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Cytogenetics Meets Phylogenetics: A Review of Karyotype Evolution in Diprotodontian Marsupials

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Abstract

We have used a combined approach of phylogenetics and cytogenetics to describe karyotype evolution in Diprotodontia. Molecular relationships of diprotodontian marsupials have been clarified using a concatenation of 5 nuclear gene sequences from multiple exemplars of all extant genera. Our well-resolved phylogenetic tree has been used as a basis for understanding chromosome evolution both within this Order, as well as in marsupials in general. It is clear that the ancestral marsupial karyotype comprised 14 relatively large chromosomes of the form retained relatively unchanged in caenolestids, microbiotherians, peramelemorphians, vombatids, and pygmy possums. Four pericentric inversions occurred in the ancestral dasyuromorphian (chromosomes 1, 2, 4, and 6) and a different 4 in the ancestral didelphimorphian (chromosomes 1, 3, 5 and 6). Within Diprotodontia, although the ancestral marsupial karyotype has been retained in some families such as the extant wombats and pygmy possums, there have been major karytoypic repatternings early in the evolution of others. Chromosome rearrangements in diprotodontia include centric fissions and fusions, translocations, and centromere shifts. Karyotypic changes are discussed in the context of current hypotheses concerning centromeres, chromosomal fragile sites, and mobile elements in marsupials and the probable repeated involvement of these elements in karyotypic restructuring.

Key words: *diprotodontia, marsupial evolution, marsupial karyotypes, molecular phylogeny*

Marsupials have held a fascination from the time of their first appearance in Europe and their subsequent description (Archer 1982). Their chromosomes are well known, having been first described in the 1930s (see Hayman 1990 for references), though bandicoot chromosomes from an unnamed species of *Perameles* were (incorrectly) reported in 1906 by Benda (see Sharman 1961). A major reason why marsupial chromosomes are so well documented lies with the twin properties of low chromosome number and large size that facilitate study. The advent of modern chromosome-staining techniques such as G-banding, and later of chromosome painting, meant that an evolutionary perspective could be more easily applied to our understanding of the relationships between the karyotypes of different marsupials. This evolutionary approach led to the proposal that the multitude of different karyotypes seen in living marsupials could easily be derived from an "ancestral" form comprising 2n = 14 essentially bi-armed chromosomes (Rofe 1979; Sharman 1982; Rofe and Hayman 1985; Hayman et al. 1987; Hayman 1990). These ideas were proposed on the basis of our knowledge of chromosome numbers and shapes found in various marsupial groups and on the operation of fairly simple karyotypic changes such as centromeric fissions/fusions, Robertsonian translocations, chromosome inversions (both peri- and paracentric), and centric shifts. The idea of a universal ancestral marsupial karyotype of 14 chromosomes is still not fully accepted, at least for some groups such as the South American didelphids, where the direction of evolutionary change has been suggested as being from an ancestral karyotype of 2n = 22 chromosomes toward lower numbers (Svartman and Vianna-Morgante 1998).

Competing hypotheses on the direction of evolutionary change in marsupial chromosomes have often been developed and pursued without any proper consideration of the phylogenetic relationships of the various groups from which the chromosomal data were drawn—relationships which were anyway difficult to properly assess on the basis of morphological character states alone. It is evident that any discussion of chromosome evolution in marsupials in general or of diprotodontians in particular can only be done in the light of a well-substantiated phylogeny for the group.

This review represents an attempt to reinterpret chromosome evolution in the order Diprotodontia based on the well-supported nuclear gene trees we have generated using a concatenated data set for multiple exemplars of each genus of australidelphian marsupials. Although it is important to consider evolutionary relationships of all the living marsupial groups to address the question of an ancestral marsupial karyotype, we will concentrate mainly on chromosome evolution in Diprotodontia, the group that represents a microcosm of all living Marsupialia.

Methods and Results

We added 5 *Macropus* species from Meredith, Westerman, and Springer (2008), 2 pseudocheirids from Meredith et al. (2010), and sequences from 3 new species (*Petrogale lateralis*, *Thylogale brunii*, and *Cercartetus concinnus*; GenBank accession numbers GU566712–GU566726) to the nuclear gene database of Meredith et al. (2009) (Supplementary Table S1). Gene segments that were amplified for all taxa included portions of exon 26 of *APOB* (apolipoprotein B), exon 11 of *BRCA*1 (breast and ovarian cancer susceptibility gene-1), exon 1 of *IRBP* (interphotoreceptor retinoid–binding protein), *RAG*1 (recombination activating gene-1), and exon 28 of *vWF* (von Willebrand Factor). DNA extraction, polymerase chain reaction amplification, DNA sequencing, and Bayesian phylogenetic analyses all followed the protocols outlined in Meredith et al. (2009). We used RAxML 7.0.4 (Stamatakis 2006) to perform a partitioned maximum likelihood (ML) analysis, using 500 replicates, randomized maximum parsimony starting trees and the fast hill-climbing algorithm; all other free parameters were estimated, and each gene was given its own model of molecular evolution. The 10 additional taxa were manually aligned to the Meredith et al. (2009) alignment. The resulting alignment was 5894 bp in length after the exclusion of the alignment ambiguous regions (273 bp) identified in Meredith et al. (2009). The resultant phylogeny shown in Figure 1 and Supplementary Figure S1 is generally concordant with the results reported in Meredith et al. (2009), although in the present analyses *Gymnobelideus* associates with *Petaurus* rather than with *Dactylopsila*. The phylogenetic tree in Supplementary Figure S1 was used to map known marsupial chromosome numbers (Figure 1). We note that most nodes in the tree were well resolved with both Bayesian and ML analyses. Some of the deeper divergences are unresolved with both methodologies. Thus, although the Bayesian tree suggested Paucituberculata as sister to Australidelphia [albeit with little support—55% Bayesian Posterior Probability (BPP)], the ML tree showed Paucituberculata as unresolved (51% bootstrap support) sister to Didelphimorphia. Similarly, although both Bayesian and ML methodologies resolve Microbiotheriidae as sister to all other Australidelphian taxa, the relationship between Diprotodontia and (Peramelemorphia + Dasyuromorphia + Notoryctemorphia) is effectively unresolved (58% BPP, 53% ML). Phylogenetic relationships within Diprotodontia are discussed in more detail in the next section.

Phylogenetic Relationships of Diprotodontia

The largest and most diverse of the 7 recognized orders of living marsupials, with more than 125 living species, Diprotodontia is characterized by the possession of an eponymous single pair of procumbent lower incisors and syndactylous hind feet. Its members include wombats and koalas (Vombatiformes), kangaroos and their relatives (Macropodiformes), and 2 distinct groups of possums (Petauroidea and Phalangeroidea). The precise interrelationships of these various groups have been the subject of much debate, especially as to whether Australian possums are monophyletic or paraphyletic (see Meredith et al. 2009). Our current molecular study of diprotodontian relationships used a concatenation of sequences from protein-coding portions of 5 unlinked nuclear genes including multiple exemplars of most polytypic genera to resolve the outstanding problems of relationships within this group (see Figure 1). We added 10 diprotodontian species to the database of Meredith et al. (2009) and confirm that, within Diprotodontia, "possums" are a paraphyletic assemblage, with Phalangeroidea (Burramyidae + Phalangeridae) being the sister group to kangaroos and their relatives (Macropodiformes) and not to the other group of possums (Petauroidea) (see Figure 1 and Supplementary Figure S1). Although the sister relationship between Phalangeroidea and Macropodiformes is completely resolved in the Bayesian analyses, the support from likelihood analyses is less strong (74% Bootstrap support). Petauroidea, comprising the 4 families Acrobatidae, Tarsipedidae, Pseudocheiridae, and Petauridae, is itself the sister group to Phalangeroidea + Macropodiformes (Australoplagiaulacoida). Wombats and koalas (Vombatiformes) are the sister group to all other living diprotodontians (see Figure 1) and despite some earlier indications, *Dromiciops gliroides,* the sole living microbiotherian species, is probably not closely related to diprotodontians.

Estimates of the times of evolutionary radiations within marsupials based on relaxed Bayesian molecular clock methods have been presented elsewhere and suggest a late Cretaceous ancestor for marsupials with interordinal divergences from a common ancestor in the early Palaeocene (see Meredith et al. 2009; Springer et al. 2009, Table 1). Diprotodontia diverged from other australidelphian groups in the late Palaeocene-early Eocene and all of the current living families, apart from Potoroidae and Macropodidae, were present in Australia by the end of the Eocene (see Meredith et al. 2009). Potoroidae and Macropodidae seem to have diverged from their common ancestor somewhat later, in the middle Miocene, with subsequent radiations within each of the 2 families occurring in the late Miocene to early Pliocene (for estimated divergence dates of taxa shown in Figure 1, see Meredith, Westerman, Case, and Springer 2008, Meredith, Westerman, Springer 2008, Table 5, Meredith et al. 2009, Springer et al. 2009, Table 1). What is clear from our molecular phylogenetic studies is that all diprotodontian superfamilies are very old, much older than previously thought. This finding has major implications for

Figure 1. Phylogenetic relationships of marsupials based on the concatenated sequences from 5 nuclear genes. Chromosome numbers of particular species (where known) are indicated by color (2n = 10 yellow, 2n = 12 light blue, 2n = 14 green, 2n = 16 red, 2n = 18 orange, 2n = 20 pink, 2n = 22 purple, 2n = 24 dark blue, 2n = 32 light green). Multiple sex chromosome systems in particular species are indicated by chromosome numbers in ♂/♀, respectively. Unknown chromosome number for particular lineages or ancestors are indicated by ?. Postulated chromosomal fusions in *Macropus* species are indicated on particular branches. Diprotodontia is indicated by light box and eutherian out-groups are indicated as black lines. Did, Didelphimorphia; Vomb, Vombatiformes; Dasyur, Dasyuromorphia; Peramel, Peramelemorphia; N, Notoryctemorphia; M, Microbiotheria; Pa, Paucituberculata, Placen, Placental out-groups.

the timings of chromosomal changes associated with the radiations that we will discuss below. Here, we consider the implications of a well-resolved phylogenetic tree of marsupial relationships (Figure 1, Supplementary Figure S1) for understanding chromosome evolution within Marsupialia.

Discussion

Diprotodontian Chromosomes and the "Ancestral Marsupial Karyotype"

Although diprotodontian genomes, like those of other marsupials, are roughly comparable in size with eutherian mammals (Hayman and Martin 1974), marsupial nuclear DNA is organized into a smaller number of relatively larger chromosomes. Thus, chromosome numbers in this large, diverse, order range from a low of 2n = 10(♀), 11(♂) in the swamp wallaby, *Macropus* (*Wallabia*) *bicolor*, to a high of 2n = 32 in the rufous rat-kangaroo, *Aepyprymnus rufescens*; numbers which are coincidentally the lowest and highest known for any living marsupial. Compared with other australidelphian marsupial groups, karyotypes of Diprotodontia show a remarkable lability both in chromosome number and morphology, a lability only approached, and then to a much lesser extent in the karyotypes of the didelphimorph subfamilies Didelphinae and Monodelphinae.

With 2 notable exceptions, the marsupial mole (*Notoryctes typhlops*; 2n = 20) and the bilby (*Macrotis lagotis*; 2n = 18), all species of the 4 remaining australidelphian orders (Microbiotheria, Notoryctemorphia, Peramelemorphia, and Dasyuromorphia) are characterized by the same general karyotype of 14 chromosomes seen in a few diprotodontian families. These latter taxa are noteworthy in being phylogenetically basal clades within their respective superfamilies, a point to which we will return. Similarly, all paucituberculate taxa and many South American didelphimorphs are also characterized by 2n = 14 karyotypes.

The results of extensive G-banding studies on australidelphian (and other) marsupials led to the proposal that this 2n = 14 karyotype, seen in so many different families, probably represents the chromosome state of ancestral marsupials (Hayman and Martin 1974; Rofe 1979; Sharman 1982; Rofe and Hayman 1985; Hayman et al. 1987; Hayman 1990; Graves and Westerman 2002). This suggestion is certainly supported by plotting chromosome numbers onto our well-resolved molecular phylogeny of marsupials (Figure 1). It is clear that this particular karyotype, with its 7 pairs of autosomes and an XX♀, XY♂ sex chromosome system, is found in all australidelphian marsupial orders in at least one genus of each constituent family, in caenolestids and in many didelphimorphs (see also Supplementary Figure S1). G-banding studies also revealed that where differences between the 2n = 14 karyotypes of these diverse groups exist; they generally represented small variations on a theme. Thus, chromosomes of dasyuromorphs are characterized essentially by the occurrence of 4 pericentric inversions that alter the morphology of autosomal pairs 1, 2, 4, and 6 relative to those seen in microbiotherians, peramelids, wombats, and some pygmy possums. Curiously, of the more than 50 dasyurid species examined, only 3 show any G-band changes from others—2 very closely related species of *Ningaui* and *Antechinomys laniger*. All 3 appear to have had a subsequent inversion in their chromosome 6 that restores the ancestral morphology (Baverstock et al. 1983; Rofe and Hayman 1985). However, this apparent G-band identity of all dasyurids masks major differences in nuclear DNA content (Westerman and Woolley 1990), differences not simply attributable to either size or occurrence of chromosomal C bands.

Although neither *D. gliroides* (Spotorno et al. 1997) nor any of the South American paucituberculate 2n = 14 karyotypes differ in G-band morphology from the proposed ancestral marsupial karyotype seen in present day wombats, bandicoots, and pygmy possums, those didelphimorphs with 2n = 14 chromosomes (caluromyids, members of the genus *Thylamys*, and many others) all differ from the "ancestral marsupial" state in also having 4 pericentric inversions. Here, the changes involve autosomal pairs 1, 3, 5, and 6 (Rofe and Hayman 1985; Spotorno et al. 1997). In both dasyurids and didelphids, the relevant inversions probably all occurred in the common ancestor of the particular group during the Eocene or earlier. Subsequent chromosomal evolution away from the postulated ancestral marsupial condition has, to a greater or lesser extent, occurred in all major marsupial lineages, the changes varying from the seemingly minor inversions of dasyurids and some didelphids, to the much more extensive chromosomal repatterning seen in Diprotodontia and Didelphidae.

Studies on the 2 "odd" non-diprotodontian karyotypes noted above, namely *Notoryctes* (2n = 20) and *Macrotis* (2n = 18/19), show them to be readily derivable from an ancestral 2n = 14 condition essentially via 3 centric fissions in *Notoryctes* or via a few centric fissions and an X-autosome translocation to give the XX♀, XY_1Y_2♂ sex chromosome system seen in *Macrotis*. To date, neither G-banding nor chromosome-painting techniques have been applied to either of these 2 species to check whether this simple scenario is correct. Unfortunately, nothing is known about the chromosomes of the recently extinct pig-footed bandicoot (*Chaeropus ecaudatus*), the lesser bilby (*Macrotis leucura*), or of the desert rat-kangaroo (*Caloprymnus campestris*).

Chromosome numbers for species for which information is available have been plotted on to the tree of marsupial phylogenetic relationships based on 5 nuclear genes (Figure 1). Such plots, as with any other morphological character, tend to be problematic and somewhat simplistic when little karyotypic information beyond simple chromosome number and shape is available for particular taxa. Thus, for example, most 2n = 16 karyotypes within the genus *Macropus* were deemed to be identical until detailed G-banding studies demonstrated that the karyotypes within each of the 3 subgenera (*Macropus*, *Notamacropus*, and *Osphranter*) were in fact derived via quite distinct chromosomal fusion events (see below). Similarly, the 2n = 14 karyotypes of tree kangaroos, although morphologically superficially similar to those of dasyurids and pygmy possums, have been shown to be secondarily derived states after fusions of separate chromosomes in a tree kangaroo common ancestor rather than being a retained plesiomorphic condition.

Chromosome-Painting Studies in Marsupials and the Concept of Evolutionarily Conserved Chromosome Segments

Classic studies on chromosome evolution in marsupials suggested an apparent conservation of karyotypes. Much of that work depended on comparisons of high-quality G-banded karyotypes of various species and resulted in the proposal, outlined above, of an ancestral marsupial karyotype comprised 14 chromosomes. All other karyotypes could be derived from this via relatively simple cytogenetic processes. The application of chromosome-painting techniques, utilizing fluorochrome-tagged DNA from flow-sorted single chromosomes which are then hybridized, in situ, to chromosomes of other species, and more especially the use of chromosome painting between divergent marsupial groups, has been of immense importance in resolving questions of chromosomal similarities and differences in mammals (see e.g., Ferguson-Smith 1997). Cross-species chromosome painting has not only confirmed most of the G-banding–based schemes for chromosome evolution in marsupials suggested, for example, by Hayman (1990), Rofe (1978, 1979), and others, but has greatly extended it. Indeed, chromosome painting has been found to be better able to resolve small karyotypic differences between taxa—differences that were not easily detected with G-banding (see Sherwin and Graves 2006 for references). More importantly, widespread use of this technique has also allowed the identification of a limited number of "conserved chromosomal elements" in the karyotypes of diprotodontians and other marsupial groups as well as in eutherian mammals (see Rens et al. 1999, 2001, 2003, and additional references therein). Cross-species painting has also been immensely important in resolving the evolutionary origins/mechanisms of some of the more complex marsupial karyotypes such as those of the macropodid species *M. (Wallabia) bicolor* (2n = 10/11) (Toder et al. 1997), *Potorous tridactylus* (2n = 12/13), and *A. rufescens* (2n = 32) (Rens, O'Brien, Fairclough, et al. 2003, Rens, O'Brien, Graves, and Fergusson-Smith 2003), as well as in extending the evolutionary approach by identifying homologous chromosomes and segments between species.

The building blocks of karyotypic evolution in macropodids, indeed all marsupials, seem to have been large chromosome segments and often whole chromosome arms—the "conserved chromosomal elements" referred to above. These conserved segments or "blocks," have been described as comprising 18 autosomal units (C1–C18) and the X chromosome (C19) in all marsupial species (Rens, O'Brien, Fairclough, et al. 2003, Rens, O'Brien, Graves, and Fergusson-Smith 2003). They are shown in Figure 2 as they might have been arranged in the ancestral marsupial karyotype (after Rens, O'Brien, Fairclough, et al. 2003) and subsequently retained in extant microbiotherians, wombats, and pygmy possums. The same 19 conserved blocks have been identified in all marsupial groups that have been included in cross-species chromosome-painting studies to date. Unfortunately, these groups do not, as yet, include any petauroids, peramelemorphians, *Notoryctes*, *Dromiciops*, 2n = 14 didelphimorphs, or paucituberculatans. These latter taxa will all need to be included in future studies to test the wider applicability of the concept of conservation of chromosome regions in all marsupials. However, positioning of some blocks may be inferred from our knowledge of G-banded karyotypes as shown for *Tarsipes rostratus* (Figure 2j) and for the 2n = 14 karyotypes of didelphimorphs such as *Thylamys* and *Caluromys* (Figure 2c).

In those taxa that have been "painted," the 19 conserved chromosomal blocks (C1–19) appear to have retained a remarkable evolutionary stability in their arrangements relative to one another in so many widely divergent groups. In the ancestral marsupial karyotype (AM), chromosome AM1 comprises conserved blocks C1–C6, AM2 has C7–C9, AM3 has C10–C12, AM4 has C13–C14, AM5 has C15–C16, AM6 has C17–C18 and the AMX chromosome constitutes C19 (Rens, O'Brien, Fairclough, et al. 2003). The (re-) arrangement of the blocks in some derived karyotypes of dasyurids, *Acrobates pygmeus*, and other species can also be seen in Figure 2.

Chromosomal Evolution in Diprotodontia

Details of the changes involved in the karyotypic evolution of constituent diprotodontian lineages are given in the Supplementary Material. Here, we will simply outline the major conclusions. The basal split in Diprotodontia (see Figure 1) is between Vombatiformes (wombats and koala) and Phalangerida (kangaroos and possums) and probably occurred in the early Eocene. Vombatidae (wombats) diverged from Phascolarctidae (koalas) in the late Eocene (Meredith et al. 2009). All vombatid species have 2n = 14 chromosomes; the koala has 2n = 16, which G-banding studies suggest is derivable from a wombat-like karyotype by means of a simple centric fission in the equivalent of the submetacentric wombat second autosomal element. The G-banded karyotype of *Vombatus* is identical to that seen in bandicoots (Rofe and Hayman 1985), the microbiotherian *D. gliroides* (Spotorno et al. 1997), and some pygmy possums such as *Cercartetus* (Rofe and Hayman 1985). As such it probably represents a retained plesiomorphic marsupial chromosome complement.

Petauroidea

This superfamily probably diverged from Australoplagiaulacoida in the Eocene. Our phylogenetic tree shows Acrobatidae as the basal petauroid family. Both of its constituent genera (*Acrobates* and *Distoechurus*) have only single living species and both are known to retain a 2n = 14 chromosome complement superficially similar to that seen in wombats and burramyids. It is not, however, identical and shows evidence of small inversions and/or centric shifts (see Supplementary Material). To date, few petauroid species have been G-banded and none have been the subject of cross-species chromosome-painting studies. These will be required to validate the chromosomal changes postulated on the basis of G-banding. The 3 remaining

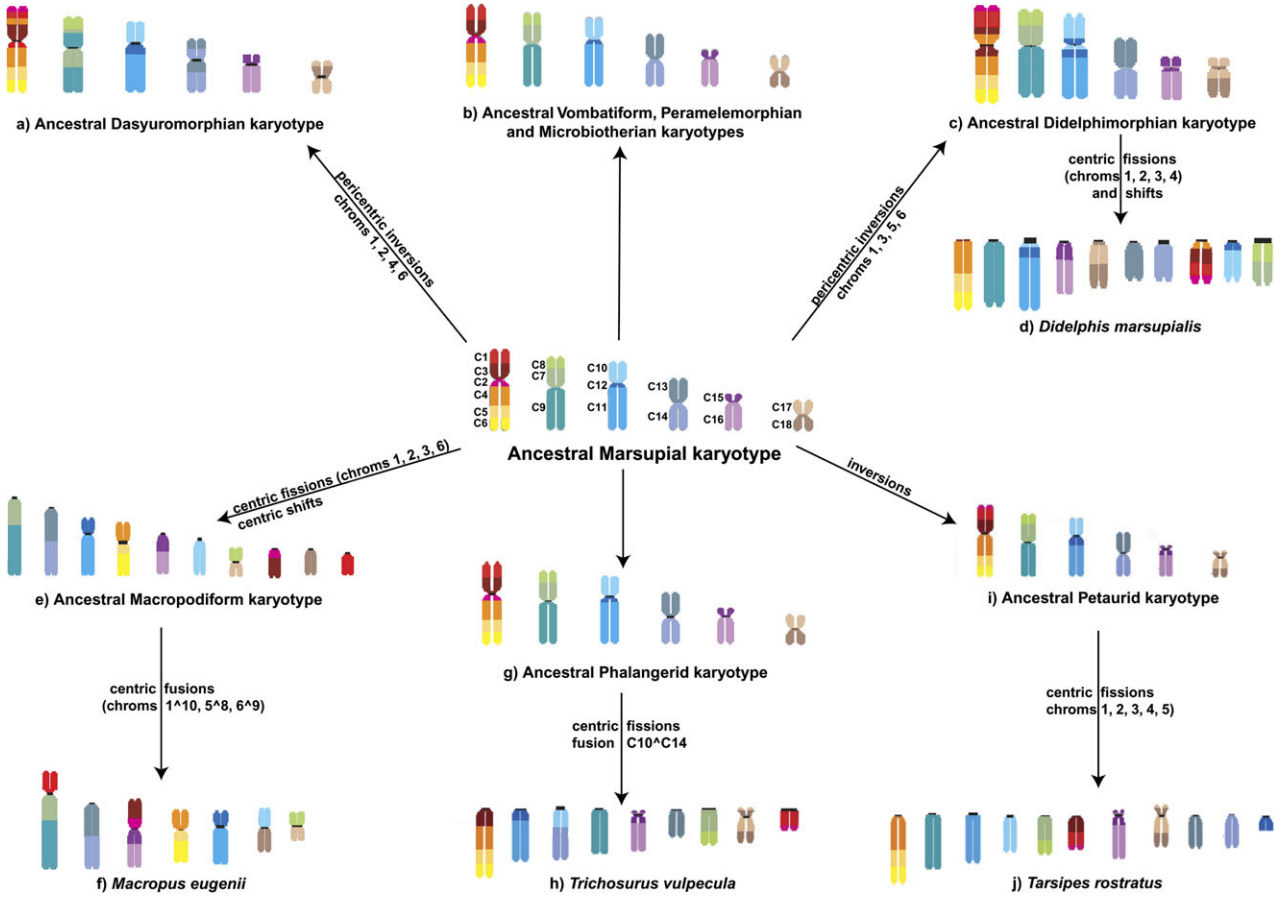

Figure 2. Ancestral karyotypes and conserved syntenic blocks for marsupial chromosomes. The ancestral marsupial karyotype comprises 18 conserved autosomal blocks C1–C18. (C19 is the X chromosome in all marsupials and is omitted from the figure) as suggested by Rens, O'Brien, Fairclough, et al. (2003). (**a**) Ancestral dasyuromorphian karyotype. (**b**) Ancestral vombatiform, peramelemorphian, and microbiotherian karyotypes (latter 2 based on G-banding only). (**c**) Ancestral didelphimorphian and (**d**) derived *Didelphis* karyotypes. (**e**) Ancestral macropodiform and (**f**) derived *Macropus eugenii* karyotypes. (**g**) Ancestral phalangerid and (**h**) derived *Trichosurus vulpecula* karyotypes. (**i**) Ancestral petauroid and (**j**) derived *Tarsipes rostratus* karyotypes based on G-banding information only. Types of chromosomal changes (centric fission/fusion) and chromosomes involved in karyotype evolution are indicated.

families—Tarsipedidae, Petauridae, and Pseudocheiridae—are all "old," probably having diverged before the end of the Eocene. All 3 show evidence of dramatic chromosomal reorganization during their evolution; the monotypic family Tarsipedidae expanding from 2n = 14 to 2n = 24 chromosomes; and the chromosome numbers of petaurid and pseudocheirid species ranging from 2n = 10 to 2n = 22 (see Figure 1). A consideration of the well-resolved phylogenetic relationships within this superfamily in which only the relative placements of *Gymnobelideus* and of *Pseudochirops (Petropseudes) dahli* relative to the 2 other *Pseudochirops* species are not resolved, strongly suggests a major reorganization of the karyotype took place in the common ancestor of Tarsipedidae and of the Petauridae + Pseudocheiridae clade shown in Figure 1. We note that the 2 petauroids not fully resolved in our current analyses, *Gymnobelideus leadbeateri* and *P. dahli*, were clearly resolved following addition of further nuclear gene sequences and extra petauroid species (Meredith et al. 2010) with *Gymnobelideus* sister to *Petaurus* (100% BPP and 98% Bootstrap ML) and *P. dahli* sister to *Pseudochirops albertisii* + *P. cupreus* +*P. corinnae* (100% BPP, 94% Bootstrap ML).

Phalangeroidea

Phylogenetic relationships in this superfamily are well resolved, apart from *Strigocuscus pelengensis* (Supplementary Figure S1) and all constituent families were distinct by the end of the Oligocene. In contrast, chromosomes of this superfamily are probably among the least well studied of any diprotodontians. Only 13 species have been karyotyped to date and very few have been the subject of either G-banding or of chromosome-painting studies. Much important information relevant to understanding chromosome evolution in the group is thus missing. All burramyids, which

represent the earliest divergent phalangeroid family, retain 2n = 14 karyotypes which differ only slightly, if at all, either from one another or from the proposed ancestral marsupial form. G-banded chromosomes of *C. concinnus* are virtually identical to those seen in *Dromiciops*, *Vombatus*, *Perameles*, and caenolestids (Hayman 1990; Spotorno et al. 1997). Chromosomes of the mountain pygmy possum, *Burramys parvus*, differ only in having some small pericentric inversions that create 3 more distinctly acrocentric pairs of autosomes in this species. Burramyids have thus essentially retained the ancestral marsupial karyotype—and probably the arrangement of conserved genome blocks (see below)—because their origin in the later Eocene.

Although 7 karyotypes of phalangerid species are known, G-banding and cross-species chromosome painting has only been done on one of them—*Trichosurus vulpecula*. The karyotype of this species, like 2 of its congeners, is 2n = 20 and is relatable to that of the proposed ancestral marsupial or ancestral phalangeroid by 4 centric fissions, a centric fusion and some pericentric inversions (see Figure 2h and Rens, O'Brien, Fairclough, et al. 2003, Figure 4). Whether this karyotypic restructuring took place in the common ancestor of the family Phalangeridae or only in *Trichosurus* (see Figure 1) is, at present, unknown. Five species of *Phalanger* all have 2n = 14 karyotypes (Donnellan 1989; Hayman 1990) which might suggests the latter alternative but we note that the morphology of these *Phalanger* chromosomes bear no obvious resemblance either to those of burramyids or to the proposed ancestral marsupial karyotype. To date, there is neither G-banding or cross-species chromosome-painting data for any species of *Phalanger* nor there any information on the karyotypes of the monotypic genera *Wyulda, Ailurops,* or of any species of *Strigocuscus* and *Spilocuscus*. Given the relative position of *Phalanger* in our phylogenetic tree, it would seem likely that the bi-armed chromosomes of this genus are the result of secondary fusion and/or translocation events subsequent to an initial round of centric fissions in the evolution of phalangerids. Consideration of the arrangement of some of the 19 conserved blocks of marsupial genomes suggests that the separation of C8 from C9 seen in *Trichosurus* (and also in the rufus rat-kangaroo *A. rufescens*) is an uncommon fission in australidelphian marsupials. These 2 conserved blocks are normally found together in all marsupial genomes. They have, independently, been separated in the ameridelphian species *Didelphis marsupialis*.

Macropodiformes

Macropodiformes diverged from Phalangeroidea 41–54 million years ago (Ma) (Meredith, Westerman, and Springer 2008) and subsequently radiated into a number of families of which only 3 are extant—Hypsiprymnodontidae, Potoroidae, and Macropodidae. Hypsiprymnodontidae (the musky rat-kangaroos) diverged from the common ancestor of all other kangaroos in the late Eocene or early Oligocene while the potoroid—macropodid split was probably an early Miocene event (Meredith, Westerman, and Springer 2008). Relationships of the 3 constituent families are well resolved, as are relationships between genera of Potoroidae. In contrast, apart from the resolution of *Lagostrophus* as sister to all other macropodids, many other macropodid relationships are not well resolved. A clade comprising *Dendrolagus*, *Petrogale*, and *Thylogale* is well supported in Bayesian and ML analyses, as is monophyly of the genera *Lagorchestes*, *Onychogalea*, and of *Dorcopsis* + *Dorcopsulus*. Monophyly of *Macropus* (plus *Wallabia*) is strongly supported by MrBayes (98% BPP) but much less so under ML criteria (66% bootstrap support). *Setonix* resolves as sister to *Macropus* + *Wallabia*, though levels of support differ markedly (100% BPP, 55% ML).

Chromosome evolution in macropodiform has been discussed in detail elsewhere and salient points are given in the Supplementary Material below. The ancestral macropodiform karyotype comprised 22 chromosomes resulting from centric fissions in 4 of the ancestral australoplagiaulocoid chromosomes, probably in the Eocene after (or concomitant with) the split between ancestral phalangeroid and macropodiform lineages. These chromosomal fissions increased the number of autosomal linkage groups from 6 to 10 in the common ancestor of all macropodiforms (AMac) and the 2n = 22 karyotype with its characteristic arrangement of the 19 conserved marsupial chromosome blocks (see Rens, O'Brien, Fairclough, et al. 2003, Figure 4 and Figure 2e below) is retained and remains essentially unchanged in *Hypsiprymnodon*, *Bettongia*, *Dorcopsis*, *Petrogale*, and *Thylogale*. Other macropodiform lineages are characterized by dramatic chromosome restructurings as detailed by Rofe (1979) and Hayman (1990, Figure 2) from their G-banding studies; restructurings which often resulted in karyotypes with the same number of morphologically similar chromosomes in different species, as evidenced by the 2n = 16 karyotypes of *Macropus* and of *Peradorcas concinna*. G-banding and cross-species chromosome painting have shown clearly that these morphologically similar karyotypes are effectively evolutionary homoplasies brought about by fusions of different conserved blocks in different lineages. This situation is similar to that reported for bats of the genus *Rhogeesa* (Baird et al. 2009), where superficially similar 2n = 34 karyotypes are generated by different centric fusions in unrelated species.

As noted, chromosome evolution in Macropodidae is probably the best understood of all marsupial groups, having been studied in detail with both G-banding and cross-species chromosome-painting techniques (see Supplementary Material for details). Indeed, our current ideas on modes of chromosome evolution within the group, and of conserved marsupial genomic blocks, stem largely from the studies by Rofe, Rens, Eldridge, and their coworkers (Rofe 1978; Hayman 1990; Eldridge and Close 1993; Glas et al. 1999; O'Neill et al. 1999; Rens et al. 1999, 2001; Rens, O'Brien, Fairclough, et al. 2003; Rens, O'Brien, Graves, and Ferguson-Smith 2003). It is clear that chromosome evolution in macropodiformes is characterized by profound rearrangements of the 19 conserved chromosomal blocks that were initially rearranged (mainly by fissions) from 14 to

22 separate chromosomes in the common ancestor of the superfamily (AMac) following its divergence from phalangeroidea. This "AMac" karyotype, retained in some genera of all 3 macropodiform families, has subsequently been altered many times by fusions or translocations to give the lower chromosome numbers seen in many genera today. (Supplementary Material for detailed discussion). These evolutionary changes include both further increases in chromosome numbers as seen in *Lagostrophus fasciatus* (2n = 24) and more spectacularly in the potoroid *A. rufescens* (2n = 32), as well as reductions in number resulting from fusions and/or translocations in various macropodids. These latter include the single AMac chromosome 1^10 fusion seen in *M. (Osphranter) rufus* (2n = 20) to the complex events giving rise to the 2n = 11♂, 10♀ karyotype of *M. (Wallabia) bicolor*.

The detailed G-banding and chromosome-painting studies also revealed that what appear to be superficially identical karyotypes (same chromosome number and morphology), even of congeners, may well be the result of different evolutionary changes. Thus, the 2n = 16 karyotypes of the 3 subgenera of *Macropus* have been independently arrived at and involve different fusions as discussed elsewhere (see Supplementary Material) and represent karyotypic homoplasies. Our tree-based approach, based as it is on a well-substantiated multi nuclear gene data set, allows us to correct some of the previously postulated pathways for chromosome evolution in macropodids which were based on much less substantial data sets. Our tree (Figure 1 and Supplementary Figure S1) shows that within kangaroos the genus *Macropus* is paraphyletic because it includes the swamp wallaby (*Wallabia bicolor*). It is also clear that *M. rufus* is a member of *Osphranter* and not sister to all *Macropus* species as suggested by Bulazel et al. (2007). Because the swamp wallaby is, phylogenetically, more closely related to *M. agilis*, *M. eugenii*, and other members of the *Notamacropus* clade (though without strong bootstrap support—see Meredith et al. 2009 and Supplementary Figure S1), then the 2n = 10♀, 11♂ chromosomes of *M. (Wallabia) bicolor* could not have been derived from those of a common ancestor with *M. giganteus* as suggested by Bulazel et al. (2007, Figure 3a), but rather from a 2n = 20 common ancestor with *M. rufus*, *M. robustus*, and *Notamacropus* species (see Supplementary Material). Inferences on the direction of chromosome change in the evolution of any group of organisms can only be correctly drawn if the phylogenetic tree underpinning them is well resolved.

What seems clear, and somewhat surprising, from a consideration of the results of both the G-banding and chromosome-painting studies of macropodiform species is that particular chromosome elements seem to be repeatedly involved in the changes to the karyotypic facies of different species. Thus ancestral macropodiform chromosomes AMac1, 5, 6, 8, 9, and 10 have been repeatedly involved in fusion, fission, or translocation events in many lineages (Hayman 1990; Sharman et al. 1990; Eldridge and Close 1993; Eldridge and Johnston 1993, Table 1; Bulazel et al. 2007). Similar nonrandom involvement of chromosomes in radiation- and chemical-induced exchanges has been demonstrated in a large number of organisms (see below) and implies either that certain chromosomal rearrangements are favored by selection or, more probably, that nonrandom involvement is a function of the nonrandom spatial arrangement of chromosomes and their parts in the 3D nuclei of eukaryotes (see below) (for references, see Eldridge and Johnston 1993; Greaves et al. 2001, 2003).

In marked contrast to the chromosomes noted above, chromosome AMac 2 has rarely been found to be involved in change, though it is involved in an exchange in *Potorous* (Rens et al. 1999). Similarly, chromosomes AMac3 and 7 are only slightly more frequently involved in chromosomal change. For example, a fusion of AMac3 with AMac6 (3^6) is also seen in *P. tridactylus*, and there is a fission of AMac3 into 2 separate chromosomal (linkage) entities in *Aepyprymnus* (Rens et al. 2003, Figure 4). The chromosome fusion AMac3^6 seen in *Potorous* seems to restore a combination of conserved segments (C10–C12) to a single linkage group found in the postulated ancestral marsupial complement. Although this phenomenon of nonrandom involvement in chromosomal change in marsupials was noted first for macropodiform species, the identification of the 19 conserved genomic blocks found in all marsupial genomes and the fairly constant association of particular combinations of blocks outlined below suggests that the same nonrandom involvement of chromosomes in karyotypic change is probably true for all marsupials.

Repeated involvement of particular conserved segments in karyotypic change in marsupial lineages does not mean that the outcome is always the same, however, because radically different linkage arrangements may ensue. Thus, in phalangerids, the ancestral marsupial C10+C12+C11 combination is rearranged differently to that seen in macropodids. The ancestral marsupial chromosome 3 that comprises these 3 blocks as a single genetic linkage group has been broken into 2 separate linkage groups (C10 and C11+C12) in the ancestral macropodiform complement. However, in *Trichosurus,* (Phalangeridae), the 2 separate linkage groups now comprise C12+C10 as one unit with C11 as a separate chromosomal element. A somewhat similar fission outcome for the same ancestral marsupial linkage unit is seen also in the karyotypes of the South American marsupials *D. marsupialis* and *Monodelphis domestica*. Clearly, these 2 latter situations represent independent fission histories for the breakup of the ancestral marsupial arrangement in didelphimorphians because the known molecular phylogenetic relationships of *Didelphis* and *Monodelphis* (Jansa and Voss 2000; Jansa et al. 2006; Voss and Jansa 2009) do not suggest a recent common ancestor for these 2 genera in which it could have occurred (see Supplementary Figure S2). These apparently identical fission states must again, therefore, represent independent occurrences and not a shared inherited character.

Similarly, conserved chromosomal blocks C13 and C14 found linked together on the ancestral marsupial chromosome 4 and in the ancestral macropodiform chromosome 2 are involved in chromosomal changes in macropodiform evolution. We have already highlighted their involvement

in potoroos and swamp wallaby. This same ancestral combination of conserved blocks, retained in so many groups of marsupials, is, however, broken up in *Trichosurus* with C13 now present as a separate chromosome and C14 combined with C10 in a new linkage structure. In South American marsupials, the ancestral 2n = 14 karyotype, retained in so many species, underwent other centric fissions in the common ancestor of the *Didelphis*, *Philander*, *Lutreolina*, and *Chironectes* clade. One of them involved chromosome 4, again producing the 2 independent chromosomes (C13 and C14) seen in these genera. The ancestral combination of C13 + C14 is retained in the *Monodelphis* lineage.

Chromosome painting has proved immensely useful in identifying 19 blocks in marsupial genomes which appear to be conserved over very large evolutionary time periods, but which have been rearranged relative to one another in different marsupial groups (Rens et al. 2003). Thus, C10 is associated with C18 in *M. eugenii* but with C12 in *M. domestica* and C14 in *T. vulpecula*; C12 is linked with C11 in *M. eugenii* and *Trichosurus* but with C10 in *Monodelphis* (Rens et al. 2003). The technique is somewhat "gross," however, in respect to its capacity to detect cytological similarities and differences. Thus, although physical mapping reveals that C10 of *M. eugenii* and *M. domestica* have been conserved genetically as well as cytologically for more than 60 million years (My) (Deakin et al. 2008), it seems that not only does C12 actually make up only a part of the pericentric region of *M eugenii* chromosome 5 and not the whole of 5p (Deakin et al. 2008) but also that the genes associated with C11 in *M. eugenii* besides having different linkage relationships in *Monodelphis* (see above) are also dramatically differently arranged (Deakin and Graves 2010). Also, genes associated with C10—a single unit on *M. eugenii* chromosome 6q—are associated not only with the long arm of *Monodelphis* chromosome 7q as suggested by Rens et al. (2003) but also with the distal end of 7p (Deakin et al. 2008).

Understanding Nonrandom Involvement of Chromosomes in Evolutionary Change in Marsupials

Having considered the dramatic deconstructions and reconstructions of diprotodontian karyotypes and of the genetic information contained therein, we must now consider what might cause the observed repeated nonrandom chromosome breakages at particular spots in the genome of marsupials. Transposable elements and nucleotide triplet repeats have been shown to be directly responsible for chromosome breakage in both insects and plants (refs in Metcalfe et al. 2007) and in mammals (Line-1 in gerbils and humans). They may also be responsible for chromosome instability at so-called fragile sites (see LeBeau and Rowley 1984; Eichler and Sankoff 2003; Waters et al. 2007) as well as in genome evolution (Kipling and Warburton 1997). Such nonrandom involvement of chromosomes in changes, which has been noted in many eutherian species including humans (Yunis 1986), implies either differential survival of certain chromosomal rearrangements or differential involvement of particular chromosomes in breakage and reunion.

The underlying causes of both of these phenomena are still largely unknown, but it is increasingly clear that particular DNA sequences may be preferentially involved in breakage and exchange and/or that the 3D arrangements of chromosomes in interphase nuclei probably plays a role in determining whether particular chromosomes will interact apparently nonrandomly. Although it had long been suggested that chromosomes might occupy defined positions within the 3D nucleus of many organisms, including humans, because involvement of chromosomes in radiation- or chemical-induced exchanges was not random, it was only following the development of chromosome painting and confocal microscopy that we gained a clear understanding that many chromosomes occupy discrete positions or territories within the nucleus (Manuelidis and Borden 1988; Nagele et al. 1999; Cremer et al. 2001; Zalenskaya IA and Zalenskaya AO 2004; Meaburn and Misteli 2007). These positions, or territories, may vary through the cell cycle (Bridger et al. 2000) and may be influenced by the gene density and/or transcriptional activity of the chromosome itself (Croft et al. 1999; Boyle et al. 2001). Cross-species chromosome painting has shown that such arrangement may well be conserved across divergent species including primates (Tanabe et al. 2002) and thus may reflect fundamental properties of the genetic material. The same phenomenon has been described in marsupials, where not only are rock-wallaby (genus *Petrogale*) chromosomes nonrandomly involved in low-dosage γ-ray–induced breakage or mitomycin-C–induced fusions (Eldridge and Johnston 1993) but also the chromosomes of dasyurid sperm nuclei are arranged in a defined order (Greaves et al. 2001). Importantly, it has also been demonstrated that the positions of chromosomes in dasyurid and wombat sperm are essentially the same, despite these organisms having diverged from one another over 50 Ma. Thus, the karyotypic repatterning (essentially 4 pericentric inversions in dasyurids) that differentiates these 2 divergent groups of marsupials has not affected chromosomal positioning within the sperm heads. Whatever the reason, there is abundant evidence from comparative G-banding, cross-species chromosome painting and gene-mapping studies that large blocks of mammalian genes and chromosomes are conserved over considerable time periods and that there is evolutionary conservation of chromosome territories in many groups including primates (Tanabe et al. 2002).

Within Diprotodontia, Eldridge, and his coworkers' extensive studies on G-banded chromosomes of rock wallabies have shown that particular chromosomes—especially 5, 6, and 10—are disproportionately involved in rearrangements in the evolution of species of *Petrogale* just as in other macropodids. Observations on radiation- or chemical-induced chromosomal changes in rock-wallaby chromosomes clearly showed that breaks were preferentially produced in the 3 chromosomes noted above, with a putative "hot spots" for breakage identified in chromosome 5. Mitomycin-C treatment of cells appeared to

preferentially involve chromosome 10 in fusion events. Thus, the very chromosomes known to be involved in naturally occurring evolutionary rearrangements in *Petrogale* species are those preferentially involved in induced damage.

It would thus appear that certain chromosomes, or chromosomal segments, are involved in karyotypic change much more than others due to overinvolvement of particular DNA sequences that are, themselves, nonrandomly arranged in the genome. This finding is supported by the findings of O'Neill and her colleagues (O'Neill et al. 1998, 1999, 2004; Ferreri et al. 2004, 2005; Ferreri 2008) that particular kangaroo endogenous retro virus (KERV) transposable sequences are involved in chromosomal breakage in rock wallabies and other macropods. Certainly, it has also been demonstrated that particular sequences of DNA on human and other primate chromosomes are often implicated in "fragile sites" and their susceptibility to breakage and that other chromosomal sites are specifically related to possible insertion or mobilization of transposable elements. Clearly there is a great need to understand more about DNA sequence arrangement in marsupial genomes as it may illuminate chromosomal evolution in this group of mammals.

We have already noted that chromosome evolution in marsupials, as with eutherian mammals (Eichler and Sankoff 2003), is frequently associated with centromere regions through fusions, fissions, centric shifts, etc. The centromere regions of chromosomes are indispensible structural regions of all eukaryotes that are responsible for attachment of tubulin fibers to the kinetochore and thus for controlling chromosome movement and precise segregation in mitosis and meiosis. Despite this vital cellular role, centromeres show considerable differences in their sequence make up not only between species but also between different chromosomes of the same nucleus. Their precise structure at the DNA level is currently unknown. However, it seems clear that the establishment and maintenance of centromeric chromatin depends on the presence of particular chromatin elements such as a variant H3 moiety (CENP-A), some repeated DNA sequences, and other molecules such as CENP-B, C, and E. CENP-A seems to be a key determinant for centromere identity and kinetochore formation rather than specific DNA sequences per se (Allshire and Karpen 2008), though the transcription of some of these centric repeats may also be essential (Carone et al. 2009). CENP-B protein seems to be essential in kinetochore formation in mammals, binding to specific 17-nucleotide elements in alpha satellite sequences at the centromere region and promoting assembly of CENP-A into chromatin (Allshire and Karpen 2008, Okada et al. 2007).

The centromere region of marsupial chromosomes has been shown to be the location of CenP-B–binding site as they are in eutherian mammals (see Kipling and Warburton 1997). The same regions also contain a number of repeat sequence moieties such as KERV (Ferreri et al. 2004, 2005; Bulazel et al. 2006; Ferreri 2008), though neither these molecules nor CENP-B sequences are by any means restricted to currently active centromeres and may also mark the location of "latent" centromeres at sites along chromosome arms. KERV sequences, named following their discovery in macropods, have subsequently been found in many other marsupial families including notoryctids, dasyurids, vombatids, pseudocheirids, and didelphids (Ferreri et al. 2005, Figure 4), suggesting that they are much more widespread in marsupials than their name implies and that they have been conserved in marsupials for more than 60 My. Similar, if not identical, sequences have been reported in several other vertebrate groups suggesting that they may be even older. Interestingly, these marsupial KERV/satellite sequences, although associating predominantly with centromeres, seem also to be associated with the junctions of most, if not all, of the 19 conserved blocks as well as with telomeres (Ferreri 2008), though apparently not between C2 and C3. Some sites at which these sequences occur are clearly internal to particular conserved blocks. This nonrandom localization of chromosome breakage-associated sites in marsupial chromosomes is similar to that of other "mobilization" sites known in mammals, including both Tigger sequences and the L1 sites of humans, at which various transposable elements can insert within the genome.

Centromere-associated sequences may not, however, be the only ones involved in chromosome evolution in marsupials. Thus, telomere sequences are, or appear to be, involved in at least some evolutionary chromosome reconstructions, especially in species with extensive C-band positive material. Such sequences when present within chromosome arms may mark the sites of previous chromosome fusions (see Metcalfe et al. 2007). Both centromere and telomere regions have long been recognized as peculiarly dynamic regions in chromosome evolution. Both are known sites of repetitive DNA sequences and are associated with large-scale rearrangements (Eichler and Sankoff 2003).

Whatever the actual mechanism of karyotypic repatterning in the evolution of both diprotodontians and other marsupial groups and whatever the role of particular (limited?) DNA sequences in this process, we must eventually also understand why such repatternings occurred when they did in particular lineages. Although karyotypic change often accompanies speciation and radiation events, it has long been clear that the latter are by no means either dependent on, or a consequence of, the former. Indeed, within Australia's marsupials, there has been massive speciation/radiation in some groups such as peramelids and the dasyurids (with more than 60 dasyurid species recognized today), yet the chromosomes show surprisingly little change over vast time periods. Within dasyurids, where the majority of species have been G-banded, the banding patterns of all species appear to be essentially identical despite the fact that there have been large (up to 30%) changes in actual amounts of DNA packed within the apparently G-band identical chromosomes of different species. These quantitative changes do not correlate with C-band content (Westerman and Woolley 1990).

Why then, for example, at some time between the median Eocene and the earliest Oligocene (see Meredith

et al. 2009), was there a truly massive upheaval in the chromosomes of the common ancestor of Macropodiformes from an ancestral marsupial 2n = 14 karyotype (retained in Burramyidae) to one comprising 2n = 22 largely acrocentric chromosomes? These changes presumably took place at or about the time that the ancestral macropodiform was moving away from a previously largely arboreal habitat into a new largely terrestrial habitat. After the early Eocene climatic optimum, the widespread warm and wet rainforests that had dominated most of Australia (or at least the southern half because the northern half of the continent may already have been somewhat drier with perhaps a more seasonal rainfall, see White 1994) began to open up. Temperatures decreased by the mid-late Eocene, *Nothofagus* replaced podocarps as the predominant vegetation type and more open, sclerophyllous habitats became available in central Australia.

If, as now seems likely, karyotypic restructuring was associated with the mobilization of transposable or retroviral-like elements in the genome and we know that even relatively innocuous environmental changes may activate transposons (Specchia et al. 2010), then we must ask just what environmental factors might have caused such mobilizations and the extensive bursts of transposable element activity in early Diprotodontia but not in other Australian Orders? What were the advantages of the massive karyotypic changes in the ancestors of Macropodiformes, apart from increasing the number of independent linkage groups in the genome, which allow for more recombination and the generation of more potential variability in the new karyotypes? Were these to do with changes to locomotory pattern, stomach structure and diet, and the production of new milk proteins, all which probably all arose at this time? What are/were the advantages of the similar breakup of the 2n = 14 karyotype in the ancestral petauroid, albeit at a slightly later time (Meredith et al. 2009), in animals that have essentially retained their arboreal lifestyle and diet? What caused the alteration of the karyotypic facies seen in the lineages leading to marsupial moles, to the bilby (*M. lagotis*)—the only perameloid not to retain an "ancestral" 2n = 14 karyotype, or to the *Didelphis* and *Monodelphis* clades of South American marsupials but not in marmosines? Whatever the "advantages" may have been, they clearly did not preclude subsequent chromosomal fusions, centric shifts, and translocations as both macropodiforms and petauroids radiated further to fill new niches created within the changing Australasian and South American climates and landscapes during the Miocene and Plio-Pleistocene.

A tree-based approach to the question of the "ancestral marsupial karyotype" and chromosome evolution is a powerful one and clearly identifies this ancestral marsupial karyotype as having had 14 chromosomes; 6 pairs of autosomes and an XX female, XY male sex chromosome system similar to those retained in many extant marsupial orders and, importantly, in the basal lineages of most extant superfamilies. These chromosomes are themselves made up of a limited number (19) of conserved genomic blocks, all of which may be bounded by retroviral-like sequences. Where larger chromosome numbers now prevail, whether in Diprotodontia, Notoryctemorphia, Peramelemorphia, or Didelphimorphia, they are all clearly derived from such an ancestral 2n = 14 form by means of relatively few kinds of chromosomal change—including centric fissions, fusions, translocations, and centric shifts. The question of "remnant" telomere sequences seen within some autosomes of didelphoids (Svartman and Vianna-Morgante 1998) is certainly interesting but by no means entails the derivation of all the lower chromosome numbers (14) seen today in this group from an ancestral higher number (20–22) as suggested initially by those authors, and also reiterated by Sherwin and Graves (2006, p. 53) and Svartman (2009). They may mark the site of recent fusions, but they may not (see Supplementary Material for discussion of karyotype evolution in didelphoids). In didelphids, as in all other marsupial orders, the direction of chromosomal change in evolution is, unequivocally, one of increase from an ancestral 2n = 14 state by means of fissions etc. in particular lineages such as Didelphinae (2n = 22) and Monodelphinae (2n = 18) and apparent chromosomal stasis in all others. Once again the well-resolved phylogenetic relationships of ameridelphian species as derived from nuclear and mitochondrial DNA sequence data sets shows clearly that the ancestral chromosome state in this group was 2n = 14 and that higher numbers were derived from this by fissions, just as they were in Australidelphia (see Supplementary Figure S2).

This, of course, is not to say that reductions in chromosome numbers have not occurred, and do not occur, in marsupial evolution. Demonstrably they do in certain groups such as the macropodid genus *Dendrolagus*. Where such reductions occur, the karyotypes can be clearly seen to be secondary derivatives from an ancestral complement—which in macropodoids comprised 2n = 22 acrocentric chromosomes. These reductions are, however, exceptions to the general trends of either numerical stasis (with or without some inversional changes) or of numerical increases via fissions and/or translocations in various marsupial families. Our tree-based approach to chromosome evolution in marsupials reveals that it is unnecessary to invoke somewhat convoluted arguments to explain current chromosome numbers such as " … karyotypes of extant Australian species could have originated from more than one ancestral stock, with different diploid numbers … The clear preponderance of the 2n = 14 'basic karyotype' in species of both continents, as well as its conservation for a very long evolutionary time period, considered a strong point in the fission hypothesis, may simply be the result of its high adaptation" (Svartman and Vianna-Morgante 1998, p. 266).

Although we may be sure that the ancestral marsupial chromosome number was 14, the precise form of this ancestral karyotype and arrangement of conserved blocks of chromatin is still moot. Whether the arrangement of the postulated 19 conserved blocks was similar to that seen in extant "basal" diprotodontian lineages such as *Vombatus* and *Cercartetus* as well as in peramelemorphians, microbiotherians, and caenolestids or whether it was similar to the arrangement seen in extant dasyurids or even to that in basal didelphimorphs, each with their own characteristic

pericentric inversions, is unknown. It is probably unknowable until we can accurately resolve the base of the marsupial phylogenetic tree.

The question of basal relationships within the marsupial tree remains one of the outstanding phylogenetic problems still to be resolved. However, it is clear that marsupials, like their placental relatives, appear to retain a limited number of conserved blocks of chromosomal material in their chromosomes that have been repeatedly shuffled/rearranged into new combinations in different lineages during evolution and speciation. The number of breakpoints at which this shuffling occurs is apparently limited, and possibly involves some special sort of sequence(s) in the chromosomal DNA that need more intensive investigation. The karyotypes seen in modern animals (and plants) represent a particular response/adaptation to environmental stress at particular stages of their evolutionary history and although probably being highly adaptive at that time, they now represent "phylogenetic relicts."

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

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