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Genetic and radiographic insights into the only known mounted specimen of Kangaroo Island Emu

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Abstract: The Natural History Museum of Geneva holds a mounted specimen of a dwarf emu, which is believed to be the only preserved skin of the extinct Kangaroo Island Emu, *Dromaius baudinianus*. We obtained new radiographs that show the absence of remaining bones in the preparation, confirming previous statements found in the museum's archives. Moreover, we sequenced the complete mitochondrial genome of this specimen and we compared it to all available emu sequences. The mitogenome of the specimen held in Geneva is very close to that of Common Emus *Dromaius novaehollandiae*. Overall, the genetic results on insular emus support a shallow divergence between the mainland population and the – now extinct – populations from King Island, Kangaroo Island and Tasmania. Based on these results, we agree with previous molecular studies that the insular emu taxa should be treated as three different subspecies of the Common Emu.

Keywords: *Dromaius baudinianus* - Casuariidae - Natural History Museum of Geneva - X-ray - complete mitochondrial genome - ancient DNA.

INTRODUCTION

Emus are iconic ratite birds endemic to Australia. Today a single extant species, known simply as the Emu *Dromaius novaehollandiae* (Latham, 1790), is widely distributed across open habitats, whereas several populations became extinct on islands surrounding mainland Australia: Tasmania, Kangaroo Island and King Island. Each insular population of emu has been recognized as a distinct taxon, mostly because of its distinct small size, at the species or subspecies level. The description of the Kangaroo Island Emu *Dromaius baudinianus* Parker, 1984 was based on bones collected in a cave in 1926 (Parker, 1984). The diagnosis includes differences of the length and shape of the tibiotarsus and tarsometatarsus. The Kangaroo Island Emu is intermediate in size between the smaller King Island Emu *Dromaius minor* Spencer, 1906 [previously *D. ater* Vieillot, 1817, see Dickinson & Remsen (2013)] and the slightly larger Tasmanian Emu *Dromaius diemenensis* Le Souëf, 1907.

Located 110 km southwest of Adelaide, Kangaroo Island is the third largest Australian island after Tasmania and Melville Island. It is separated from the mainland by the

13 km wide Backstairs Passage, which is currently 40 m deep. The island is 145 km long east-west and 54 km long at its widest north-south section, covering 4405 km². Its climate is Mediterranean, having mild winters and dry summers. Compared to mainland South Australia, the island has been relatively spared from anthropogenic degradation with almost 40% of the island still being covered by native vegetation (Robinson & Armstrong, 1999). The island's earliest human occupation dates from ca. 16,000 before present (BP; Lampert, 1981). At the beginning of the 18th century, when European expeditions reached the island (see below), evidence suggests that Aboriginal people were not permanently settled on the island but that they used the land occasionally (Draper, 2015). In 1802-1803, a British expedition under Matthew Flinders and a French naval expedition commanded by Nicolas Baudin each sailed close to South Australia. Both expeditions landed on Kangaroo Island to explore and replenish supplies. The French Expedition captured two living emus there on 31st January 1803 (see details in Jansen, 2014, 2018). One died *en route* to Europe and the other was kept captive in Paris until its death

in 1822. The skeleton of the latter was retained at the Muséum National d'Histoire Naturelle of Paris (MNHN) (registration number MNHN-ZO-AC-A3525), and it was believed that its skin corresponds to the specimen from the Muséum d'histoire naturelle of Geneva (MHNG) (registration number MHNG-OIS-629.041; Fig. 1).

A report from the archives of the Natural History Museum of Geneva from 26th January 1828 states that the mounted specimen was bought in Paris for 150 Swiss francs in December 1827 by M.-E. Moricand, who was then the administrator of the “*Musée Académique de Genève*”. Jouanin (1959) found in the registers in Paris that “*a Cassowary from New Holland (without beak)*”, of a value of 60 francs, was indeed “given” (“*donné*”) to Moricand in December 1827. The specimen was examined by F. de Schaeck (assistant curator at the MHNG), and registered, apparently for the first time, in September 1892 during curatorial activities at the collection. A torn piece of paper, which seemed to read “*Ile Decrès*” (=Kangaroo Island), was then found

associated with the specimen. The specimen was exhibited until 1925 and then removed from its socle. It was restored in 1955 and mounted again in February 1958. The specimen, which is still on exhibition in Geneva, was not examined by S. Parker (Parker, 1984). Jouanin (1959) concluded that it was possible that this specimen corresponded to the skeleton preserved in Paris [*contra* Hume (2017) who misinterpreted Jouanin's text], in particular because the measurements of the two specimens matched perfectly. The taxidermists in 1955 indicated that the specimen has no bones at all, and that the bill was artificial. This was confirmed later by a radiography commanded by François Poplin (François Baud in litt.), which was unfortunately not preserved in the archives of the MHNG. Balouet & Jouanin (1990) pointed out that a bone could still remain in one claw of the Geneva specimen, but the evidence for this claim was uncertain. In the same article, Balouet & Jouanin (1990) confirmed the identification of the skeleton in Paris as *D. baudinianus*, according to measurements and bone details found in Parker (1984). They considered that the mounted Geneva specimen was very likely the skin of the mounted Paris skeleton. They also identified as “*D. ater*” = *D. minor* two other specimens; one other mounted specimen held in Paris (MNHN-ZO-2012-610) and another skeleton in Museo di Storia Naturale di Firenze, Italy (C.G.U. 9588; Barbagli & Violani, 2010). Worthly *et al.* (2014) analyzed the skeletal characters of *D. novaehollandiae* and *D. baudinianus* in comparison to an Oligo–Miocene fossil taxon *Emuarius gidju*. They concluded that, apart from their difference in size, the two *Dromaius* taxa did not present major qualitative skeletal differences and that subspecific rank should apply to *D. baudinianus*. Two genetic studies have also been conducted recently on the insular dwarf emus. Heupink *et al.* (2011) investigated the phylogenetic relationships between King Island and mainland emus. They sequenced two partial mitochondrial DNA (mtDNA) regions (Control Region and Cytochrome c oxidase subunit 1) and a small region of a nuclear gene (Melanocortin 1 receptor) for five bone remains from King Island. They found that for these genetic markers King Island emus fall within the diversity of modern samples of mainland emus. Thomson *et al.* (2018) sequenced more samples for mtDNA (Control Region gene only) including ancient bones from the extinct Tasmanian emus (five bones) and Kangaroo Island emus (11 bones). Their conclusions were similar and they suggested that all insular taxa are subspecies of *D. novaehollandiae*.

The first goal of this study was to re-evaluate the phylogenetic placement of the specimen held in Geneva relative to all emus previously sequenced, sampled from Kangaroo Island, the other islands, and mainland Australia. To achieve this, we used High-Throughput Sequencing to reconstruct the complete mitochondrial genome for this specimen and compared it to all sequences available for emus. Our second objective was



Fig. 1. The Geneva mounted specimen of Kangaroo Island Emu (MHNG-OIS-629.041). Photo P. Wagneur/MHNG.

to shed light on the origin of the Geneva specimen in relation with the skeleton held in Paris Museum. Unfortunately, we were not able to sample this skeleton for genetic analyses. However, the main uncertainty in the chronicle regarding the Geneva specimen is the potential presence of bones within the mount that would disqualify it from being the same individual as the Paris skeleton. Therefore, we obtained new radiographs to test whether bones were left inside the mounted specimen during the taxidermy preparation.

METHODS

DNA Extraction, Library preparation, sequencing, assembly and bioinformatics

DNA was extracted from a toe-pad sample taken from specimen MHNG-OIS-629.041 following the protocol described in Irestedt *et al.* (2006) for historical specimens. Genomic libraries were prepared using the Meyer & Kircher (2010) protocol, with slight modifications as detailed in Johansson *et al.* (2018), and included four unique index libraries to reduce PCR duplicates. All four libraries were pooled together with an additional avian sample (Paradise Parrot *Psephotellus pulcherrimus* – not part of the current study) and sequenced on a single Illumina HiSeq X lane at SciLifeLab Stockholm. Following sequencing, each library was individually processed using a custom designed workflow (available at https://github.com/mozesblom/NGSdata_tools/clean_up_raw_reads.py), which removes adapter contamination, PCR duplicates, merges overlapping read-pairs and excludes low-complexity reads and low-quality bases.

To avoid any putative bias in mitogenome reconstruction, an iterative baiting and mapping strategy was employed to reconstruct the mitogenome for the Geneva specimen. Using a subsample of polished reads (15 million) and the extant emu as a seed reference (Genbank - AF338711), we used MITObim (Hahn *et al.*, 2013) for mitogenome reconstruction and subsequently corrected the resultant sequence by mapping the complete dataset against this initial reference. Each library was mapped using BWA – mem (Li, 2013), the four corresponding BAM files being merged with Picard (<https://broadinstitute.github.io/picard/>) and variants called using FreeBayes (Garrison & Marth, 2012). Moreover, we masked sites with coverage above or below 3X the mean coverage to avoid the inclusion of putative NUMT calls and low coverage regions. Finally, uncalled sites were manually edited using Geneious R10 (<https://www.geneious.com>) by lowering the consensus threshold.

Phylogenetic analysis

We compared the complete mitochondrial genome (hereafter DromGE) of the Geneva specimen to the three available complete or near complete mitochondrial genomes of extant continental emus (for clarity hereafter called Common Emu): AF338711 and NC_002784 (both

16,711 bp; origin of the samples unknown) (Haddrath & Baker, 2001), and AY016014 (12,280 bp; captive bird) (Cooper *et al.*, 2001). We also included DromGE to previously published data sets that included both extant and extinct emus for two mitochondrial genes: COI (Heupink *et al.*, 2011) and Control Region (Heupink *et al.*, 2011; Thomson *et al.*, 2018). The COI and Control Region sequences from the complete mitogenomes of Common Emus were added to these data sets.

Divergence time

We used the complete mitogenomes to infer the most recent time of divergence of the Kangaroo Island Emu from the Common Emu. We first applied Lerner *et al.*'s (2011) average rate of sequence divergence of 1.8% per million years for the complete mtDNA genome (0.009 s/s/myr). However it has been suggested that the rates of mitochondrial genes vary among bird species and correlate with life history traits, such as body mass and generation time (Eo & DeWoody, 2010; Nabholz *et al.*, 2009; Pereira & Baker, 2006). The Common Emu is a large and long-lived species, sexual maturity is usually achieved at 2-3 years and longevity is 10-16 years in captivity (Flower, 1938; Del Hoyo *et al.*, 1992). Size varies from 150 to 190 cm, weight from 30 to 55 kg, males being smaller than females (Folch *et al.*, 2018). The life history of the Kangaroo Island Emu is unknown but, although a “dwarf” emu, it was a rather large and probably long-lived bird. We assume that DromGE was a full-grown adult, based on its 19 years in captivity (see introduction). Nabholz *et al.* (2016) suggested that body mass could be used as a proxy to estimate corrected molecular rates for molecular dating studies. We calculated a mass-corrected molecular rate of mitochondrial coding genes following the procedure described in Nabholz *et al.* (2016), using an extrapolated weight for DromGE (23 kg) based on the height of the mounted specimen (116 cm) and on the value for the smallest Common Emus (30 kg for 150 cm). We also used the equation proposed by Nabholz *et al.* (2016) for the mitochondrial coding genes (10,869 bp), based on all codon positions and the two sets of calibrations proposed in this study (“calibrations 2 and 4”, see the original article for details).

Radiography

The specimen MHNG-OIS-629.041 was X-rayed using a portable GIERTH TR 90/20 X-ray unit (OR Technology), equipped with a Toshiba tube D-0814/0.8 mm. The unit was connected to a Canon Digital Radiography System CXDI-80C. The images were visualized using a Canon Dicomacs Acquisition Software. Because of its large size, only selected sections of the mounted specimen were examined: the feet, legs, head and back.

The complete mitogenome of the Geneva specimen (DromGe, 16,713 bp after final editing) was deposited in GenBank, accession number MK625178. On average DromGE differs from the three Common Emu mitogenomes by $0.090\% \pm 0.008$. Lerner's *et al.* (2011) rate applied to emus suggested that DromGE diverged ca. 100,000 years ago from this set of three Common

Emus (mean $93,074 \pm 23,029$ years). The rates for emus corrected for size, used as a proxy for generation time, varied from 0.004455221 s/s/myr (calibration 2) to 0.007377136 s/s/myr (calibration 4). Applied on the coding genes of the mitochondrial genomes, it suggested that DromGE and the three Common Emus diverged between 206,510 years ago (calibration 2) and 124,716 years ago (calibration 4).

Fig. 2. Haplotype networks. (A) Control Region (563 bp), modified from Thomson *et al.* (2018). (B) COI (1,544 bp), modified from Heupink *et al.* (2011). Circles are proportional to the number of individuals but the scales are different for the two genes (see details in the original studies). The black circles represent intermediate or unsampled haplotypes. “DromGE “indicates the specimen from Geneva Museum.

Control region and COI data sets

We added DromGE's sequence to the genetic haplotype network provided by Thomson *et al.* (2018) for the Control Region (partial gene, 563 bp) (Fig. 2A). This individual has a new haplotype, different by one substitution to haplotypes H7 (predominantly found in Common Emus) and H1 (Common Emus, including AF338711 and NC_002784; AY016014 was not sequenced for this gene). It differs by two to three substitutions to the three haplotypes found in bones from Kangaroo Island: H6, also found in mainland samples; H2, found also in mainland and Tasmanian samples; and H5, found in a single Kangaroo Island bone.

Regarding the COI gene (Fig. 2B), DromGE shares Haplotype A with Common Emus. It differs by two mutations from the haplotype GH found in King Island Emus. Bones from Kangaroo Island Emus were not sequenced for this gene. Common Emus also share three other haplotypes, DE (including AF338711, NC_002784 and AY016014), I and J.

Radiography

Images were taken at different sections of the specimen: all showed that no bones were conserved in the preparation, in particular in the toes (Fig. 3). No elements of the skull were conserved either, and the bill is artificial and supported by wires.

DISCUSSION

The origin of the Geneva specimen

The genetic data do not provide definitive elements regarding the origin of the Geneva specimen, DromGE. Because no unique haplotypes exist for Kangaroo Island or King Island populations, the putative geographic origin of Kangaroo Island for this specimen can be neither confirmed nor refuted. The new radiographs show the absence of remaining bones in the preparation, confirming previous statements found in the museum's archives. Balouet & Jouanin's (1990) claim of a remaining bone in a toe of the Geneva specimen might have been based on the fact that a bone was missing from the Paris skeleton (left foot, digit II, proximal phalanx; A. Cibois pers. obs.). It is common practice to keep the bones in the legs and feet of birds, even in large birds such as ratites (Davie, 1894; Larsen, 1945). However, skin and skeleton for an individual large bird could also have been sold separately, resulting in boneless mounted specimens. For instance, we checked the foot of another old mounted specimen in Geneva (a Common Emu acquired in 1926; MHNG 837.071) and no bones were preserved in that one either. Thus, the lack of bones in the Geneva specimen is not absolute proof that it is the same individual as the Paris skeleton. Nonetheless, we conclude that after considering the historical evidence and correspondence of the measurements of both specimens, the hypothesis

of Kangaroo Island provenance for DromGE remains the most likely.

Divergence time and isolation of the Kangaroo Island Emu

Previous studies on the extinct emus showed that the island populations had a subset of the mitochondrial genetic diversity found in Common Emus. The numbers of extant emus on mainland Australia have increased ca. tenfold since colonization by Europeans, with some fluctuation in numbers, as they benefited from increased water supplies and the erection of fences to exclude predators like dingos (*Canis lupus dingo*) (Folch *et al.*, 2018; Pople *et al.*, 2000). Their numbers are currently considered as stable, and no recent bottleneck has led to the erosion of genetic diversity. The mitogenome of the specimen held in Geneva is very weakly divergent from that of Common Emus. Taken together, all the mitochondrial results on island populations of emus support a shallow divergence between the extant mainland population and the extinct populations from King Island, Kangaroo Island and Tasmania. An alternative explanation could be that these results are biased by introgression events that led to the capture of the mainland mitogenome (the most important population in number) by that of the three island populations. However, the topology of the largest haplotype network (for the Control Region, Fig. 2A) based on 134 individuals, is consistent with incomplete lineage sorting, the island taxa having both shared and unique haplotypes. Based on these results, we agree with Heupink *et al.* (2011) that all emu taxa should be considered as subspecies of *Dromaius novaehollandiae*, *D. n. diemenensis* (Tasmania), *D. n. minor* (King Island) and *D. n. baudinianus* (Kangaroo Island).

Heupink *et al.* (2011) and Thomson *et al.* (2018) discussed the morphological differences between the insular dwarf populations and the mainland emus. They based their analyses on the hypothesis of a very recent isolation of these islands during the Holocene, when sea-level rose after the Last Glacial Maximum and flooded the straits connecting the islands: 10,000 years ago for Kangaroo Island and the mainland, 12,000 years ago for King Island and Tasmania, and 14,000 years ago for Tasmania and the mainland (Lambeck *et al.*, 2014). These short time frames would imply that the significant reduction of size occurred very rapidly for such long-lived birds. Our divergence time analysis, although based on a single insular individual and on Common Emus of unknown origin, suggests on the other hand that the isolation of the emus of Kangaroo Island, or some population structure on nearby parts of mainland Australia, took place during the Pleistocene, at least ca. 100,000 years ago. Probably the most parsimonious scenario implies the mid-Pleistocene episodes of sea-level variations that could have triggered the isolation of emus on Kangaroo Island. During the glacial Marine Isotope Stage 6 (which began 190,000 years ago), sea-level dropped ca. 100 m below present sea level, permitting a land connection

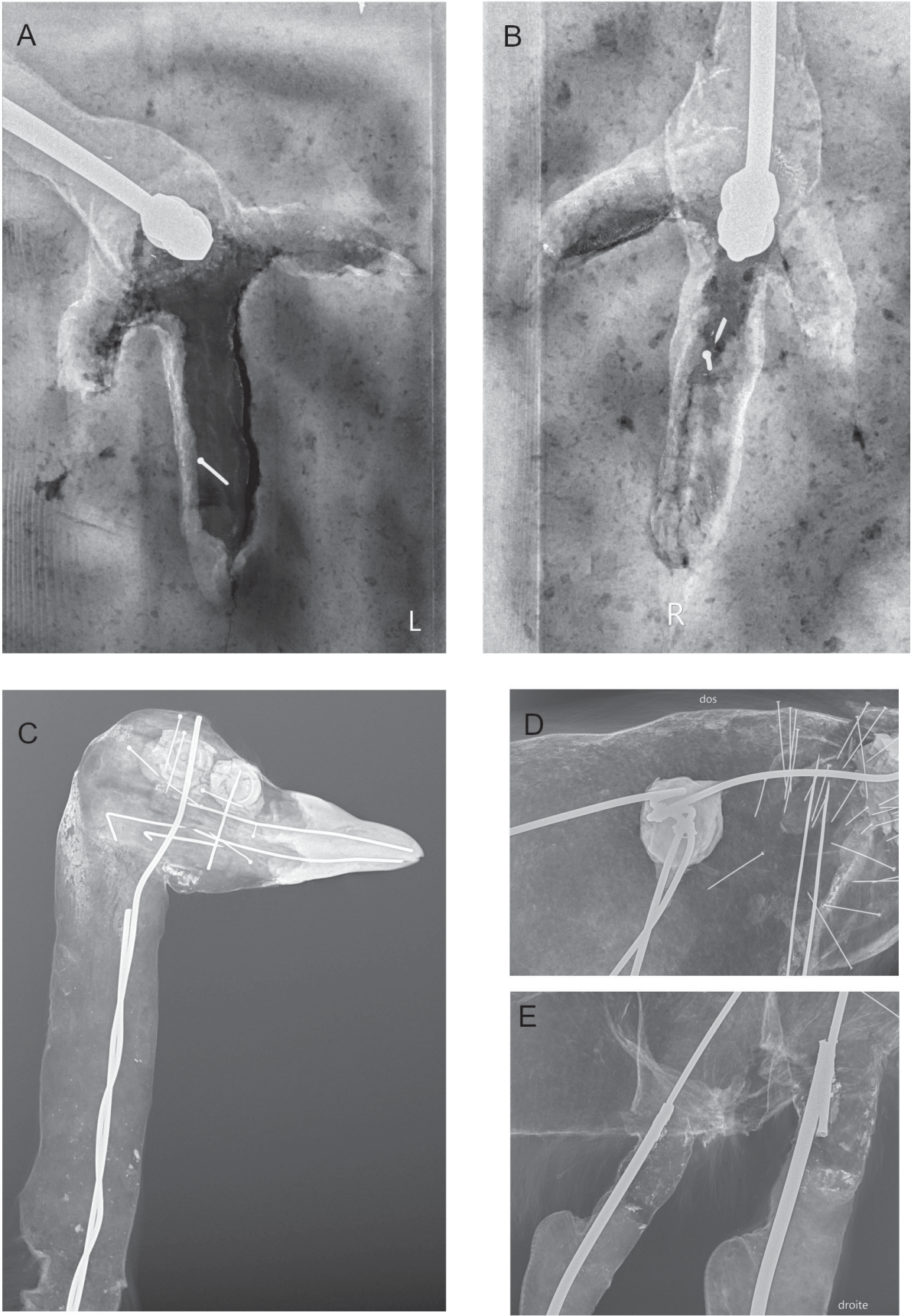


Fig. 3. Radiographs of the specimen MHNG-OIS-629.041. (A) Left foot. (B) Right foot. (C) Head and neck. (D) Back. (E) Thighs and upper legs.

between Kangaroo Island and Australia (Elderfield *et al.*, 2012). During the Last Interglacial Maximum (Marine Isotope Stage 5e, which began 130,000 years ago and ended about 115,000), sea level was up to 6 m or 9 m above present sea surface in the Pacific Ocean (Dickinson, 2001; Hearty *et al.*, 2007), inundating the 40 m deep Backstairs Passage between Kangaroo Island and Australia and leading to the isolation of the former's emu population. This period resulted in the inundation of many land surfaces in the Pacific Ocean, and in particular on low atolls, leading for instance to the extirpation of landbird populations in the Eastern Pacific (Cibois *et al.*, 2010). Our new estimation of the divergence time for the Kangaroo Island Emu, based on a molecular clock, suggests then that the morphological differentiation (i.e. dwarfing) of this insular population, due to selection or genetic drift, took place over a longer period of time than proposed by previous studies.

Kangaroo Island, a biodiversity refugium in past and recent times

Kangaroo Island is one of the six major biodiversity hotspots identified across the state of South Australia for plants (Guerin *et al.*, 2016), with 45 endemic species being inventoried by Kinnear *et al.* (1999). The island also includes three recently described species: two lichens (Kantvilas & Kondratyuk, 2013) and one fungus (Catchside *et al.*, 2015). Along with other parts of southern Australia, Kangaroo Island is thought to have acted as a refugium for Mediterranean and semi-arid plants during colder and drier periods (Byrne, 2008). However, such a high level of endemism has not been found for animals. The only endemic mammal, the Kangaroo Island Dunnart (*Sminthopsis aitkeni*), presents shallow morphological and genetic differentiation from closely related mainland taxa and it has recently been reclassified as a subspecies of the Sooty Dunnart (*Sminthopsis fuliginosus*) by Kemper *et al.* (2012). The situation is similar for birds, in which the only endemic species, the Kangaroo Island Emu, has been reevaluated as warranting only subspecific rank based on its shallow genetic difference from mainland populations (Thomson *et al.*, 2018; this study). In fact, the majority of organisms for which genetic studies have been conducted show a weak differentiation between Kangaroo Island and mainland Australia: Western Grey Kangaroos (*Macropus fuliginosus*) (Neaves *et al.*, 2009), Tawny Dragon Lizard (*Ctenophorus decresii*) (McLean *et al.*, 2014), *Labiosimplex australis* (a parasitic nematode) (Chilton *et al.*, 2009), Narrow-leaf Hopbush (*Dodonaea viscosa angustissima*) (Christmas *et al.*, 2017). A more complex genetic pattern was found in the Crimson Rosella complex (*Platycercus elegans*), in which the subspecies endemic to Kangaroo Island showed introgression from nearby mainland populations (Joseph *et al.*, 2008). 17 subspecies of birds are endemic to Kangaroo Island (Schodde & Mason, 1999) but most of them have not been subjected to molecular analyses.

Because some pristine habitats are still present on Kangaroo Island, the island acted as a recent refugium for species endangered or extirpated from mainland Australia. For instance, the last population of an endangered subspecies of the Glossy Black-Cockatoo (*Calyptorhynchus lathami halmaturinus*) is now restricted to Kangaroo Island. The diet of this bird is specialized to the seeds of sheoaks (*Allocasuarina verticillata*). Extensive tracts of this tree and the bird have both disappeared from nearby parts of mainland Australia, the bird last having been recorded in the late 1970s (Joseph, 1989; Berris *et al.*, 2018; Schodde *et al.*, 1993). Species on Kangaroo Island have also been preserved from alien predators or competitors that have not reached the island. It is the case for the Bush Rat (*Rattus fuscipes greyii*), which populations on Kangaroo Island present higher genetic diversity than those on the mainland (Hinten *et al.*, 2003).

The entomofauna of Kangaroo Island is poorly known, but a recent discovery brought to light an endemic moth species. This new species, called the “enigma moth”, exhibits a unique combination of morphological characters and it was placed in a new family Aenigmatineidae (Kristensen *et al.*, 2015). A molecular analysis showed that it was basal in the phylogenetic tree of the moth families, suggesting an old origin. The current restricted distribution of such an ancient family suggests that the Kangaroo Island moth could be either a paleo-endemic that had a wider distribution in the past and disappeared from most of its range due to climate modifications – or a pseudo-endemic (i.e. a species with a large distribution anthropogenically reduced by habitat loss). These alternative hypotheses, which might also apply to several endemic plants, both support the idea that Kangaroo Island acted more as a museum than as a cradle for biodiversity [*sensu* Stebbins (1974)]. Most of its diversity did not evolve *in situ*, but the island, by its ancient and recent history, has been a refugium for many plants and animals.

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