Molecular Phylogenetics and Evolution 62 (2012) 97–108

Contents lists available at SciVerse ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Phylogenetic relationships of living and recently extinct bandicoots based on nuclear and mitochondrial DNA sequences

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ARTICLE INFO

Article history:
Received 4 November 2010
Revised 6 September 2011
Accepted 12 September 2011
Available online 11 November 2011

Keywords:
Marsupial
Bandicoot
Peramelemorphia
Phylogeny

ABSTRACT

Bandicoots (Peramelemorphia) are a major order of australidelphian marsupials, which despite a fossil record spanning at least the past 25 million years and a pandemic Australasian range, remain poorly understood in terms of their evolutionary relationships. Many living peramelemorphians are critically endangered, making this group an important focus for biological and conservation research. To establish a phylogenetic framework for the group, we compiled a concatenated alignment of nuclear and mitochondrial DNA sequences, comprising representatives of most living and recently extinct species. Our analysis confirmed the currently recognised deep split between *Macrotis* (Thylacomyidae), *Chaeropus* (Chaeropodidae) and all other living bandicoots (Peramelidae). The mainly New Guinean rainforest peramelids were returned as the sister clade of Australian dry-country species. The wholly New Guinean Peroryctinae was sister to Echymiperinae. The poorly known and perhaps recently extinct Seram Bandicoot (*Rhynchomeles*) is sister to Echymipera. Estimates of divergence times from relaxed-clock Bayesian methods suggest that living bandicoots originated in the late Oligocene or early Miocene, much earlier than currently thought based on fossils. Subsequent radiations within Peramelemorphia probably took place on the Australian mainland during the Miocene, with diversification of rainforest taxa on the newly emergent New Guinean landmasses through the middle-late Miocene and complete establishment of modern lineages by the early Pliocene.

1. Introduction

Although the evolutionary relationships of Peramelemorphia (commonly known as bandicoots) within Marsupialia have been clarified by recent molecular studies (Meredith et al., 2008), the taxonomy of its various genera and species is still confused. The 23 species of living or recently extinct bandicoots are currently classified into three families (Groves, 2005); Chaeropodidae, Thylacomyidae and Peramelidae. Peramelidae is the most diverse, comprising three subfamilies – the mainly New Guinean forest-dwelling Echymiperinae and Peroryctinae, and the largely Australian, dry-country Peramelinae. Echymiperinae contains *Echymipera*, *Rhynchomeles* and *Microperoryctes*; Peroryctinae includes only *Peroryctes*; Peramelinae accommodates *Isoodon* and *Perameles*.

In their concept of peramelemorphian relationships based on morphology, Groves and Flannery (1990) placed the endemic New Guinean *Peroryctes* together with *Microperoryctes*, *Echymipera* and *Rhynchomeles* in a distinct family (Peroryctidae) under the caveat that "…. three features… in which *Peroryctes* resembles the *Echymipera* clade… are most likely the result of convergence" (Groves and Flannery, 1990, p. 6). Since then, the broader genus-level evolution of these taxa has been studied using mitochondrial (mtDNA) and nuclear (nDNA) gene sequences (Westerman et al., 1999, 2001; Meredith et al., 2008). In contrast, comparatively little attention has been paid to peroryctin and echymiperin species, in part due to the inherent difficulty of obtaining useable tissue samples for DNA extraction. For example no tissues and few museum samples are presently available for the Mouse Bandicoot (*Microperoryctes murina*) or the recently described *Echymipera davidi* and *Echymipera echinista*. A similar problem exists for the enigmatic *Rhynchomeles prattorum*, which is known from a total of seven specimens collected in 1920 from the Mt. Mansuela area of central Seram. This island taxon has not been captured or seen since that time and may be extinct (Flannery, 1995). Tate (1948) and Groves and Flannery (1990) aligned *Rhynchomeles* with *Echymipera*, the latter citing several derived morphological characters uniting them.

Species identity, relationships and distributions within *Microperoryctes* are unclear. In addition to the rare *M. murina* (known only

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doi:10.1016/j.ympev.2011.09.009

from three specimens from the Weyland Range of West Papua), *Microperoryctes longicauda* and *Microperoryctes papuensis* are also recognised. Helgen and Flannery (2004) recently described a new form (*Microperoryctes aplini*) from the Vogelkop Peninsula of New Guinea and advocated the separation of *Microperoryctes ornata*, from *M. longicauda*.

The Giant Bandicoot *Peroryctes broadbenti* is the largest of all living bandicoots, weighing up to 5 kg, and is restricted to hill forests on the southeastern peninsula of Papua New Guinea. This bandicoot has been variously treated as a subspecies of the more common and widespread *Peroryctes raffrayana* (Tate, 1948; Laurie and Hill, 1954) or as a distinct species (Ziegler, 1977; George and Maynes, 1990; Flannery, 1995). Aplin et al. (2010) recently documented its distribution and morphological characters.

The primarily Australian genera *Perameles* and *Isoodon* are readily distinguishable using morphological criteria (e.g. Tate, 1948), but taxonomic boundaries within each are poorly defined. Currently three species of *Isoodon* are recognised – *I. auratus*, *I. macrourus* and *I. obesulus* – although up to ten have been established in the past (Westerman and Krajewski, 2001). Most of these geographically defined forms are regarded as subspecies (Strahan, 1995), but recent molecular work suggests that some (e.g. *I. o. peninsulae*) may warrant species status (Close et al., 1990; Pope et al., 2001). On the other hand, Pope et al. (2001) and Zenger et al. (2005) suggested that *Isoodon auratus* might be subordinate to *Isoodon obesulus* based on variation in a ~500 nucleotide mitochondrial D-Loop dataset.

Four species of *Perameles* are presently considered valid (*P. bougainville*, *P. eremiana*, *P. gunnii* and *P. nasuta*) one of which (*P. eremiana*) is recently extinct. *P. eremiana* has sometimes been considered as a sub-specific form of *Perameles bougainville* (Strahan, 1995) which itself, though formerly widespread with two or more distinct subspecies (Friend, 1990), is now restricted to a few localities in Shark Bay (Western Australia).

This paper provides the first concatenated nuclear and mitochondrial gene-sequence dataset designed to evaluate current evolutionary/biogeographical hypotheses relating to species within Peramelemorphia.

2. Materials and methods

2.1. Taxon sampling

We obtained tissue samples (Table 1) from most living bandicoot species together with the recently extinct taxa *R. prattorum*, *Perameles eremiana*, and *Chaeropus ecaudatus* (see Westerman et al., 1999; Meredith et al., 2008 for tissue source details on *C. ecaudatus*). An ear clip from *P. broadbenti* captured by hunters near Ajoa, New Guinea was stored in 95% ethanol after collection and prior to extraction. Dried muscle and connective tissue were obtained from the cranium of the *P. broadbenti* holotype (AM A3238), a scraping from a museum skin of *Rhynchomeles* (AM M29415), and a specimen of *P. eremiana* (C5864) from the National Museum of Victoria. For some geographically widespread species of *Peroryctes*, *Echymipera* and *Microperoryctes*, we utilised multiple exemplars from across the species' ranges in order to assess genetic variation. The four rare echymiperins *E. davidi* (Flannery, 1990), *E. echinista*, *Microperoryctes longicauda* (*sensu* Helgen and Flannery, 2004) and *M. murina* proved difficult to source and were therefore not included in the study (see Table 1). However, we did include a *Microperoryctes* specimen captured at Tembagapura (Snow Mts, West Papua) which may represent a novel species (K. Helgen, pers. commun.). We here note that animals identified as *M. longicauda* in previous studies (Westerman et al., 2001; Meredith et al., 2008) have since been referred to *M. ornata* as defined by Helgen and Flannery (2004); *M. longicauda* (*sensu* Helgen and Flannery, 2004) is thus restricted to specimens from the Vogelkop Peninsula.

2.2. Molecular protocols and dataset assembly

Tissues were placed in 1.5 ml microfuge tubes with 0.4 ml 1 mM Tris-EDTA, to which 40 μl of 10% SDS was added. Following total digestion with proteinase K at 55 °C, the solution was shaken with chloroform: isoamyl alcohol (24:1) and centrifuged to separate aqueous and organic layers, DNA was precipitated from the aqueous phase with 2.5 volumes of ice-cold absolute ethanol. The precipitate was dried and resuspended in 1 mM Tris-EDTA and digested for 1 h with RNAse, then with Proteinase K for a further hour before re-extraction as above. Final DNA precipitates were resuspended in a small volume of 1 mM Tris, 1 mM EDTA. PCR amplification, direct sequencing, and sequence alignment procedures were as detailed in Krajewski et al. (1997), Burk et al. (1998), and Westerman et al. (1999). The nuclear protein coding genes ApoB, BRCA1, IRBP, RAG1, vWF have consistently been shown to resolve marsupial intergeneric relationships (Meredith et al., 2009) so these were sequenced along with the mitochondrial genes (12S rRNA, cytochrome *b* and the 3′ portion of 16S rRNA) from all taxa following the methodologies of Meredith et al. (2008) and Westerman et al. (2001), respectively.

Ingroup monophyly and the phylogenetic position of Peramelemorphia relative to various outgroup marsupial lineages has been demonstrated by Westerman et al. (2001) and Meredith et al. (2008). Consequently, except for molecular dating analyses, we employed only a limited number of outgroup taxa all from Dasyuromorphia (species of *Antechinus*, *Dasyurus*, *Phascogale*, *Phascolosorex*, *Planigale Myrmecobius* and *Thylacinus*). We constructed a dataset of 9979 nucleotides for representatives of each available bandicoot species incorporating five nuclear (6137 nucleotides) and three mitochondrial genes sequences (~3600 nucleotides representing almost 23% of the mitochondrial genome). 12S rRNA sequences (958 nucleotides) from a much larger sampling of *Microperoryctes* and *Peroryctes* as well as from a number of the available subspecies of *Isoodon* and *Perameles* were included in order to cover as much of their species' ranges as possible. Sequences were aligned as described in Meredith et al. (2008); protein-coding genes were conceptually translated to check for premature stop codons, frameshifts and indels. No indications of pseudogene amplifications were found. Alignments of incomplete sequences were filled out with the missing data code ("?") for phylogenetic analyses.

2.3. Phylogenetic analyses and statistical tests

Our rationale for combining nuclear genes in phylogenies of marsupials has been dealt with elsewhere (Meredith et al., 2010). Maximum parsimony (MP) was implemented with TNT (Goloboff et al., 2008) distributed by the Willi Hennig Society in order to generate bootstrap support values for 1000 pseudoreplicates of the large dataset. Maximum likelihood (ML) and Bayesian analyses were performed with RAxML 7.2.8 (Stamatakis, 2006), and MrBayes 3.1.2 (Huelsenbeck and Rondquist, 2001; Rondquist and Huelsenbeck, 2003), respectively. ML and Bayesian analyses were performed with unpartitioned and partitioned datasets. In the latter case we allowed each of seven partitions (ApoB, BRCA1, IRBP, RAG1, vWF, cytochrome b, mt rRNA) to have its own model of sequence evolution. Partitioned RAxML analyses employed a GTR + Γ model for each partition. Best-fit models of molecular evolution for Bayesian analyses were chosen using the Aikake Information Criterion as implemented by jModelTest (Posada, 2008) and were as follows: GTR + Γ (ApoB, BRCA1, RAG1, cytochrome b, mt rRNA); HKY + Γ (IRBP); and SYM + Γ (vWF). Support for nodes on