1998; 7(6):679–86. doi: 10.1046/j.1365-294x.1998.00376.x PMID: 9640648

33. Piggott MP, Taylor AC. Extensive evaluation of faecal preservation and DNA extraction methods in Australian native and introduced species. Aust J Zool. 2003; 51(4):341–55. doi: 10.1071/ZO03012

34. Nicholls JA, Double MC, Rowell DM, Magrath RD. The evolution of cooperative and pair breeding in thornbills Acanthiza (Pardalotidae). Journal of Avian Biology. 2000; 31(2):165–76. doi: 10.1034/j.1600-048X.2000.310208.x

35. Fumagalli L, Pope LC, Taberlet P, Moritz C. Versatile primers for the amplification of the mitochondrial DNA control region in marsupials. Mol Ecol. 1997; 6(12):1199–201. doi: 10.1046/j.1365-294X.1997.00298.x PMID: 9421920

36. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution. 2011; 28(10):2731–9. doi: 10.1093/molbev/msr121 PMID: 21546353

37. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic acids research. 1994; 22(22):4673–80. Epub 1994/11/11. PMID: 7984417; PubMed Central PMCID: PMC308517.

38. Tajima F. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics. 1989; 123(3):585–95. PMID: 2513255

39. Fu YX, Li WH. Statistical tests of neutrality of mutations. Genetics. 1993; 133(3):693–709. PMID: 8454210

40. Librado P, Rozas J. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. Bioinformatics. 2009; 25(11):1451–2. doi: 10.1093/bioinformatics/btp187 PMID: 19346325

41. Fu Y-X. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics. 1997; 147(2):915–25. PMID: 9335623

42. Excoffier L, Lischer HEL. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources. 2010; 10(3):564–7. doi: 10.1111/j.1755-0998.2010.02847.x PMID: 21565059

43. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003; 19(12):1572–4. doi: 10.1093/bioinformatics/btg180 PMID: 12912839

44. Posada D. jModelTest: Phylogenetic model averaging. Molecular Biology and Evolution. 2008; 25(7):1253–6. doi: 10.1093/molbev/msn083 PMID: 18397919

45. Guindon S, Gascuel O. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol. 2003; 52(5):696–704. doi: 10.1080/10635150390235520 PMID: 14530136

46. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution. 1993; 10(3):512–26. PMID: 8336541

47. Rambaut A. FigTree. 1.3.1 ed. Edinburgh: Institute of Evolutionary Biology, University of Edinburgh; 2009.

48. Bandelt HJ, Forster P, Röhl A. Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution. 1999; 16(1):37–48. PMID: 10331250

49. Polzin T, Daneshmand SV. On Steiner trees and minimum spanning trees in hypergraphs. Operations Research Letters. 2003; 31(1):12–20. doi: 10.1016/S0167-6377(02)00185-2

50. Malekian M, Cooper SJB, Norman JA, Christidis L, Carthew SM. Molecular systematics and evolutionary origins of the genus *Petaurus* (Marsupialia: Petauridae) in Australia and New Guinea. Mol Phylogenet Evol. 2010; 54(1):122–35. doi: 10.1016/j.ympev.2009.07.026 PMID: 19647084

51. Pavlova A, Walker FM, Ree R, Cesarini S, Taylor AC. Threatened populations of the Australian squirrel glider (*Petaurus norfolcensis*) show evidence of evolutionary distinctiveness on a Late Pleistocene timescale. Conserv Genet. 2010; 11(6):2393–407. doi: 10.1007/s10592-010-0125-5

52. Grant WAS, Bowen BW. Shallow population histories in deep evolutionary lineages of marine fishes: insights from sardines and anchovies and lessons for conservation. J Hered. 1998; 89(5):415–26. doi: 10.1093/jhered/89.5.415

53. Honnen A-C, Hailer F, Kenntner N, Literák I, Dubská L, Zachos FE. Mitochondrial DNA and nuclear microsatellites reveal high diversity and genetic structure in an avian top predator, the white-tailed sea eagle, in central Europe. Biol J Linn Soc. 2010; 99(4):727–37. doi: 10.1111/j.1095-8312.2010.01373.x

54. Commonwealth of Australia. Kangaroo population estimates 2011 [cited 2015 9 March 2015]. Available from: http://www.environment.gov.au/biodiversity/wild-harvest/kangaroo-wallaby-statistics/kangaroo-population.

55. Tanner Z, Hocking G. Status and management of the forester kangaroo in Tasmania, 2000. Hobart: Department of Primary Industries, Water and Environment, 2001.

56. Jarman P, Taylor R. Ranging of eastern grey kangaroos and wallaroos on a New England pastoral property. Wildl Res. 1983; 10(1):33–8. doi: 10.1071/WR9830033