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Analysis of Phylogenomic Tree Space Resolves Relationships Among Marsupial Families

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Abstract.—A fundamental challenge in resolving evolutionary relationships across the tree of life is to account for heterogeneity in the evolutionary signal across loci. Studies of marsupial mammals have demonstrated that this heterogeneity can be substantial, leaving considerable uncertainty in the evolutionary timescale and relationships within the group. Using simulations and a new phylogenomic data set comprising nucleotide sequences of 1550 loci from 18 of the 22 extant marsupial families, we demonstrate the power of a method for identifying clusters of loci that support different phylogenetic trees. We find two distinct clusters of loci, each providing an estimate of the species tree that matches previously proposed resolutions of the marsupial phylogeny. We also identify a well-supported placement for the enigmatic marsupial moles (Notoryctes) that contradicts previous molecular estimates but is consistent with morphological evidence. The pattern of gene-tree variation across tree-space is characterized by changes in information content, GC content, substitution-model adequacy, and signatures of purifying selection in the data. In a simulation study, we show that incomplete lineage sorting can explain the division of loci into the two tree-topology clusters, as found in our phylogenomic analysis of marsupials. We also demonstrate the potential benefits of minimizing uncertainty from phylogenetic conflict for molecular dating. Our analyses reveal that Australasian marsupials appeared in the early Paleocene, whereas the diversification of present-day families occurred primarily during the late Eocene and early Oligocene. Our methods provide an intuitive framework for improving the accuracy and precision of phylogenetic inference and molecular dating using genome-scale data. [Mammals; marsupials; multispecies coalescent; phylogenomics; tree space.]

Genome-scale data have helped to resolve some stubborn phylogenetic problems across the tree of life, including the deep relationships among placental mammals (Meredith et al. 2011; Song et al. 2012; Tarver et al. 2016). Nevertheless, heterogeneity in the phylogenetic signal across loci has led to persistent uncertainty in estimates of the evolutionary timescale and relationships of marsupials, the sister group of placental mammals (Phillips et al. 2006; Mitchell et al. 2014). Heterogeneous signals across loci can be the product of rapid diversification (Degnan et al. 2006), low data quality, or poor substitution-model fit (Gatesy and Springer 2013; Arcila et al. 2017). However, there are few available methods for characterizing and evaluating the phylogenetic signal across loci in genome-scale data. This methodological gap undermines the reliability of phylogenetic inference and our understanding of the causes of incongruent signals across the genome (Gatesy and Springer 2014; Springer and Gatesy 2015). One common approach is to evaluate the strength of support for specific phylogenetic hypotheses across a given set of loci (Song et al. 2012; Linkem et al. 2016; Arcila et al. 2017). But as the numbers of taxa and loci in phylogenetic data sets increase, so does the need for intuitive methods for exploring the information content across the data.

Australasian marsupials are a group of about 334 species found in Australia and parts of South-East Asia. They underwent rapid diversification, which has complicated efforts to resolve their relationships. Some molecular systematic studies identify the clade Eomarsupialia, which excludes all American marsupials (Archer and Hand 1984). But Australasian marsupials have also been reported to be paraphyletic (Cardillo et al. 2004; Bininda-Emonds et al. 2007). Many other deep divergences in the marsupial phylogeny have been resolved inconsistently across studies. One example is that of the possums, which are generally acknowledged to comprise two separate superfamilies: Phalangeroidea (brushtail possums, cuscuses, and pygmy possums) and Petauroidea (gliders and ringtail possums). However, studies have disagreed on whether the closer relative of Macropodiformes (bettongs, kangaroos, potoroos, wallabies, and allies) is Phalangeroidea (Szalay 1994; Meredith et al. 2008, 2009; Phillips and Pratt 2008) or Petauroidea (Meredith et al. 2011; Mitchell et al. 2014). The marsupial moles (*Notoryctes*; family Notoryctidae) and the American monito del monte (*Dromiciops*, family Microbiotheriidae) have also proven to be difficult cases, being placed in various positions using different molecular data sets (Szalay 1994; Kirsch et al. 1997; Springer et al. 1998; Horovitz and Sanchez-Villagra 2003; Asher et al. 2004; Beck 2008; Meredith et al. 2008; Beck et al. 2016).

To describe the patterns of diversification and lineage sorting across marsupial taxa, we aimed to visualize the trees supported by different loci across the genome as points in phylogenetic tree space (Hillis et al. 2005; Matsen 2006; Höhna and Drummond 2012; Gori et al.

2016; Huang et al. 2016). In this space, trees inferred from the most informative loci might be placed close to each other and potentially to the species tree. In contrast, gene trees that differ markedly from the species tree might be placed in their own, separate clusters. In practice, however, the distances between gene trees can be distorted when visualized in Euclidean space (Hillis et al. 2005; Matsen 2006; Höhna and Drummond 2012; Gori et al. 2016; Huang et al. 2016). Owing to this distortion and the unknown drivers of gene-tree clustering, the potential for such visualization methods to serve as objective tools for describing genomic information is unclear (Stockham et al. 2002; Nye 2008; Darlu and Guénoche 2011). If such methods are effective, gene trees in separate clusters can be compared for the strength of their statistical support and for any biochemical or other characteristics that might be shared by the corresponding loci. Loci that carry strong and congruent phylogenetic signals can then be investigated in detail, with the intention of improving the accuracy and precision of phylogenomic inference.

There is also an increasing interest in improving the accuracy and precision of molecular dating using genome-scale data (Tong et al. 2017). This presents a number of challenges, including accounting for heterogeneous evolutionary signals across loci (Ho 2014; Angelis et al. 2017; Foster and Ho 2017). Many methods for molecular dating assume that the underlying tree topology is known, which can lead to substantial amounts of error in phylogenomic dating (e.g., Yang 2007). One way to relax this assumption is to account explicitly for phylogenetic uncertainty in estimates of evolutionary timescales (Drummond et al. 2006; Ronquist et al. 2012). Alternatively, using loci that are known to support the same underlying tree topology can maximize the precision in date estimates, potentially at the cost of introducing bias during the selection of loci. A few studies have explored objective approaches for selecting loci that share congruent evolutionary signals in a phylogenomic framework (Gori et al. 2016; Huang et al. 2016), but none has provided a framework for combining this type of data selection with molecular dating.

In this study, we investigated the relationships and divergence times among marsupial families using a novel phylogenomic data set, representing the largest data set assembled for this group of mammals. Using simulations, we investigated the potential of multidimensional scaling in phylogenetics (Hillis et al. 2005; Gori et al. 2016) and an algorithm for identifying clusters of gene trees across the genome. We then assessed whether various proxies for information content can be used to describe the differences in phylogenetic signals across loci when observed in Euclidean space.

By applying these methods to our marsupial data, we identify two clusters of gene trees with different amounts of statistical support and with topologies that support different sister groups to Macropodiformes. We investigate the impact of using each of the two clusters independently for estimating the divergence times among marsupial families and find that a minor form of bias in date estimates can arise from this type of data selection. Using simulations of gene-tree evolution, we find that incomplete lineage sorting along the branch leading to the Macropodiformes, and its sister group is sufficient to explain the clustering patterns seen in the data.

Materials and Methods

Sample Collection, Targeted Nuclear Enrichment, and Bioinformatics

We sampled 45 species of marsupials, representing 18 of the 22 extant families. Our data set did not include the South American families Caenolestidae, Caluromydiae, and Glironiidae, and our samples from the Australian family Petauridae failed to yield sequence data of sufficient quality. Our samples were mostly obtained from the Australian Museum Research Institute frozen tissue collection (Supplementary Table S1; data available on Dryad http://dx.doi.org/10.5061/dryad.353q5). We used a custom in-solution capture approach to target loci in marsupials. The approach we used to identify target exons is outlined by Bragg et al. (2016a), and a criterion was imposed such that targets were greater than 220 bp and could be identified in at least six out of eight previously sequenced genomes and transcriptomes (Supplementary information Section 1 available on Dryad). Genomic libraries (1400 ng total) were prepared for each sample following the methods by Meyer and Kircher (2010), with the modifications of Bi et al. (2013). Exon sequences were assembled *de novo* from the cleaned sequencing reads, following an approach described by Bragg et al. (2016b). Haplotype sequences were aligned using MASCE v1.01b (Ranwez et al. 2011).

We located orthologues of our exon targets (Bragg et al. 2016a) on the chromosomes of the *Monodelphis domestica* (opossum) reference genome (Mikkelsen et al. 2007; Ensembl release 74; Flicek et al. 2013), using the criterion of a single blastn hit with bit score >380 (blastall 2.2.26, Altschul et al. 1990). The target sequences, code, and results for the bioinformatics and phylogenetic analyses in this study are available online (github.com/duchene/marsupial_family_phylogenomics). Our exon-capture approach produced a phylogenomic data set of 1550 loci comprising 867,000 aligned nucleotide sites with 98% completeness. This is the largest genetic data set so far assembled for marsupials, providing unprecedented power for resolving the relationships among families.

Clustering Gene Trees

One method of describing the relative distances between gene trees is to approximate these distances in Euclidean space. This representation of tree space can be made using multidimensional scaling (MDS)

and relies on a metric for describing the pairwise distances between trees (Hillis et al. 2005; Matsen 2006; Höhna and Drummond 2012). MDS finds the Euclidean positions of gene trees that minimize the sum of the distances between them (Mardia et al. 1979). We began by estimating gene trees using maximum likelihood, assuming a GTR+Γ model of substitution, in the software PhyML v3.0 (Guindon et al. 2010). We then calculated the distance between all pairs of trees. In order to disentangle the effects in inferences of tree topology and branch lengths, we focused on distances in topology inferences by calculating unweighted Robinson–Foulds pairwise distances (Penny and Hendy 1985) using the R package APE (Paradis et al. 2004). MDS was used to represent these distances in a varying number of dimensions to reflect tree-topology space.

To select clusters of loci with similar evolutionary histories, we used the partitioning around medoids (PAM) algorithm (Kaufman and Rousseeuw 1990) using the R package CLUSTER (Maechler et al. 2017). We first selected the optimal number of clusters (k) from a number between 1 and the number of loci minus 1 (i.e., $1 < k < n-1$). For each k we calculated the gap statistic, which is a standardized measure of dispersion within clusters (Tibshirani et al. 2001). The optimal k is that which provides the largest reduction in the gap statistic (Supplementary information Section 1).

Study of Multidimensional Scaling

Before using MDS and PAM to study the phylogenomic data set of marsupials, we investigated whether using MDS with 1, 2, or 3 dimensions can provide a useful representation of tree space and for recovering topology clusters in the genome. To make this assessment, we simulated sequence evolution under a number of scenarios and used different numbers of MDS dimensions to represent the space of possible tree topologies (Supplementary information Section 1, Fig. S1). Simulated data sets included 200 loci with varying numbers of taxa (20, 50, or 100), numbers of gene-tree clusters (1, 5, or 10), and distances between clusters in terms of subtree-prune and regraft (SPR) events (1 or 5 SPR events).

We used three measures to investigate the performance of MDS clustering under different conditions. The error in cluster identification was calculated as the difference between the simulated number of clusters and the detected number of clusters. Next, we calculated the error rate for each scenario as the proportion of replicates in which the wrong number of clusters was identified. Lastly, we investigated the gap statistic of the optimal number of clusters across scenarios, which allowed us to compare the fit of clustering across scenarios. The scenarios with low error in cluster identification, low error rate, and high gap statistics can be considered to provide a good clustering performance.

Marsupial Phylogenomic Analysis

For each of the 1550 loci, we obtained sequences that represented 45 marsupial species. We estimated gene trees using maximum likelihood and calculated the branch support of gene trees using a highly efficient, nonparametric measure with a similar behavior to the nonparametric bootstrap (approximate likelihood-ratio test), using the software PhyML (Guindon et al. 2010). Using these gene trees, we performed species-tree inference using the summary-coalescent method and quartet-based branch support (local posterior probabilities) in ASTRAL v4.10 (Mirarab and Warnow 2015).

We also performed phylogenetic analyses using a concatenated data set comprising the sequences of all 1550 loci. Each locus was assigned an independent GTR+Γ substitution model. The tree was inferred and bootstrap support was evaluated with maximum likelihood using RAxML v8.1.1 (Stamatakis 2014). We also repeated all of the gene-tree and species-tree analyses in this study using two subsets of the data: the first and second codon sites, and the third codon sites. To compare the phylogenetic information among groups of loci with congruent phylogenetic information, we calculated PH85 pairwise distances (equivalent to the Robinson–Foulds pairwise distances; Robinson and Foulds 1981; Penny and Hendy 1985) across loci and used MDS and PAM to identify gene-tree clusters, as described for our simulation study above. We then performed summary-coalescent and concatenation analyses for each cluster identified. We used a distance metric based solely on tree topology, excluding branch lengths, in order to disentangle and compare topology inferences with those of overall branch lengths.

For each gene, we calculated measures of mean branch support, GC content, number of parsimony-informative sites, tree length, difference between base composition in the data, and that predicted under the model used for inference (Foster 2004), and selective pressure or variation in population sizes (dN/dS; Yang 2007). We used a nonparametric measure of effect size, Spearman's ρ correlation coefficient, to investigate whether each of these metrics was associated with the MDS dimensions used for representing gene-tree space. Similarly, we used a nonparametric effect-size metric based on the probability of the values from each variable in one cluster occurring in the other cluster (correspondence probability, or c-index). We set the assessment using the c-index such that a value above 0.5 indicates that the cluster with more loci has higher values of a variable, whereas a value below 0.5 indicates that the cluster with fewer loci has higher values. We also investigated whether clustering was associated with chromosomal scaffold assignment. Specifically, we tested whether each chromosome was equally represented in the two clusters. For each chromosome, we performed a test for equality of two proportions.

Molecular Dating

We inferred the evolutionary timescale of marsupials using the full data set of 1550 loci. However, the error in divergence-time estimates might be inflated by incongruence between gene trees, such that using only data from a single gene-tree cluster might improve the precision of molecular dating. This procedure is also known as data filtering, whereby a chosen criterion is used to select a subset of the genomic data for inferring evolutionary relationships and timescales (Jarvis et al. 2014; Doyle et al. 2015). However, there is little understanding of the potential bias introduced when using different criteria to filter genomic data. To explore the impact of filtering data by the signals of tree topologies in the genome, we inferred the evolutionary timescale of marsupials in separate analyses of each of the subsets of the data identified in our gene-tree clustering in MDS space. In every molecular dating analysis, we used the species-tree topology inferred from the complete data set. Our estimate was calibrated using 12 fossil-based age constraints on internal nodes in the tree, specified as uniform priors with soft bounds, based on calibrations used in a recent phylogenetic study of marsupials (Supplementary Table S2; analysis available on Dryad; Mitchell et al. 2014).

To account for differences in overall rates of molecular evolution, we partitioned the molecular clock into each of the two topology clusters and each of the three codon positions (six partition subsets). We performed an additional analysis with data partitioned only by codon position (three partition subsets). We performed Bayesian dating analyses using a GTR+Γ substitution model and an uncorrelated-gamma relaxed clock model in MCMCtree, part of PAML v4.8 (Yang 2007). Approximate likelihood computation was used to improve the efficiency of the analysis (Thorne et al. 1998). The posterior distribution was estimated using Markov chain Monte Carlo (MCMC) sampling. Samples were drawn every 10^3 MCMC steps over a total of 10^7 steps, following a burn-in of 10^5 steps. We checked for convergence to the stationary distribution by comparing parameter estimates from two independent runs. Effective sample sizes were above 200 for all estimated parameters.

Assessing Incomplete Lineage Sorting

To investigate the causes of separation of gene trees into clusters, we tested whether clusters contained different proportions of loci supporting particular topologies. We focused on the three possible topologies arising from each of the four shortest branches in the dated species tree (the "time-tree"). These four triplets of taxa were: (i) *Wallabia bicolor*, *Onychogalea fraenata*, and *Dorcopsulus vanheurni*; (ii) Macropodiformes, Petauroidea, and Phalangeroidea; (iii) *Notoryctes*, Peramelemorphia, and Dasyuromorphia; and (iv) Diprotodontia, Dasyuromorphia, and *Notoryctes* + Peramelemorphia. For each gene tree, we randomly pruned the data to leave a single representative of each of the three taxa in each triplet. If none of the three possible relationships occurred in more than half of 100 trees randomly pruned in this way, the gene tree was considered to be indecisive for the triplet in question. We repeated the process for each gene tree and each triplet and used a test for equality of two proportions to compare whether gene-tree clusters contained the same proportion of gene trees supporting a given relationship.

Using a simulation study, we investigated whether incomplete lineage sorting contributed to the observed pattern of tree clustering. Gene trees were simulated within the time-tree of marsupials using the R package phybase (Liu and Yu 2010), and their clustering pattern was compared with that of the phylogenomic data. These simulations assume that our estimates of the species tree and divergence times are accurate representations of marsupial evolutionary history. We simulated the same number of gene trees as in our phylogenomic data set (1550) under five different population sizes, spread uniformly between 2×10^5 and 2×10^6 individuals (assuming a generation time of 5 years). Simulated gene trees were represented in 2D space using MDS and clustering using PAM, as done in our analysis of the genomic data. In each population-size scenario, we explored whether the patterns of clustering in MDS space were associated with discordance at particular branches, focusing on the four shortest branches in the marsupial time-tree, as done for the phylogenomic data.

Results

Performance of Multidimensional Scaling in Representing Tree Space

Under the conditions explored, we found that the power to detect different numbers of gene-tree clusters depends on the number of taxa (Gori et al. 2016), as well as the number of MDS dimensions used (Supplementary Figs. S2 and S3). The number of topology clusters was detected most accurately when the data set contained 100 taxa and when using two or three MDS dimensions (Supplementary Fig. S2). Clustering using a single MDS dimension will lead to underestimation of the number of clusters when there are actually multiple clusters. In contrast, using multiple dimensions can lead to overestimation of the number of topology clusters when there is actually only a single cluster (Supplementary Figs. S3 and S4). These biases are most pronounced for smaller numbers of taxa, so this approach is expected to perform best for large data sets ($\geqslant$100 taxa) and when the number of clusters identified is consistent across analyses based on several different numbers of MDS dimensions.

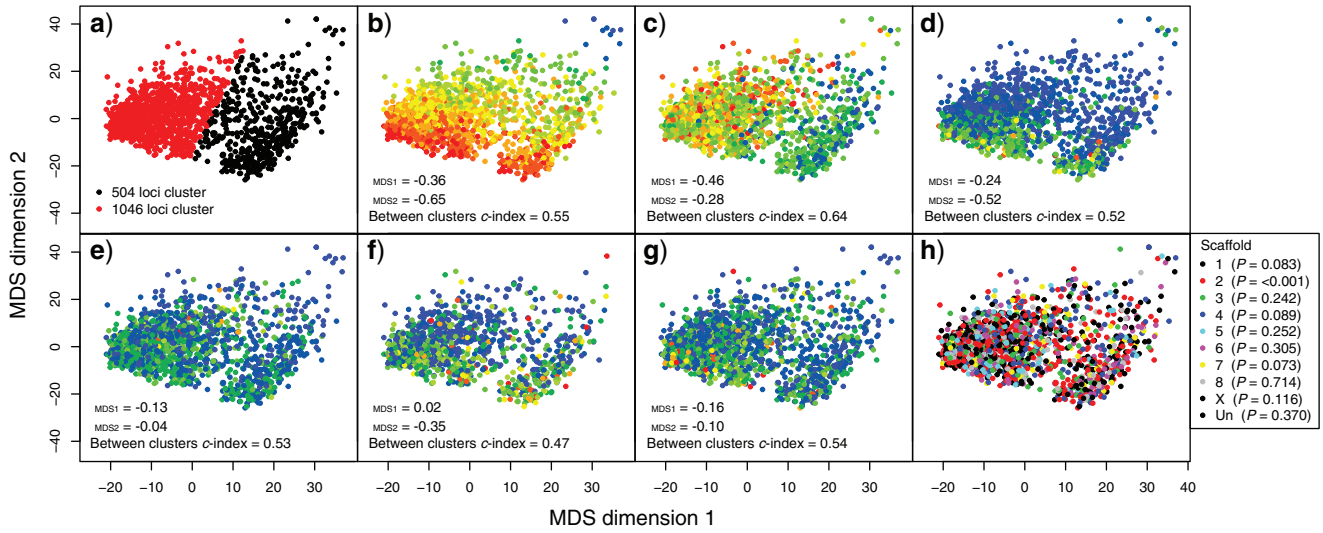

FIGURE 1. Two-dimensional MDS representation of gene-tree space for a sample of 1550 loci from marsupials. Data points are coloured by: (a) estimated gene-tree clusters; (b) overall branch support; (c) mean GC content; (d) number of parsimony-informative sites; (e) gene-tree length; (f) *dN/dS* calculated for each locus (missing loci are those in which every codon contained missing data); (g) difference between the base composition across taxa and that predicted under the substitution model, a metric of model adequacy (see Supplementary information Section 1); and (h) genomic scaffold. In panels (b) to (g), high and low values are shown by the colour spectrum from red (darkest shade), respectively. Panels (b) to (g) show the *c*-index (or concordance probability) for each of the variables, which is the probability that the values from a locus in one cluster could arise in the other cluster. These panels also show Spearman's ρ correlation coefficients, which are measures of effect size of the association between a descriptive variable and each of the two MDS dimensions. Panel (h) shows two-sample tests for equality of proportions for the data for each scaffold, assessing whether loci from a given scaffold occur with higher probability in one of the two clusters.

Marsupial Phylogeny and Gene-Tree Clustering

We identified two topology clusters in the data when using either two or three MDS dimensions (Supplementary Fig. S5 available on Dryad). Only a single cluster was found when using a single-MDS dimension. This difference might occur because using a single MDS dimension often leads to underestimation of the number of clusters, as seen in our simulation study (Supplementary Figs. S3 and S4). Alternatively, identifying the clusters in these data might be difficult when using only a single MDS dimension, because there are gene trees that lie between the two clusters identified with two and three MDS dimensions. Nonetheless, the statistical support for topological clustering, according to the gap statistic, was larger when using an increasing number of MDS dimensions (Supplementary Fig. S5).

We performed species-tree inferences using the results based on two MDS dimensions, which clustered almost the same loci as when using three dimensions (with the exception of seven loci). One of the clusters contains around two-thirds of the loci (1046), and has gene trees with higher mean overall branch support (mean approximate likelihood-ratio test support = 0.70) than the gene trees in the other cluster (504 loci, mean aLRT support = 0.64). The tree topology estimated from the 1046-locus cluster is identical to that inferred from the complete data set (Supplementary Fig. S6). The topologies of the gene trees in the 504-locus cluster are more distant from each other (silhouette value = 0.36) than those in the 1046-locus cluster (silhouette value = 0.53, Fig. 1a).

Correlation analysis shows that both MDS dimensions are associated with a decrease in overall branch support, number of informative sites, and estimated tree length (Fig. 1b–d). These gradients are evidence that loci with greater information content support trees that are in similar regions of tree space, and the same is the case for loci with low information content. We also calculated the ratio of nonsynonymous to synonymous substitutions for each locus (dN/dS), which can be used as a proxy for the strength of selection at each locus. We found that loci with dN/dS closest to 1 also had a tendency to support trees in a similar region of tree space as those with high branch support (Fig. 1e). This suggests that loci under strong negative selection have a weaker phylogenetic signal. Loci that have more variable base composition across taxa also supported trees in a similar region of tree space as loci that yielded trees with the highest branch support (Fig. 1f). However, the departures from the substitution model assumptions in these data are unlikely to lead to biased phylogenetic inferences (Duchêne et al. 2017) and are due to differences in overall rates of evolution across loci.

In addition to concordance probabilities, we compared characteristics of the two clusters using Spearman's ρ correlation coefficient across MDS dimension 1, which best separates the two clusters. These metrics show that loci in the 1046-locus cluster have higher overall branch support, lower substitution-model adequacy,

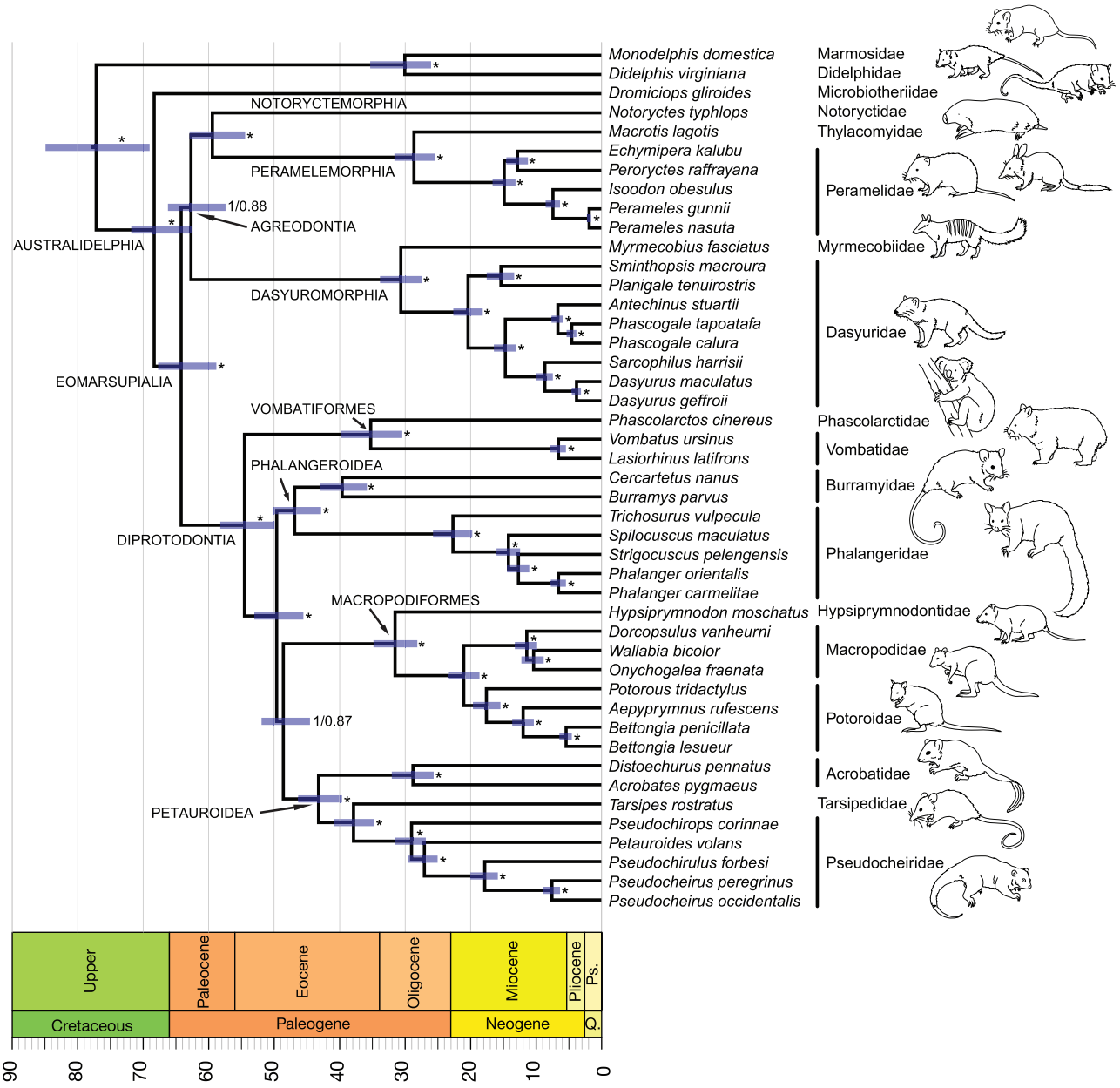

FIGURE 2. Estimated time-tree for 45 species of marsupials. The species tree and divergence times were inferred from the complete data set of 1550 loci. Blue bars indicate 95% credibility intervals of the estimates of divergence times. Family names are given on the right. All nodes received likelihood bootstrap support of 1 and local posterior probability ≥ 0.9. Line drawings are based on images by Toni Llobet in *Handbook of the Mammals of the World*, volume 5 (Wilson and Mittermeier 2015).

fewer parsimony-informative sites, and longer trees (Fig. 1). However, all of these associations have small effect sizes. The smallest effect size was that of dN/dS, suggesting that the two clusters have been subject to similar selective pressures.

Strikingly, the greatest effect size is shown by GC content, with the 1046-locus cluster containing higher values. Previous studies have found that GC-rich regions yield trees with low branch support and have suggested that these regions might be severely affected by recombination (Romiguier et al. 2013; Jarvis et al. 2014). However, regions with high GC content yield trees with strong branch support and that have greater overall estimated lengths (Supplementary Fig. S12), suggesting these regions are highly informative in our data set. We also investigated whether clustering was associated with assignment to chromosomes of the opossum reference genome. This association can be expected if loci with similar phylogenetic signals occur in genomic regions with low recombination and are therefore linked (Pollard et al. 2006). Gene regions in a particular scaffold might also be associated with drivers of diversification, such

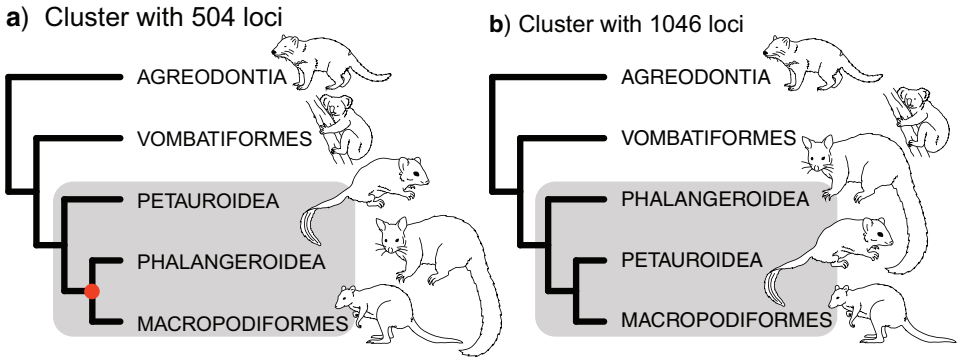

FIGURE 3. Summary of phylogenetic estimates using ASTRAL v4.10 for suborders in Eomarsupialia and superfamilies for the two tree-topology clusters identified. Despite these being the best trees recovered, the red node in (a) had a local posterior probability of 0.4 (Supplementary Fig. S6). All of the nodes shown in (b) had local posterior probabilities ≥ 0.9. Line drawings are based on images by Toni Llobet in Handbook of the Mammals of the World, volume 5 (Wilson and Mittermeier 2015).

as chromosome rearrangement in marsupials (Eldridge and Close 1993). Instead of belonging to a single cluster, highly supported gene trees are spread along a gradient in tree space, which is split between the two clusters identified (Fig. 1).

Species-tree inferences for the 1046-locus cluster and the complete data set had high levels of branch support (local posterior probability, LPP > 0.85; bootstrap support, BS $= 1$; Fig. 2; Supplementary Fig. S7). These trees are largely consistent with those inferred in recent molecular phylogenetic studies of marsupials (Beck 2008; Meredith et al. 2008, Meredith et al. 2011; Mitchell et al. 2014). The species tree estimated from the 504-locus cluster has a single difference from the tree from the 1046-locus cluster, supporting Phalangeroidea rather than Petauroidea as the sister clade to Macropodiformes (Fig. 3). This resolution has also been found in previous molecular studies of marsupial relationships (Szalay 1994; Meredith et al. 2008, 2009; Phillips and Pratt 2008). A grouping of Petauroidea and Macropodiformes based on the 504-locus cluster had lower statistical support (LPP $= 0.4$). The 504-locus cluster also contains fewer loci and its gene trees have lower overall levels of branch support.

All of our data sets strongly supported a sister relationship between the marsupial mole *Notoryctes* (Notoryctidae) and Peramelemorphia (LPP > 0.95 and BS $= 1$), the latter comprising bandicoots (Peramelidae) and the bilby (Thylacomyidae). Our data also support the placement of the American monito del monte (*Dromiciops*) as the sister taxon to Australasian marsupials, which is a resolution that has received strong support in some recent molecular phylogenetic studies (Beck 2008; Meredith et al. 2008, 2009; Nilsson et al. 2010; Mitchell et al. 2014).

Molecular Dating

The inferred evolutionary timescale of marsupials using the complete data set shows, with an unprecedented degree of precision, that most of the ordinal divergences occurred in the late Paleocene to early Eocene (Fig. 2). Several families from every order diverged in the late Eocene and Oligocene. We find that divergence-time estimates using data from either the 1046-locus cluster or the 504-locus cluster overlap with the times inferred using the complete data set (Supplementary Fig. S10). Compared with the complete data set, however, age estimates from the 1046-locus and 504-locus clusters are consistently younger and older, respectively. In addition, analyses of the 504-locus cluster or in which the data are not partitioned by gene-tree topology produce age estimates with consistently greater uncertainty. This might occur because loci supporting discordant topologies provide disproportionate amounts of information about branch lengths.

Causes of Multiple Gene-Tree Clusters

We found that both of the gene-tree clusters identified in the phylogenomic data set contained every possible resolution of the relationships determined by the four shortest branches in the inferred time-tree. Nonetheless, the 1046-locus cluster contained a significantly greater proportion of loci supporting the grouping of Macropodiformes with Petauroidea ($X^2 = 4.99, P = 0.02$), and a significantly smaller proportion of loci that were indecisive for that resolution ($X^2 = 9.64, P < 0.01$). Other characteristics were similar between the clusters and are unlikely to drive the separation of the clusters in tree space (Supplementary Table S3).

Simulations of incomplete lineage sorting show that when populations are large, there are greater numbers of distinct gene trees (Supplementary Fig. S11). When populations are small, yet large enough to generate discordance among gene trees (2×10^5 individuals), multiple clusters of gene trees are identified despite the topological differences being relatively small (Fig. 4). The number of clusters in this scenario might be overestimated, as observed when comparing similar

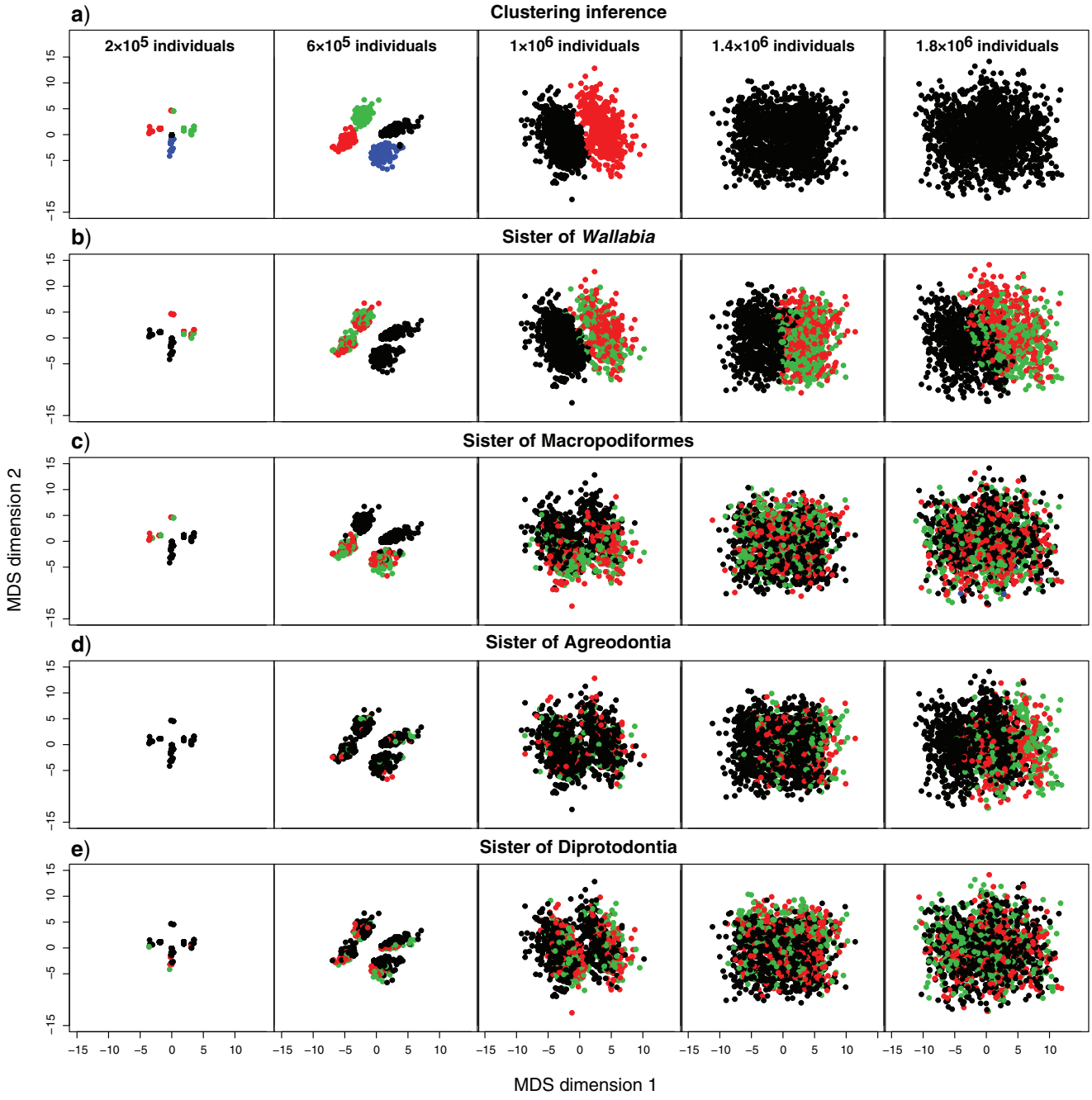

FIGURE 4. Estimated 2-dimensional MDS representation of gene trees simulated along the marsupial time-tree. A sample of 1550 gene trees were simulated under five different population sizes. The clustering inference and relationships supported by each gene tree are mapped by colours, with the darkest shade indicating (a) the first cluster, and (b–e) the sister relationship in the species tree inferred using the complete phylogenomic data set.

trees and using more than a single MDS dimension for representing tree space (Supplementary Figs. S2 and S3). Four gene-tree clusters are identified at the next population size (6×10^5), with separation between clusters driven by incomplete lineage sorting at two of the shortest branches in the time-tree. These branches correspond to the grouping of *Wallabia* and its sister clade, and the grouping of Macropodiformes with its sister clade (Fig. 4).

The different resolutions of the sister group to the Macropodiformes are the same form of discordance as found in the species trees inferred from each of the two clusters in the phylogenomic data. This suggests that incomplete lineage sorting is a plausible explanation for

the clustering pattern in the data. In simulations with intermediate population sizes (10^6), two distinct clusters are identified, corresponding to different resolutions for the sister clade to *Wallabia*. At intermediate population sizes, the clustering approach no longer identifies the separation of clusters caused by the resolution of the sister clade to Macropodiformes as observed in the phylogenomic data (Fig. 4). Under the largest population sizes that we explored (1.8×10^6), only a single topology cluster is identified, with regions of tree space distinguishable by resolutions of the sister groups to *Wallabia* and to *Notoryctes*.

Discussion

Evaluating the phylogenetic information across loci is an important step in phylogenomic analysis. To answer evolutionary questions such as the relationships among marsupial families, it is critical to identify loci that give anomalous signals (Degnan and Rosenberg 2009) or that support trees that differ from the species tree because of stochastic error or bias (Huang et al. 2010; Salichos and Rokas 2013; Doyle et al. 2015). Here we have used an intuitive method to visualize and group loci by their phylogenetic estimate, by using MDS to place them in Euclidean space (Hillis et al. 2005). The MDS distribution of gene trees and clustering in our marsupial data set suggest that loci have varying degrees of topological conflict, which can arise from incomplete lineage sorting. This is congruent with the hypothesis of a rapid ordinal diversification in marsupials (Meredith et al. 2008, 2011; Mitchell et al. 2014). The varying degree of lineage sorting across gene trees explains why some studies have produced different resolutions of the evolutionary relationships among several marsupial taxa, often with short branches and low support for the nodes near the base of the group.

Our data set provides a strongly supported phylogenetic estimate for marsupials (Fig. 2) that is robust under both summary-coalescent and concatenation approaches to species-tree inference. A grouping of Petauroidea and Macropodiformes, supported by the complete data set, has been identified in some recent studies (Meredith et al. 2011; Mitchell et al. 2014). However, the gene-tree cluster with around one-third of the loci provides weak support for an alternative grouping of Macropodiformes with Phalangeroidea (Supplementary Fig. S6), which has also been identified in previous studies but with only minor support (Szalay 1994; Meredith et al. 2008, 2009; Phillips and Pratt 2008). These results suggest that the divergences between the two clades of possums and Macropodiformes occurred within a short space of time, involved large population sizes, or both. Our findings suggest that incomplete lineage sorting and anomalous loci are likely be common in the gene-tree cluster with fewer loci and lower overall branch support. It seems surprising that only one of the rapid divergences in marsupials has led to incomplete lineage sorting that can be observed in the MDS representation of tree space. One possible explanation is that population sizes were particularly large during the diversification of Macropodiformes, Phalangeroidea, and Petauroidea, leading to more incomplete lineage sorting in the branches among these taxa than for other deep divergences in the marsupial phylogeny.

Strikingly, our data provide strong support for a placement of the marsupial moles (Notoryctidae) that has not been inferred using molecular data in the past, as the sister lineage to Peramelemorphia (bandicoots and bilby). Previous molecular studies have placed the marsupial moles as the sister lineage to a group comprising Dasyuromorphia and Peramelemorphia (Phillips et al. 2006; Meredith et al. 2011; Mitchell et al. 2014), or as the sister lineage to only the former (Beck 2008; Meredith et al. 2008). In contrast, our finding of a grouping of *Notoryctes* with Peramelemorphia has been supported by morphological data (Szalay 1994; Horovitz and Sanchez-Villagra 2003; Beck et al. 2016). The discordance across data sets and short branches between marsupial lineages suggest that the clade Agreodontia, comprising *Notoryctes*, Dasyuromorphia, and Peramelemorphia, experienced a rapid series of divergences (Beck 2008; Meredith et al. 2008, 2011; Mitchell et al. 2014). Nevertheless, all of the data sets and methods of analysis that we used here support the same placement of *Notoryctes*.

One explanation for the discrepancy between our inferred placement of *Notoryctes* and those of previous studies is that the latter were primarily based on mitochondrial genes, whereas our data set is entirely based on the nuclear genome. Several studies have suggested that the mitochondrial genome of many marsupials has been subject to substantial introgression or incomplete lineage sorting, and that phylogenetic information in mitochondrial DNA tends to differ from that in nuclear loci (Bee and Close 1993; Potter et al. 2017). Our results using multiple nuclear loci echo previous cautions against the heavy reliance on mitochondrial data for inferring marsupial relationships (Phillips et al. 2013). On the other hand, our results stand in contrast with previous findings that exome data lead to phylogenetic inferences with low support (Romiguier et al. 2013; Jarvis et al. 2014). The strong phylogenetic support that we found in data from regions with high GC content might be associated with these regions having high rates of evolution compared with recombination. A potential avenue for future research would involve the use of more densely sampled data sets (e.g., at the genus level) for examining the contribution of recombination to phylogenetic patterns across marsupial genomes.

Our estimate of the evolutionary timescale of marsupials is consistent with those of other molecular dating studies in placing the origin of most Australasian families in the late Eocene and Oligocene (Case 1989; Beck 2008; Meredith et al. 2008; Mitchell et al. 2014). The timing of the origin of Australasian marsupials (Eomarsupialia) appears to be later than suggested by some previous studies, in the early Paleocene instead of

closer to the Cretaceous-Paleogene boundary. This result is congruent with suggestions that the fossil Tiupampa fauna from Bolivia, which contains several marsupial specimens (Marshall and DeMuizon 1988), is from the mid instead of early Paleocene (Benton and Donoghue 2007). This finding might also explain the limited presence of Australasian marsupials in the Tingamarra Local Fauna fossil record from early Paleocene (Godthelp et al. 1992). The orders of Australasian marsupials appeared in the early- to mid-Eocene, when Australia was beginning to separate from Antarctica, leaving the temperate Weddellian Biogeographical Province and becoming covered with broad-leaf rainforests (Martin 2006). Several marsupial families and superfamilies underwent periods of rapid diversification in the late Eocene and Oligocene (Fig. 2), times at which rainforests in Australia were widespread but had started to be replaced by sclerophyll forests.

When evaluating clusters of gene trees in phylogenomic data, a gradient of branch support is expected to occur from the center to the edge of each cluster. This is because loci that lead to inferences with more stochastic error will support tree topologies that are less common and therefore occur in less populated regions of tree space. The marsupial data show a gradient in statistical support primarily in one direction, such that trees with low support are similar to each other. This can occur if loci yield trees that have poor precision across the same or nearby nodes. In addition, gene trees with high statistical support lead to a large range of similar resolutions of topology, suggesting that there is a large number of lineage-sorting patterns in the data. Our data show that gene trees in genomic data might not frequently be clustered around a single topology, such that highly supported trees are not necessarily similar to each other and to the species tree. Our simulations of gene trees within the marsupial species tree show that incomplete lineage sorting can explain this differentiation, but other processes such as introgression and hybridization might lead to similar patterns of gene-tree incongruence.

Separation of gene trees as visualized using MDS seems to be consistently driven by differences in a small number of branches. This occurs because multiple nodes interact when there is substantial incomplete lineage sorting, such that the alternative topologies are no longer sufficiently unique to form distinct clusters. Visualization of phylogenomic data might thus be particularly useful when there is a small number of discordant nodes. Furthermore, it can shed light on taxa that diversified particularly rapidly or with large population sizes, as in the case of the Macropodiformes and their relatives. Other phylogenomic studies using a larger sample of marsupial taxa are likely to provide a better understanding of the patterns of lineage sorting across the genome.

Conclusions

In this study, we have used a novel phylogenomic data set and analytical approach to reconcile the results of past studies of marsupial phylogenetics, and have provided a strongly supported estimate of the relationships among marsupial families. We have identified a group of loci that lead to highly supported phylogenetic inferences but have potentially been subject to incomplete lineage sorting. We have also found strong support for the placement of the genus *Notoryctes* as the sister taxon to Peramelemorphia, in agreement with past morphological studies. Our results show that Australasian marsupials started diversifying from the early Paleocene, during a time when Australia was undergoing a major climatic and floral transformation.

Our phylogenomic and dating analyses highlight the importance of exploiting the full power of genome-scale data, while emphasizing the potential bias of using subsets of the data when estimating evolutionary relationships and divergence times. The approaches for phylogenomic analysis described here have the principal advantage of objectively inferring the number of distinct evolutionary signals across loci in the genome. Combining a tree-clustering approach with proxies of information content could also be used for data filtering and model improvement in the future. These results demonstrate the considerable power of phylogenomic data sets when variation in the evolutionary process across loci is explored and taken into account.

Supplementary Material

Data available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.353q5.

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