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Abstract.   Southern brown (*Isoodon obesulus*) and golden (*Isoodon auratus*) bandicoots are iconic Australian marsupials that have experienced dramatic declines since European settlement. Conservation management programs seek to protect the remaining populations; however, these programs are impeded by major taxonomic uncertainties. We investigated the history of population connectivity to inform subspecies and species boundaries through a broad-scale phylogeographic and population genetic analysis of *Isoodon* taxa. Our analyses reveal a major east–west phylogeographic split within *I. obesulus*/ *I. auratus*, supported by both mtDNA and nuclear gene analyses, which is not coincident with the current species or subspecies taxonomy. In the eastern lineage, all Tasmanian samples formed a distinct monophyletic haplotype group to the exclusion of all mainland samples, indicative of long-term isolation of this population from mainland Australia and providing support for retention of the subspecific status of the Tasmanian population (*I. o. affinis*). Analyses further suggest that *I. o. obesulus* is limited to south-eastern mainland Australia, representing a significant reduction in known range. However, the analyses provide no clear consensus on the taxonomic status of bandicoot populations within the western lineage, with further analyses required, ideally incorporating data from historical museum specimens to fill distributional gaps.

Additional keywords: *Isoodon auratus*, *Isoodon obesulus*.

Introduction

It is often assumed that the taxonomy (species and subspecies classifications) and distribution of mammal species are now well established. However, even for well known species of conservation significance this assumption can be incorrect, and, in such cases, inaccuracies in taxonomic designations may have

negative consequences for conservation management (Mace 2004; Gutiérrez and Helgen 2013; Zachos *et al.* 2013; Burgin *et al.* 2018). For example, poor taxonomy can fail to identify that an apparently widespread and low-risk species consists of multiple species or subspecies, some of which are rare or threatened (e.g. Adams *et al.* 2014; Ely *et al.* 2017). On the other

hand, significant conservation resources may be applied to the protection of taxa that are conspecific with a common and widespread species (e.g. Laerm *et al*. 1982; Zachos *et al*. 2013).

The importance of resolving taxonomic uncertainties for conservation management of mammals is particularly relevant in Australia, where more than 10% of mammal species have become extinct since European settlement around 220 years ago, and a significant proportion (~21%) are now assessed to be threatened under International Union for Conservation of Nature Red List criteria (Woinarski *et al*. 2015). Ground-dwelling mammals in the critical weight range of 35 g to 5.5 kg have been particularly impacted (Burbidge and McKenzie 1989; McKenzie *et al*. 2007), largely through predation by introduced feral cats (*Felis catus*) and red foxes (*Vulpes vulpes*) (Radford *et al*. 2018). Consequently, populations of some species now survive only on islands or in fenced exclosures on the mainland of Australia (e.g. Moseby *et al*. 2009; Woinarski *et al*. 2015; Legge *et al*. 2018). There is an ongoing need for conservation management of these remnant populations, with translocations currently being employed in several states of Australia to establish new populations and augment genetic diversity levels in existing populations (Weeks *et al*. 2011, 2017; Morris *et al*. 2015).

Two Australian species in the critical weight range that have seen dramatic declines in abundance and distribution are the southern brown bandicoot (*Isoodon obesulus* (Shaw, 1797)) and the golden bandicoot (*Isoodon auratus* (Ramsay, 1887)) (Paull 1993, 1995; Department of Environment and Conservation (NSW) 2006; Coates *et al*. 2008; Warburton and Travouillon 2016). These declines led to the National listing of the subspecies *I. o. obesulus* (Shaw, 1797) (southern and eastern mainland Australia) as Endangered, and the subspecies *I. o. nauticus* Thomas, 1922 (Franklin and St Francis islands of South Australia (SA)) as Vulnerable (*Australian Environment Protection and Biodiversity Conservation* (EPBC) *Act Status 2012*). The mainland population of *I. auratus* is also listed as Vulnerable (EPBC Act 2012), with subspecies restricted to small populations on several offshore islands. Population translocations for *I. auratus* and *I. o. obesulus* have recently been enacted or are planned (e.g. Ottewell *et al*. 2014; Pedler *et al*. 2018; Robinson *et al*. 2018). Given these current and future conservation efforts, it is important that all key management decisions reflect a robust taxonomic framework, underpinned by the detailed population genetic analyses required to ensure that any deliberate mixing of populations does not result in outbreeding depression (loss of fitness due to crossing genetically divergent populations: Frankham *et al*. 2011). However, resolving the conservation status of these species and subspecies and developing strategies for their conservation management is hampered by significant and ongoing taxonomic uncertainty.

The taxonomy of species and subspecies within the genus *Isoodon* Desmarest, 1817 has been in a state of flux since the 19th century (Pope *et al*. 2001), with a range of taxonomic views continuing to be expressed in recent times, particularly for subspecies of *I. obesulus* (reviewed by Jackson and Groves 2015). Jackson and Groves (2015) listed four subspecies within *I. obesulus*: *I. o. obesulus*, *I. o. fusciventer* (J. Gray, 1841) from south-west Western Australia (WA), *I. o. affinis* (Waterhouse,

1846) from Tasmania and the insular *I. o. nauticus* (see Fig. 1 distribution map). More recently, *I. o. fusciventer* has been proposed as a distinct species, *I. fusciventer*, by Travouillon and Philips (2018), based mainly on phylogenetic position and teeth morphology. The related species *I. peninsulae* Thomas, 1922 from north Queensland was previously considered a subspecies of *I. obesulus*, but was also recently given specific rank following genetic studies by Westerman *et al*. (2012) and both morphological (skull characteristics) and genetic studies by Pope *et al*. (2001). However, to date, the World listing of mammal species (https://www.departments.bucknell.edu/biology/resources/msw3/) recognises just two subspecies of *I. obesulus* (*I. o. obesulus* and *I. o. nauticus*), based originally on proposals by Groves (2005), and this taxonomic scheme has been utilised in several recent publications (Woinarski *et al*. 2014; Driessen and Rose 2015). Within the golden bandicoot, *I. auratus* (Ramsay, 1887), Jackson and Groves (2015) listed three subspecies: *I. a. auratus* (Ramsay, 1887) from the Kimberley region of WA, *I. a. arnhemensis* Lyne & Mort, 1981 from the Northern Territory (NT), and *I. a barrowensis* (Thomas, 1901) from Barrow Island, WA, but the taxonomic status of *I. a. arnhemensis* has also been questioned (Westerman *et al*. 2012).

The recent taxonomic changes have been strongly influenced by the genetic studies of Westerman and Krajewski (2000), Pope *et al*. (2001), Zenger *et al*. (2005), Westerman *et al*. (2012) and, more recently, Travouillon and Philips (2018). With the exception of the latter two studies, which utilised nuclear gene markers, these studies were based on mitochondrial DNA (mtDNA) sequence data. The three earlier studies indicated that there was little genetic differentiation between *I. obesulus* and *I. auratus* and suggested that they should be synonymised as a single species (Pope *et al*. 2001; Zenger *et al*. 2005). In contrast, morphological data, indicate that *I. obesulus* and *I. auratus* can be distinguished by a range of characters, including size, skull and teeth characters and fur colour (Lyne and Mort 1981; Pope *et al*. 2001; Menkhorst and Knight 2011; Travouillon and Philips 2018). Zenger *et al*. (2005) suggested that there was little support for the genetic distinction of *I. o. nauticus* (suggesting its inclusion within *I. o. obesulus*), and also considered that *I. o. affinis* would most likely be placed within *I. o. obesulus* based on the allozyme data of Close *et al*. (1990). These taxonomic suggestions have major implications for the national conservation status of the endangered subspecies *I. o. obesulus*, because the Tasmanian subspecies, *I. o. affinis*, is not currently listed, and together these populations would be considered secure and of sufficient size to revoke the Endangered status of *I. o. obesulus* (e.g. see Woinarski *et al*. 2014). It is, therefore, important to consider the reliability of these existing genetic analyses for assessing species and subspecies boundaries of *Isoodon* bandicoots.

A major issue with the above conclusions on subspecies/species status is that each study included very few samples (e.g. Close *et al*. 1990; Westerman *et al*. 2012; Travouillon and Philips 2018) and, generally, very little sequence data (e.g. mtDNA Control Region data (~600 bp) only in the studies by Zenger *et al*. (2005) and Pope *et al*. (2001)). In particular, there have been limited mtDNA sequence data available for the Tasmanian subspecies, *I. o. affinis*, as no samples of this taxon were included in the studies by Pope *et al*. (2001) and Zenger
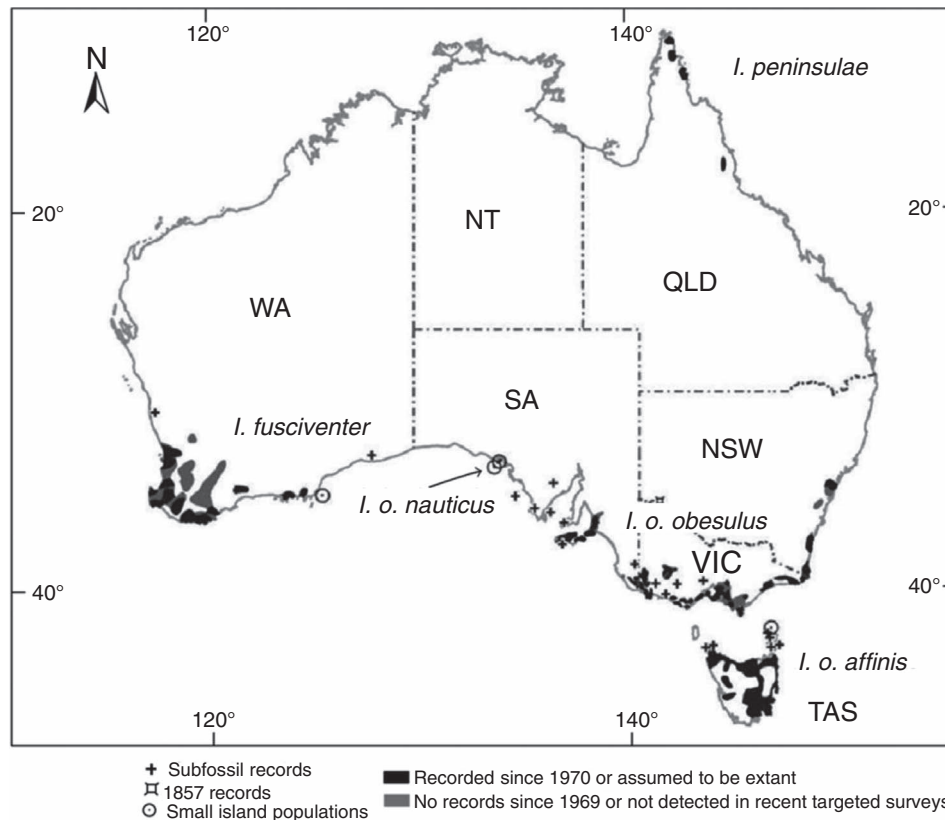
Fig. 1. Historical and extant distribution of subspecies of *I. obesulus* and closely related species *I. fusciventer* and *I. peninsulae*. The map was adapted from Paull *et al.* (2013).

et al. (2005), while the analysis by Westerman *et al*. (2012) included only one Tasmanian sample that grouped with a single *I. o. obesulus* individual from eastern Australia. Ideally, studies to inform taxonomic status should sample broadly from across the distribution of each species or subspecies and use multiple genetic loci (mtDNA and nuclear) to assess whether there is population differentiation associated with each discrete taxonomic unit. Long-term isolation of populations, either through allopatry (geographic) or reproductive isolation, leaves distinct patterns in gene trees over time, with a progression from polyphyly to paraphyly and then reciprocal monophyly of the discrete allopatric populations or reproductive units, although the timing of this progression is strongly influenced by population size (Avise 1994; Kingman 2000). Hence, resolution of taxonomic entities in southern brown and golden bandicoots still requires genetic studies that include appropriate sampling of each purported taxon, covering their full distribution in Australia, to assess their phylogeographic and population genetic structure and to determine whether they show long-term isolation from other taxa.

In comparison with studies at the taxon level, Li *et al*. (2014) recently carried out a genetic (microsatellite and mtDNA sequence) study of SA populations of *I. o. obesulus*, including populations from the Mt Lofty Ranges, Kangaroo Island and the south-east of SA. This study found that the Mt Lofty Ranges/Kangaroo Island populations were genetically highly distinct from a population in the south-east of SA and western Victoria;

the populations showed reciprocal monophyly and were considered to represent distinct Evolutionarily Significant Units (ESUs) based on the criteria of Moritz (1994). Furthermore, the Mt Lofty Ranges/Kangaroo Island populations were found to group with samples of *I. fusciventer* from WA, to the exclusion of all the east coast *I. o. obesulus* individuals, suggesting that the current recognised distribution of the latter subspecies may not represent an evolutionary unit (Li *et al*. 2014). Here, we have broadened the study of Li *et al*. (2014) to assess the phylogeographic structure of *Isoodon* taxa from across Australia and determine whether the subspecies and species are associated with distinct phylogeographic lineages (mtDNA sequence data) or fixed nuclear gene haplotypes. We also include evaluation of an earlier large-scale population genetic analysis based on allozyme electrophoresis for comparison with the phylogeographic analyses.

Methods

Sample overview

The analyses presented in this paper incorporate two largely independent genetic studies that have good coverage of samples from across the range of *Isoodon* in Australia: a recently gathered dataset based on DNA sequence analyses of mtDNA and nuclear gene markers, and an earlier one based on allozyme electrophoresis conducted between 1990 and 2013. Several of the same samples were analysed in both studies (*n* = 13; see

supplementary file 1; Table S1, Supplementary Material), but as allozyme electrophoresis requires fresh tissue samples, it was not feasible to use this technology on many of our recent samples (notably all the Tasmanian tissues), which included road kill tissues, ear tissue samples from ecological studies (Li *et al*. 2015, 2016), or old samples of DNA from past genetic studies (e.g. Pope *et al*. 2001). With respect to naming taxa within *Isoodon*, we have largely followed the taxonomic scheme of Jackson and Groves (2015), but have tentatively accepted *I. fusciventer* as a distinct species (Travouillon and Philips 2018). This scheme lists *I. o. obesulus* from east coast and southern Australia (including the Mt Lofty Ranges and Kangaroo Island in SA), *I. fusciventer* from south-west WA, *I. o. affinis* from Tasmania, *I. o. nauticus* from islands off the Eyre Peninsula in SA, *I. peninsulae* in northern Queensland, *I. auratus barrowensis* from Barrow Island in WA and *I. auratus auratus* from northern WA and the NT (see Fig. 2).

DNA sequencing analyses

DNA sequence analyses were based on a total of 158 samples from populations of *I. obesulus*, *I. fusciventer*, *I. auratus*, and *I. peninsulae* covering the distribution of the species and subspecies in Australia. These samples include 21 samples from Tasmania (including Flinders Island), 24 samples from south-eastern mainland Australia (New South Wales (NSW), Victoria and south-east SA), 12 samples from the Franklin and St Francis

Islands (SA), 11 samples from the Mt Lofty Ranges (SA), 3 samples from Kangaroo Island (SA), 5 samples of *I. peninsulae* from north Queensland, 28 samples of *I. auratus* from WA and the NT, and 35 samples of *I. fusciventer* from WA (Fig. 2; Table S1, Supplementary Material). A further 17 samples from the related species *Isoodon macrourus* were also included in the genetic analyses. DNA was extracted from skin or liver tissue using the Gentra Puregene extraction kit and methods specified by the manufacturer (Gentra Systems Inc.), or by using the salting-out method described in MacDonald *et al*. (2011).

Two mitochondrial gene segments and three nuclear gene fragments were amplified: the noncoding control region (*CR*), the NADH dehydrogenase subunit 2 (*ND2*); protein-coding portions of the breast and ovarian cancer susceptibility gene (*BRCA1*, exon 11), recombination activating gene-1 (*RAG1*, intronless) and vonWillebrand factor gene (*vWF*, exon 28). The primers used to amplify these genes and their annealing temperatures are listed in Table 1. PCR amplifications were carried out in 25-μL volumes containing 0.1 U AmpliTaq Gold® polymerase (Applied Biosystems), 1 × Gold Buffer (Applied Biosystems), 0.20 mM dNTPs, 2.5 mM MgCl₂, 0.5 μM of each primer and ~100 ng genomic DNA. Thermocycling conditions were: initial activation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 48–55°C for 45 s, and extension at 72°C for 60 s; and a final extension at 72°C for 3 min. PCR products were either (a) purified using Millipore
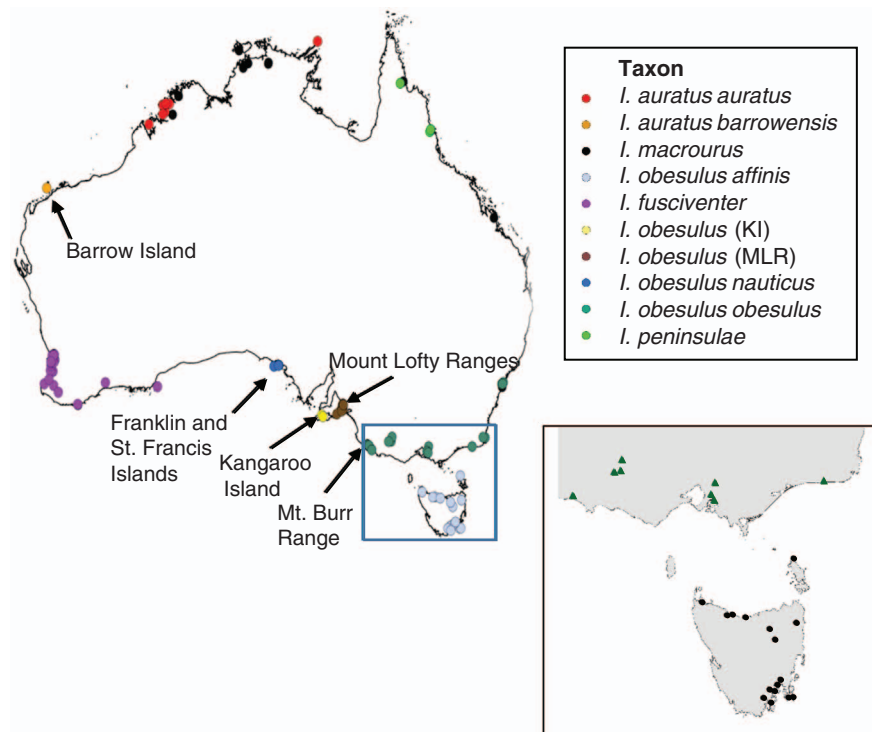
Fig. 2.   Location of samples used for DNA sequence analyses collected from across Australia, including representatives of each of the major *Isoodon* taxa. The inset shows the locations of samples collected from Victoria (green triangles) and Tasmania (black circles). Further locality details are given in Table S1 (Supplementary Material). Symbols for population groups are: KI, Kangaroo Island; MLR, Mount Lofty Ranges.

Table 1.   Primers and annealing temperatures (Tm) used to amplify segments of *CR*, *ND2*, *BRCA1*, *RAG1* and *vWF* from *Isoodon* taxa

Gene	Primer name	Source	Sequence (5′–3′)	Tm
CR	m989 (L15999M)	Fumagalli *et al.* (1997)	ACCATCAACACCCAAAGCTGA	55°C
	m990 (H16498M)	Fumagalli *et al.* (1997)	CCTGAAGTAGCAACCAGTAG	55°C
ND2	m635 (mmND2.1)	Osborne and Christidis (2001)	GCACCATTCCACTTYTGAGT	48°C
	m636 (mrND2c)	Osborne and Christidis (2001)	GATTTGCGTTCGAATGTAGCAAG	48°C
BRCA1	G1800 (F9)	Meredith *et al.* (2008)	AGTTCTGAAAGTGGATTCTTT	50°C
	G1801 (R-1MAC9-20)	Meredith *et al.* (2008)	CTGACCTRCAGCCTGAGGATTTCAT	50°C
RAG1	G2311 (F2204)	Amrine-Madsen *et al.* (2003)	GCTTCTGGCTCWGTCTACATYTGTAC	50°C
	G2312 (R2794)	Amrine-Madsen *et al.* (2003)	AAACGCTGTGARTTGAAACT	50°C
vWF	G2313 (MF119)	Amrine-Madsen *et al.* (2003)	GACTTGGCYTTYCTSYTGGATGG	55°C
	G2314 (MR1140)	Amrine-Madsen *et al.* (2003)	TTGATCTCATCSGTRGCRGGATTGC	55°C

MultiScreen PCR$_{384}$Filter Plates (Millipore) and capillary Sanger-sequenced by the Australian Genome Research Facility or (b) purified using an ethanol/EDTA precipitation method and capillary Sanger-sequenced by the Australian Cancer Research Foundation Biomolecular Resource Facility (Australian National University, Canberra).

DNA sequences were edited and aligned using the Geneious alignment option within Geneious 6.1.4 (www.geneious.com). Pairwise distances (p-distances) among mitochondrial haplotypes were determined using Geneious. Geneious was also used to construct Neighbour Joining (NJ) trees, using the HKY-85 (Hasegawa *et al.* 1985) model of sequence evolution. Before concatenating the two mitochondrial genes (*CR* + *ND2*), we constructed separate phylogenetic trees to check their concordance. The robustness of nodes in the NJ trees was assessed by 1000 bootstrap replicates. A maximum likelihood (ML) analysis was also conducted using the program RAxML and the WEB-based RAxML 7.7.1 'black box' (http://phylobench.vital-it.ch/raxml-bb/; Stamatakis *et al.* 2008; provided by the Vital-IT Unit of the Swiss Institute of Bioinformatics). A single model of evolution, the General Time Reversible (GTR) model (Rodríguez *et al.* 1990) with unequal variation at sites modelled using a Gamma (G) distribution (Yang 1996), was applied to the concatenated sequence data. Robustness of branches on the tree was assessed using 100 bootstrap pseudoreplicates. Trees were rooted using *I. macrourus* as an outgroup; its sister lineage relationship to other *Isoodon* taxa was supported in the most recent multigene analysis of the genus by Travouillon and Philips (2018). Trees were visualised and prepared for publication using FigTree 1.4.2 (http://tree.bio.ed.ac.uk/).

Average sequence divergence levels among haplotypes from subspecies and populations were determined using MEGA 6.0 (Tamura *et al.* 2013).

Because of the low sequence variation for the nuclear genes, we constructed haplotype networks to visualise the relationships among haplotypes. The phase of multiple ambiguities in the sequence data were resolved into distinct haplotypes using the program PHASE 2.1.1 (Stephens *et al.* 2001; Stephens and Scheet 2005). The distance-based (NJ) method and p-distances between sequences were used to derive a gene tree in Newick format, using Geneious, which was then used as input into the

program Haploviewer (developed by G. Ewing: http://www.cibiv.at/~greg/haploviewer) to generate a haplotype network.

An assessment of how genetic variation was partitioned among hypothetical groups of populations was determined using Analyses of Molecular Variation (AMOVA), as implemented in the program Arlequin 3.5.2.2 (Excoffier and Lischer 2010), using default options based on frequency of, and p-distances among, haplotypes in populations. This approach was used to compare different hypotheses of species and subspecies boundaries for both mtDNA data and nuclear gene data. Tests of selective neutrality of the data were undertaken using Fu's FS (Fu 1997) and Tajima's D (Tajima 1989), as implemented in Arlequin.

Allozyme analyses

Allozyme electrophoresis was undertaken on cellulose acetate gels (Cellogel$^{©}$) using the methods employed by Southgate *et al.* (1996) for an earlier study focussing on *I. auratus*. Frozen tissues (liver, kidney or whole blood; blood chosen only when no other tissue type was available) were sourced from the SA Museum's Australian Biological Tissues Collection for 137 ingroup *Isoodon* and four outgroup (*n* = 3 *Perameles nasuta*; *n* = 1 *P. pallescens*) specimens (Table S2, Supplementary Material). The following 47 presumptive loci displayed interpretable allozyme patterns in at least one tissue type: *Acon1*, *Acon2*, *Acp*, *Acyc*, *Ada*, *Ak1*, *Ak2*, *Alb*, *Ca*, *Dia1*, *Dia2*, *Enol*, *Est*, *Fdp*, *Fum*, *G6pd*, *Gapd*, *Gda*, *Glo*, *Got1*, *Got2*, *Gpd*, *Gpi*, *Idh1*, *Idh2*, *Ldh1*, *Ldh2*, *Mdh1*, *Mdh2*, *Me1*, *Me2*, *Mpi*, *Ndpk*, *Np*, *PepA1*, *PepA2*, *PepB*, *PepD*, *Pgam*, *6Pgd*, *Pgk*, *Pgm*, *Pk1*, *Pk2*, *Sod*, *Sordh*, and *Tpi*. Details of enzyme/locus nomenclature and abbreviations, enzyme commission numbers, electrophoretic conditions, and stain recipes are presented elsewhere (Richardson *et al.* 1986; Southgate *et al.* 1996).

The allozyme data were analysed using two complementary approaches. Initially, all ingroup individuals were subjected to stepwise Principal Coordinates Analysis (PCoA) following the rationale and methodology of Adams *et al.* (2014). This approach facilitates the identification of all diagnosable lineages/taxa from first principles, without assuming the taxonomic identity of individuals. Here we regard discrete PCoA groups as diagnosable lineages if they differed from one another by a minimum of two fixed differences (*sensu* Adams *et al.* 2014).

Having confirmed that there were no discrepancies between an individual's *a priori* taxonomic identification and our PCoA findings, we then assessed the genetic affinities among all regional populations and diagnosable PCoA lineages within all described species or subspecies. This was achieved by constructing a NJ tree from a pairwise matrix of unbiased Nei's distances, rooted using the two outgroup taxa. All methodological details for producing this NJ tree are presented in Adams *et al.* (2013).

Results

MtDNA sequence analyses

MtDNA sequence data from *CR* (~550 bp) and *ND2* (629–698 bp) regions were obtained from 69 *I. obesulus* (including 21 *I. o. affinis* from Tasmania), 35 *I. fusciventer* samples, 27 *I. auratus* samples and 17 *I. macrourus* samples. Separate NJ analyses of *CR* and *ND2* revealed concordant phylogenetic trees and, therefore, the data were concatenated for further phylogenetic analyses using ML methods. There were no indels or premature

stop codons evident in the *ND2* data, suggesting that it was most likely mitochondrial DNA in origin rather than a nuclear copy of mtDNA. NJ and ML analyses of the concatenated data revealed similar phylogenetic trees, so we describe and present the ML tree only (Fig. 3; Fig. S1, Supplementary Material). Overall, there was evidence for considerable phylogenetic structure, with at least three major evolutionary lineages evident, one comprising samples of *I. peninsulae* from north Queensland, a second 'western and southern group' comprising *I. fusciventer*, *I. auratus* and *I. obesulus* (Mt Lofty Ranges, Kangaroo Island and Franklin and St Francis Islands) and a third comprising 'east coast' *I. o. obesulus* and Tasmanian *I. o. affinis*. The latter subspecies formed a distinct, well supported (99% bootstrap value) monophyletic group that was in paraphyly with *I. o. obesulus* from the east coast of Australia (Fig. 3). Additional divergent mtDNA lineages were also evident within the 'east coast' group (Fig. 3; Fig. S1, Supplementary Material): one occurring at Mt Burr in south-east SA, one from St Francis Island in SA, and one from Cranbourne in Victoria. Within the 'western and southern' group, considerable mtDNA diversity was evident,
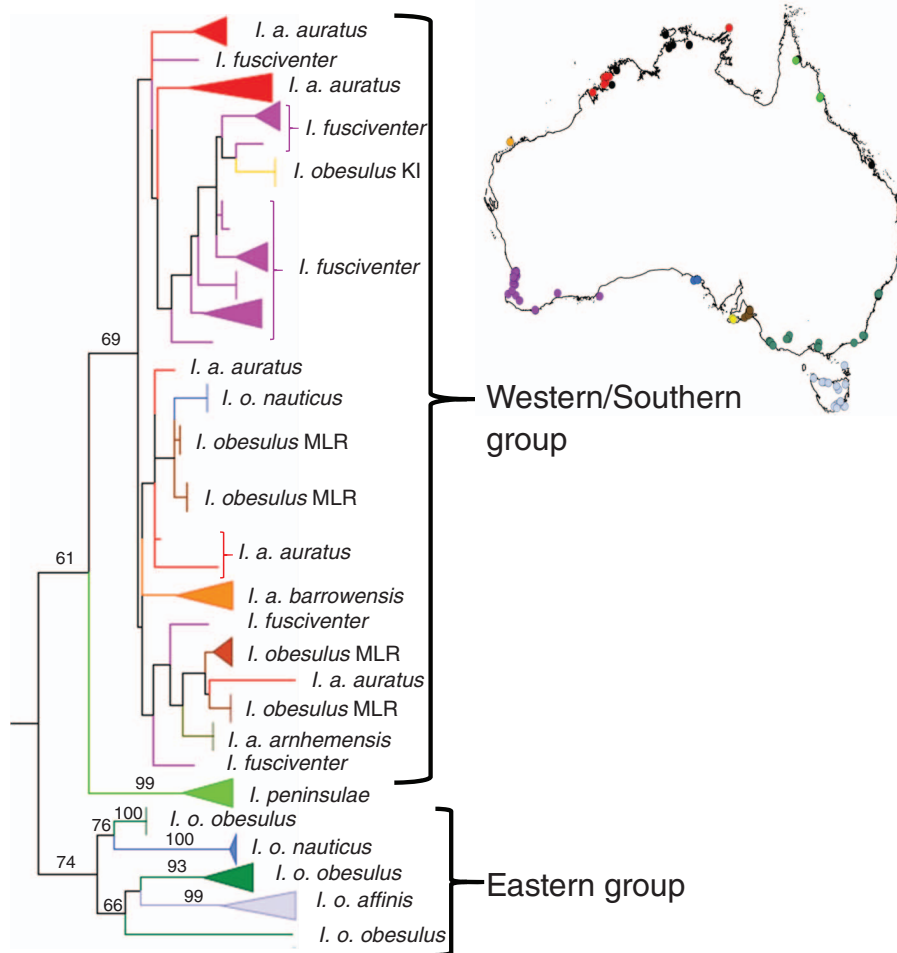
Fig. 3. Maximum likelihood phenogram derived using RAxML based on concatenated mtDNA sequence data from the NADH dehydrogenase subunit 2 gene (*ND2*) and Control Region (*CR*) of *Isoodon* taxa. The tree was rooted using *I. macrourus* as an outgroup. Monophyletic groups of individuals from the same taxon have been collapsed to improve visualisation of the phenogram. The full trees are shown in Fig. S1 (Supplementary Material). Numbers above branches represent bootstrap values from 100 pseudoreplicates.

with groups of divergent mtDNA haplotypes associated with each of the species, populations or subspecies, but no sharing of specific mtDNA haplotypes between entities. However, only *I. auratus barrowensis* formed a discrete monophyletic group, albeit with only three specimens sequenced, and the *I. auratus auratus* exemplars were polyphyletic with *I. fusciventer* and *I. obesulus* from the Mt Lofty Ranges (Fig. 3).

Estimates of average p-distances (*ND2* and *CR* data combined) among species/subspecies/populations ranged from 0.013 between the Kangaroo Island population of *I. obesulus* and *I. fusciventer*, to 0.038 between the Kangaroo Island population of *I. obesulus* and *I. o. affinis* from Tasmania (Table 2). The latter (*I. o. affinis*) showed a divergence level of 0.023 from *I. o. obesulus* from the south-east mainland of Australia. The Mt Lofty Ranges and Kangaroo Island populations showed the lowest divergences with *I. fusciventer* and *I. auratus* from WA, and diverged from east coast *I. o. obesulus* by p-distances of 0.035–0.036 (*CR*: 0.046–0.05; *ND2*: 0.025: Table S3, Supplementary Material).

Nuclear sequence analyses

Nuclear sequence data were derived from three genes, *vWF*, *BRCA1* and *RAG1*, from 113, 81, and 116 *Isoodon* samples covering the range of each species (including *I. macrourus*) and subspecies in Australia (Fig. 2; Table S1, Supplementary Material). Low levels of divergence among sequences were observed for each gene, with a total of nine haplotypes identified for *RAG1*, 11 haplotypes identified for *BRCA1*, and 21 haplotypes identified for *vWF*. Owing to the low levels of nucleotide variation, ancestral haplotypes may coexist with multiple descendent haplotypes and generation of bifurcating trees using standard phylogenetic analyses may not be appropriate. Hence a haplotype network approach was used to infer relationships among haplotypes and their associated populations (Fig. 4). We highlight below the distribution of private haplotypes among taxa and populations, but note that the privacy and frequency of haplotypes is likely to be influenced by sample size, which is not uniform across the distribution of taxa. Of the markers sequenced, *vWF* was most informative, showing several private haplotypes that were fixed in different

populations. For example, *I. obesulus* from the Mt Lofty Ranges, Kangaroo Island and the Franklin and St Francis Islands showed a fixed haplotype difference from all other populations/ subspecies; *I. o. affinis* (Tasmania) shared a haplotype with *I. o. obesulus* (east coast) and *I. peninsulae*. *I. auratus* had five private *vWF* haplotypes and shared one common haplotype with *I. peninsulae*. *I. fusciventer* had two private haplotypes and a common one that was shared with *I. a. barrowensis*. *I. macrourus* had eight private haplotypes and shared no haplotypes with other *Isoodon* taxa. For *BRCA1*, although one common haplotype was shared among *I. auratus*, *I. fusciventer*, *I. obesulus* and *I. macrourus*, other haplotypes were private in different geographic regions. One *BRCA1* haplotype was found at high frequency in both *I. o. obesulus* (east coast) and *I. o. affinis* (Tasmania), one distinct haplotype was fixed within *I. peninsulae* and one was fixed within *I. obesulus* from the Mt Lofty Ranges, Kangaroo Island and the Franklin and St Francis Islands. In contrast, there was considerable haplotype sharing among species, subspecies and populations for two common *RAG1* haplotypes, with five private haplotypes detected in *I. a. auratus* and two private haplotypes detected in *I. macrourus*. However, one *RAG1* haplotype was almost exclusively found in the Mt Lofty Ranges, Kangaroo Island, the Franklin and St Francis Islands, and *I. fusciventer* (WA).

AMOVA

For all mtDNA and nuclear gene markers, the grouping of samples from the Mt Lofty Ranges, Kangaroo Island and *I. o. nauticus* with *I. o. obesulus* and *I. o. affinis* (consistent with current classification of these populations) led to non-significant ($P > 0.05$) negative variances being estimated. In contrast, grouping samples from Mt Lofty Ranges, Kangaroo Island and *I. o. nauticus* together as a distinct group, or with *I. fusciventer*, gave positive and significant estimates of variance (Table 3). For two of the three nuclear gene markers (*vWF* and *BRCA1*), variation among groups was maximised for the tested species hypotheses when samples from Mt Lofty Ranges, Kangaroo Island and *I. o. nauticus* were grouped together as a group distinct from all other taxa (Table 3). For mtDNA data, variation among groups was maximised for the tested species hypotheses

Table 2. Estimates of average sequence divergence (p-distances) over sequence pairs (*ND2* and *CR* data combined) within (boxed on diagonal) and between (below diagonal) *Isoodon* species, subspecies and populations
Symbols for subspecies/population groups are as follows: Im, *I. macrourus*; Ioa, *I. obesulus affinis*; Ioo, *I. obesulus obesulus*; Ion, *I. obesulus nauticus*; Iaa, *I. auratus auratus*; If, *I. fusciventer*; Iab, *I. auratus barrowensis*; Iok, *I. obesulus* Kangaroo Island SA; Iomlr, *I. obesulus* Mt. Lofty Ranges SA; Ip, *I. peninsulae*

	Im	Ioa	Ioo	Ion	Iaa	If	Iab	Iok	Iomlr	Ip
Im	0.031									
Ioa	0.094	0.008								
Ioo	0.092	0.023	0.009							
Ion	0.091	0.033	0.031	0.017						
Iaa	0.086	0.036	0.035	0.021	0.013					
If	0.089	0.036	0.035	0.023	0.016	0.009				
Iab	0.089	0.036	0.033	0.022	0.015	0.018	0.008			
Iok	0.089	0.038	0.036	0.024	0.016	0.013	0.020	0.000		
Iomlr	0.086	0.037	0.035	0.020	0.014	0.015	0.015	0.016	0.009	
Ip	0.087	0.037	0.035	0.030	0.027	0.025	0.026	0.028	0.026	0.003
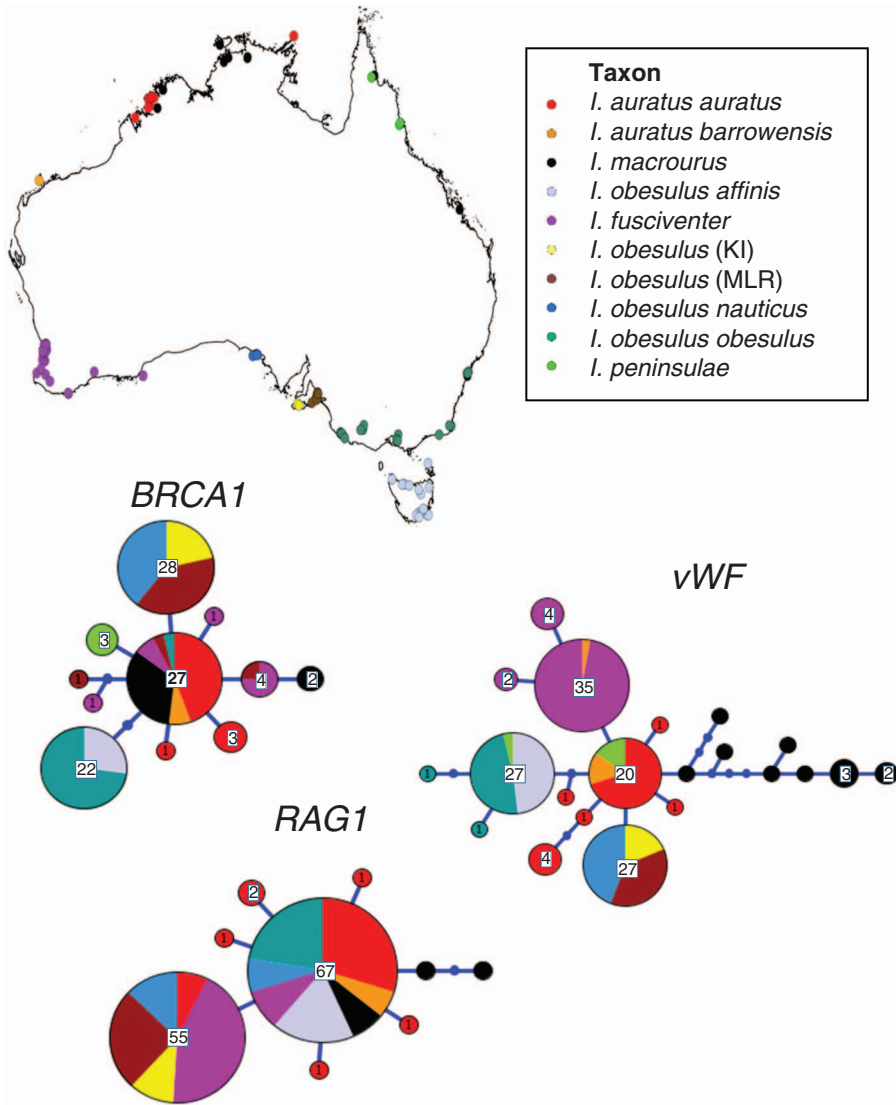
Fig. 4. Haplotype networks for three nuclear genes, *vWF*, *BRCA1* and *RAG1*, based on Neighbour Joining trees and derived using the program Haploviewer (G. Ewing, http://www.cibiv.at/~greg/haploviewer). Colour codes for each of the populations are given in the key and their distribution is shown in the map. The frequency of the different haplotypes is shown within the circle. Small blue circles represent inferred haplotypes, not sequenced in the current study.

Table 3.   Among-group genetic variation estimates from AMOVA analyses using Arlequin 3.5.2.2 for different species hypotheses

*, significant at the 5% level; **, significant at the 1% level. Symbols for subspecies/population groups are as follows: Ioa, *I. obesulus affinis*; Ioo, *I. obesulus obesulus*; Ion, *I. obesulus nauticus*; Iaa, *I. auratus auratus*; If, *I. fusciventer*; Iab, *I. auratus barrowensis*; Iok, *I. obesulus* Kangaroo Island SA; Iomlr, *I. obesulus* Mt. Lofty Ranges SA; Ip, *I. peninsulae*

Species hypothesis	Population groupings	Genetic markers			
		mtDNA	vWf	BRCA1	RAG1[A]
Current classification: 4 species	(Ioo,Ioa,Iomlr,Iok,Ion) (If) (Ip) (Iaa,Iab)	−4.3%	7.46%	−12.01%	−12.23%
New taxon in SA (Iomlr,Iok,Ion): 5 species	(Ioo,Ioa) (Iomlr,Iok,Ion) (If) (Ip) (Iaa,Iab)	24.07%	85.25%**	83.03%**	53.10%
I. fusciventer in SA: 4 species	(Ioo,Ioa) (Iomlr,Iok,Ion,If) (Ip) (Iaa,Iab)	33.34%*	39.75%	70.50%**	61.19%*
East–west and Ip: 3 species	(Ioo,Ioa) (Iomlr,Iok,Ion,If,Iaa,Iab) (Ip)	47.08%**	53.06%*	68.60%**	29.55%
7 taxa: subspecies and species	(Ioo) (Ioa) (Iomlr,Iok,Ion) (If) (Ip) (Iaa) (Iab)	32.07%	86.24*	81.16%*	42.86%
If and Ia synonymised: 4 species	(If,Iaa,Iab) (Iomlr,Iok,Ion) (Ip) (Ioo,Ioa)	37.22%**	67.91%**	81.28%**	25.08%

[A]No RAG1 data were obtained from *I. peninsulae*.

with a 3-taxon grouping based on the east–west split of *I. o. obesulus* + *I. o. affinis* versus Mt Lofty Ranges, Kangaroo Island, *I. o. nauticus*, *I. fusciventer* and *I. auratus*, and with *I. peninsulae* representing the third group (Table 3). Fu's FS and Tajima D tests of selective neutrality for mtDNA data were all non-significant ($P > 0.05$; results not shown).

Allozyme analyses

The final dataset comprised the allozyme profiles of 137 *Isoodon* (excluding *I. o. affinis* and *I. peninsulae*), three *Perameles nasuta*, one *P. pallescens* at 47 loci (data summarised in Table S2, Supplementary Material). Stepwise PCoA of all ingroup individuals (Fig. 5), coupled with complementary assessments of cluster diagnosability (Table S4, Supplementary Material), revealed the presence of five diagnosable groups, namely: NT/WA *I. macrourus*; Queensland *I. macrourus*; *I. auratus*; *I. o. obesulus*; and a composite of *I. fusciventer* and all other western and southern subspecies/regional populations of *I. obesulus*. These diagnosable taxa are also evident in the NJ tree (Fig. 6), which shows a single group comprising individuals from *I. fusciventer*, *I. o. nauticus* and *I. o. obesulus* from the Mt Lofty Ranges and Kangaroo Island.

Discussion

Taxonomic assessment of entities in species complexes is often informed by multigene molecular analyses, which should include adequate sampling across the distribution of putative taxa. Here we have demonstrated this approach to evaluate taxonomic boundaries in the southern brown and golden bandicoots, a suite of related marsupial species of conservation significance that are the subject of major ongoing taxonomic uncertainty. The genetic analyses presented here considerably extend previous genetic analyses of the southern brown bandicoot (*I. obesulus*) and related species (*I. fusciventer*, *I. peninsulae* and *I. auratus*), particularly incorporating many additional samples from Tasmania and South Australia, which were poorly represented in past genetic studies (Pope *et al.* 2001; Zenger *et al.* 2005; Westerman *et al.* 2012). The results have important taxonomic and conservation management implications, particularly for eastern and southern Australian populations of *I. obesulus*. However, our data were still not sufficient to characterise the relationships among *I. auratus*, *I. fusciventer* and southern populations, previously recognised as *I. o. obesulus*. Further work, building on the analyses presented here, will be required to resolve these relationships.

The taxonomic status of the Tasmanian and east coast populations of I. obesulus

Our study included 21 samples of *I. obesulus* from across the range of the species in Tasmania (including Flinders Island). Phylogenetic analyses revealed that the Tasmanian exemplars were monophyletic for mtDNA haplotypes relative to *I. o. obesulus* from the south-east coast of mainland Australia. However, the latter did not show reciprocal monophyly of mtDNA haplotypes; a single haplotype from Mt Burr in south-east SA was more closely related to a haplotype from St Francis Island in SA, and a haplotype from Cranbourne, Victoria, also grouped outside the main *I. o. obesulus* east coast mtDNA clade. These findings indicate that *I. o. obesulus* (east coast) is a paraphyletic taxon based on analyses of mtDNA. The monophyly of the Tasmanian population and moderate level of sequence divergence (~2.3%) from *I. o. obesulus* provides evidence of long-term isolation of this population from the nearest mainland population. Given an observation that a 9.4% mtDNA divergence level (*ND2* and *CR* combined for *I. macrourus* versus *I. o. affinis*: Table 2) here equates to an ~2.4
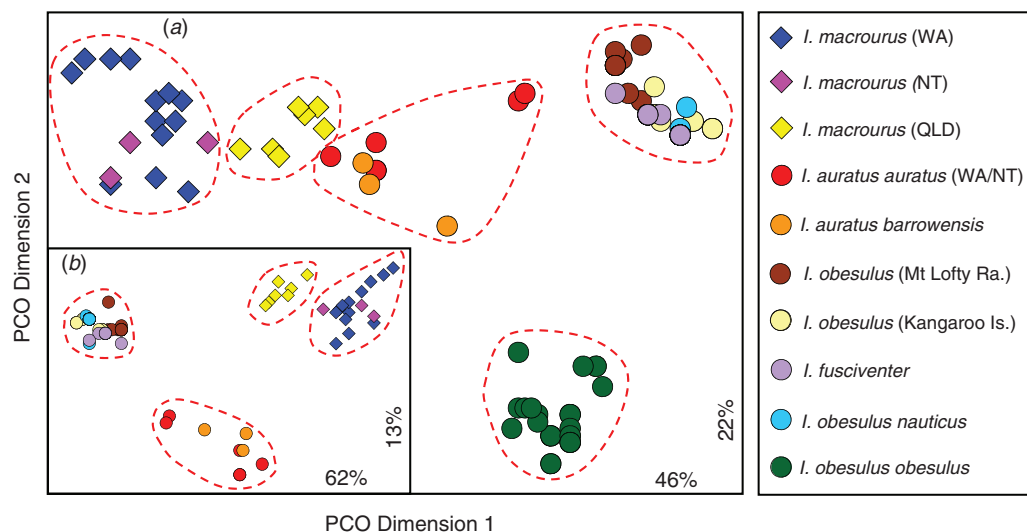
Fig. 5. Stepwise Principal Coordinates Analysis of the 137 *Isoodon* genotyped in the allozyme study. Relative PCoA scores have been plotted in the first two dimensions for (*a*) the initial PCoA on all individuals, and (*b*) a follow-up PCoA on the subset of individuals created by removing the most distinctive PCoA cluster (*I. obesulus obesulus*; $n = 24$). Individuals are labelled by taxon name or geographic lineage, according to the symbols provided (which match those used in other figures for *Isoodon*). Clusters enclosed within a dotted line are diagnosable from others by at least two fixed differences (Table S3, Supplementary Material). Note that individuals with identical multilocus genotypes will overlie each other.
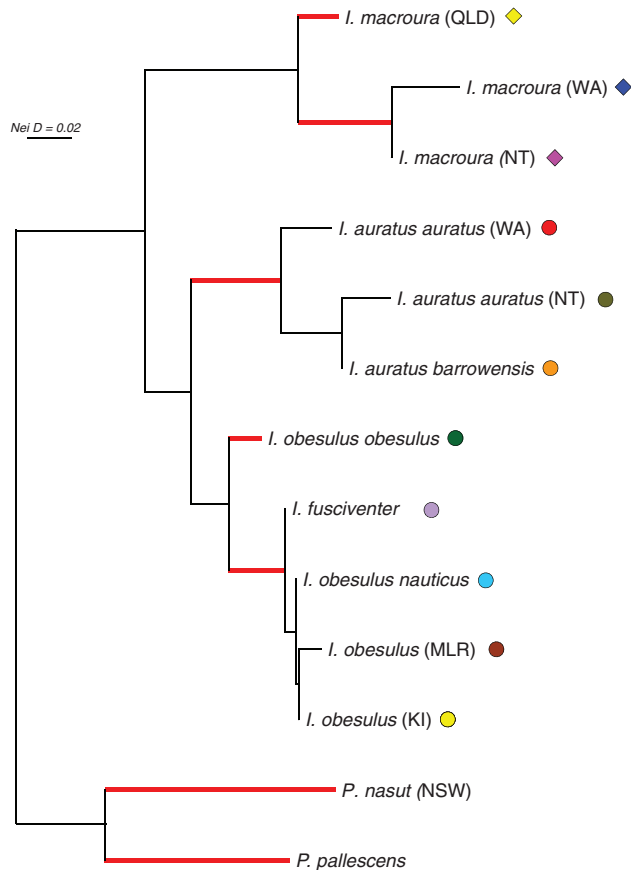
Fig. 6. Neighbour-joining tree for allozyme data, based on a matrix of pairwise unbiased Nei's distances among all regional populations and diagnosable PCoA lineages identified for species and subspecies of *Isoodon*. The tree was rooted using the two outgroup taxa of *Perameles*. Lineages that are diagnosable by a minimum of two fixed differences are identified by the thick (red) lines.

among Tasmanian and mainland Australian populations and no evidence of monophyly of the Tasmanian population, suggesting that population connectivity occurred during the most recent land-bridge connection (MacDonald and Sarre 2017). The sugar glider (*Petaurus breviceps*) also shows low divergence of mtDNA between Tasmanian and mainland populations; however, in contrast, this is indicative of a recent invasion history (Campbell *et al.* 2018). Other marsupial taxa, for example, *Potorous tridactylus*, show phylogeographic structure and considerable genetic differentiation among Tasmanian and mainland populations, indicative of long-term isolation of their gene pools, via a barrier that most likely predated the recent flooding of Bass Strait (Frankham *et al.* 2016). This latter pattern is more in line with that found for *I. obesulus*.

For the nuclear DNA markers, *vWF*, *RAG1* and *BRCA1*, there was no evidence for differentiation of east coast *I. o. obesulus* and the Tasmanian population of *I. obesulus*, with each population sharing identical haplotypes. However, the three genes are relatively slowly evolving and, for *vWF* and *RAG1* loci, the haplotypes observed in the Tasmanian population were also shared with *I. peninsulae* and *I. macrourus* respectively, suggesting that they represent ancestral haplotypes that became fixed in the Tasmanian population. Therefore, these two loci are essentially uninformative about whether there has been long-term genetic isolation of the Tasmanian population from *I. o. obesulus* (south-east coast). Similarly, the finding that these two populations shared an alternative *BRCA1* haplotype may reflect a historical connection, but should not be considered as evidence for recent population connectivity.

The monophyletic status and significant divergence of the Tasmanian population of *I. obesulus* from east coast *I. o. obesulus*, based on analyses of mtDNA, strongly supports their genetic distinction and long-term isolation. Past morphological analyses by Lyne and Mort (1981) also support the distinction of *I. o. affinis* from *I. o. obesulus*, though sample sizes for these comparisons were relatively low: Tasmanian individuals are larger with respect to their skull length, but skull width is notably smaller for Tasmanian males when compared with those found in Victoria and NSW, and so is ramus width for both males and females. Currently, a morphological re-evaluation is in progress for the genus *Isoodon* (Travouillon, pers. comm.). Together with a lack of information about population differentiation from the nuclear markers, we cannot support the recommendation of Zenger *et al.* (2005) to subsume *I. o. affinis* within *I. obesulus*, as adopted in the Action Plan for Australian Mammals (Woinarski *et al.* 2014). Indeed 'affinis' may represent a distinct species within *Isoodon*, but further analyses are required with more informative nuclear markers to help resolve the appropriate taxonomic designation for this population.

(1.5–2.7) million years divergence time estimate based on the study of Westerman *et al.* (2012), and assuming a strict molecular clock, a 2.3% level of divergence would equate to a coalescent time of ~0.6 (0.4–0.7) million years, representing a Pleistocene divergence period between the Tasmanian and south-east mainland populations. It should be noted that population divergence time is often substantially less than the coalescent time of individual genes and there can be considerable stochastic variation in coalescent time estimates at recent time scales (Ho *et al.* 2011). Hence, there is a need for additional nuclear gene analyses and alternative approaches to time estimation that model the isolation of populations (e.g. Isolation–Migration model: Hey 2010). However, given the above coalescent time estimates, it would seem unlikely that there has been any recent mtDNA gene flow between the Tasmanian population of *I. obesulus* and the mainland population of *I. o. obesulus*, despite having the opportunity to come into contact across the land bridge between Tasmania and the mainland during the last glacial maximum (~15 000 years ago). This finding is in contrast to observations from the eastern barred bandicoot (*Perameles gunnii*), which shows low *ND2* sequence divergence (<0.43%)

The taxonomic status of South Australian populations of I. obesulus

In a previous study, Li *et al.* (2014) proposed that the Mt Lofty Ranges and Kangaroo Island populations of *I. obesulus* represented a distinct ESU based on the criteria of Moritz (1994), compared with *I. obesulus* from south-eastern SA and western Victoria. Given the expectation that subspecies should represent discrete evolutionary lineages, or, at least, genetically

differentiated populations (Avise and Bull 1990), the previous study suggested that the Mt Lofty Ranges and Kangaroo Island populations should be regarded as a separate taxon different from *I. o. obesulus*. The current study verified this conclusion and showed that animals from the Mt Lofty Ranges, Kangaroo Island and Franklin and St Francis islands of South Australia, currently recognised as *I. o. obesulus*, are more closely related to *I. fusciventer* and *I. auratus* from WA than to the east coast subspecies *I. o. obesulus* and Tasmanian *I. o. affinis*. On the basis of mtDNA sequence data, both the Mt Lofty Ranges and Kangaroo Island populations comprised distinct groups of haplotypes that are embedded within the 'western/southern group' that includes *I. fusciventer* and *I. auratus*. The high level of divergence in mtDNA (>3.5% combined data; >4.6% for *CR* and 2.5% for *ND2*) from populations of *I. o. obesulus* from south-eastern SA and east coast Australia, suggests that there has been long-term isolation of these populations.

The distinction of the Mt Lofty Ranges and Kangaroo Island populations from animals currently recognised as *I. o. obesulus* was also supported by the nuclear gene analyses, where they showed fixed haplotype differences, when compared with *I. o. obesulus* and *I. o. affinis*, for *vWF*, *BRCA* and *RAG1*. Indeed, the nuclear data revealed a close association of the Mt Lofty Ranges/Kangaroo Island populations with *I. o. nauticus* from the Franklin and St Francis Islands, with each of these populations sharing a distinct *vWF* and *BRCA1* haplotype that was not found in any other *Isoodon* population in Australia. They also shared a *RAG1* haplotype that was detected at high frequency in *I. fusciventer*. Similarly, the allozyme analyses also support a close association between the Mt Lofty Ranges/Kangaroo Island/ *I. o. nauticus* group and *I. fusciventer*, the taxa only showing several allelic differences and no fixed differences (Table S4, Supplementary Material). Overall, the mitochondrial and all nuclear data confirm the close association of the Mt Lofty Ranges, Kangaroo Island and Franklin and St Francis Island populations and suggest that, together, they may also represent a distinct evolutionary lineage that could warrant separate taxonomic status. Such a taxon should retain the name *nauticus*, either at the specific or subspecific level, given that is the oldest available name. Furthermore, we propose that SA has two species of *Isoodon* bandicoots, given the close genetic relationship of this lineage with *I. fusciventer*, and the presence of *I. o. obesulus* in the south-east of SA.

Within the 'western and southern group', multiple divergent mtDNA haplotype lineages were evident, some shared between the Mt Lofty Ranges and Franklin Islands populations, but others representing distinct lineages more closely related to haplotypes found in *I. fusciventer*, *I. auratus* and *I. o. obesulus* (south-east SA). These apparent genetic relationships most likely represent ancient connections, possibly by gene flow across the intervening landscape between these populations during suitable climatic periods, and the chance fixation of haplotypes by genetic drift in island populations. For example, St Francis Island has a fixed mtDNA haplotype that is most closely related to a haplotype from Mt Burr in the south-east of SA, but these haplotypes diverge by ~1.7% (combined mtDNA data: Table 2), suggesting that it represents an ancient connection across the landscape between these populations and retention of an ancestral mtDNA lineage, and not recent gene flow across southern Australia. As reported

by Li *et al.* (2014), individuals containing these divergent mtDNA haplotypes were part of the eastern ESU (Mount Burr subcluster) based on microsatellite markers, indicating that the divergent mtDNA haplotypes are not representative of an additional cryptic species present in the south-east of South Australia. Furthermore, the St Francis Island animals shared identical nuclear gene haplotypes with the Franklin Island, Kangaroo Island and Mt Lofty Ranges populations, distinct from those found in the south-east of SA, thus supporting its close genetic association with these former populations, rather than an association with the latter. The fixation of divergent mtDNA lineages within St Francis Island and the Franklin Islands populations, despite their close proximity (~30 km), reflects their potential long-term isolation, or a lack of maternal gene flow among them, despite the likelihood of a land bridge between these islands being present during previous glacial maxima. Further investigation of nuclear gene data and morphology of these island populations is warranted to determine whether the mtDNA differences are associated with any additional genetic differences or whether they possibly result from male-biased dispersal.

Taxonomic status of I. fusciventer *and* I. auratus

Our genetic analyses support the distinction of *I. fusciventer* from *I. o. obesulus*, as recently proposed by Travouillon and Philips (2018), but uncertainties remain in the genetic distinction of *I. fusciventer* from *I. auratus*, and, as stated above, whether the range of *I. fusciventer* extends to SA (Mt Lofty Ranges, Kangaroo Island, Franklin and St Francis Islands). Mitochondrial DNA analyses provide evidence for polyphyly of the mtDNA haplotypes from different populations of both *I. fusciventer* and *I. auratus*, as well as populations from the Mt Lofty Ranges and other island populations in SA. It would be expected that each species would show reciprocal monophyly for mtDNA sequences if there was long-term isolation due to reproductive incompatibilities or geographic isolation (Avise 1994). However, coalescent theory also predicts that the progression from polyphyly to paraphyly to monophyly is strongly influenced by population size, with large populations taking much longer to achieve reciprocal monophyly than small populations (Kingman 2000). Therefore, given the possibility of historically high population sizes of *Isoodon* taxa (Short and Smith 1994) and relatively recent speciation (Westerman *et al.* 2012), it is likely that what are currently regarded as individual species/subspecies may not be reciprocally monophyletic with respect to other taxa (Kizirian and Donnelly 2004). Such a pattern of non-monophyly of mtDNA was observed for recently speciated rock-wallabies (Potter *et al.* 2015, 2017). However, signatures of isolation may still be evident from either paraphyly or the fixation of divergent haplotypes in different populations. In the case of *I. fusciventer* and *I. auratus*, although monophyly was not present, there were no shared mtDNA haplotypes and divergence among their mtDNA haplotypes was a minimum of 0.8% and, on average, >1.6%.

In contrast to the mtDNA phylogeographic analyses, the allozyme data provided some level of support for the genetic distinction of *I. fusciventer* and *I. auratus*, with each being discriminated from the other on both PCoA plots and NJ analyses, and also by fixed allozyme differences (Table S4,

Supplementary Material). The analyses of nuclear gene sequence data also revealed the presence of distinct private haplotypes associated with *I. fusciventer* and *I. auratus*, supporting their genetic distinction. However, given that both species are currently allopatric, their genetic distinction based on the frequency of nuclear gene alleles is not entirely surprising. Future exon-capture genetic analyses, incorporating 1000s of nuclear gene loci (Bragg *et al*. 2017), and including museum specimens from the former range of *I. auratus*, which came close to the current and historical ranges of both *I. obesulus* and *I. fusciventer*, would be of considerable value for resolving whether there has been hybridisation among the taxa leading to introgression of mtDNA and other loci, an alternative explanation for the observed patterns. This approach is currently being employed through the Oz Mammals Genomics initiative, supported by Bioplatforms Australia (Eldridge *et al*. 2019).

Recommendations for conservation management of Isoodon *taxa*

Our results provide support for continued recognition of the subspecific status of Tasmanian *I. o. affinis*, and strongly suggest that the subspecies *I. o. obesulus* is restricted in its distribution to eastern mainland Australia, extending to the south-east of SA. Taxonomic resolution of *I. fusciventer* and *I. auratus* is yet to be fully resolved and requires additional nuclear gene analyses in the future. The proposed delineation of *I. o. obesulus* suggests that the distribution, and hence the population size, of this taxon is significantly reduced compared with previous estimates and we recommend that the evaluation of conservation status under the EPBC Act be made on this basis, with the likely retention of the threat status of Endangered. Given the genetic distinctness of the animals from Mt Lofty Ranges, Kangaroo Island and Franklin and St Francis Islands localities of SA, the taxonomic status of these animals needs revision. However, until these data are available, we recommend managing these combined populations as a separate conservation unit.

On the basis of mtDNA data, the Tasmanian population of *I. obesulus* is genetically distinct from east coast mainland *I. obesulus*, supporting its current subspecies status as *I. o. affinis*, and represents the most closely related taxon to the endangered subspecies *I. o. obesulus*. For endangered taxa that have low genetic diversity, genetic rescue strategies can be employed through translocation of animals to increase genetic diversity within populations. Such a strategy is currently being planned for eastern barred bandicoots (*Perameles gunnii*). In this species, Tasmanian individuals are not genetically differentiated from those on the mainland and are being translocated to exclosures in Victoria, following the success of a similar genetic rescue program in mountain pygmy possums (*Burramys parvus*) in Victoria (Weeks *et al*. 2017; Aulsebrook 2018). In the case of *I. o. obesulus*, this study has confirmed that any implementation of a genetic rescue strategy should, in the first instance, focus on translocation within the delineated taxon, and not involve the differentiated Tasmanian *I. o. affinis* without further evaluation.

Conclusions

With expanded geographic sampling our analyses have provided a comprehensive assessment of phylogenetic patterns within southern brown and golden bandicoots of the genus *Isoodon* to support taxonomic assessment of the group. In doing so, we demonstrate the value of appropriate sampling and a multigene-based molecular analysis in resolving evolutionary relationships to provide a basis for taxonomic resolution. We also highlight the importance of an integrated approach to the conservation of closely related species, particularly in complexes where taxonomy may be unresolved, as a basis for effective management strategies.

Conflicts of interest

The authors declare no conflicts of interest.

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