CSIRO PUBLISHING

Reproduction, Fertility and Development
https://doi.org/10.1071/RD18201

Review

Marsupial chromosomics: bridging the gap between genomes and chromosomes

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Abstract. Marsupials have unique features that make them particularly interesting to study, and sequencing of marsupial genomes is helping to understand their evolution. A decade ago, it was a huge feat to sequence the first marsupial genome. Now, the advances in sequencing technology have made the sequencing of many more marsupial genomes possible. However, the DNA sequence is only one component of the structures it is packaged into: chromosomes. Knowing the arrangement of the DNA sequence on each chromosome is essential for a genome assembly to be used to its full potential. The importance of combining sequence information with cytogenetics has previously been demonstrated for rapidly evolving regions of the genome, such as the sex chromosomes, as well as for reconstructing the ancestral marsupial karyotype and understanding the chromosome rearrangements involved in the Tasmanian devil facial tumour disease. Despite the recent advances in sequencing technology assisting in genome assembly, physical anchoring of the sequence to chromosomes is required to achieve a chromosome-level assembly. Once chromosome-level assemblies are achieved for more marsupials, we will be able to investigate changes in the packaging and interactions between chromosomes to gain an understanding of the role genome architecture has played during marsupial evolution.

Additional keywords: comparative genomics, cytogenetics, DNA sequence, epigenomics, genome architecture.

Received 4 June 2018, accepted 5 December 2018, published online 11 January 2019

Introduction

Marsupials possess features for which comparative genomics could provide an understanding of the steps leading to their evolution. Among these unique features is their mode of reproduction, where emphasis has been placed on development *ex utero* in the presence of a more complex lactation system rather than *in utero*, as in their eutherian counterparts. For the altricial marsupial young, development of their brain, immune system, reproductive system and thermoregulation occurs while attached to a teat and typically within the confines of the pouch, a distinctive feature of marsupials from which their name is derived. Comparative genomics has the ability to determine the genomic features contributing to these unique marsupial attributes. Already, comparative genomics has identified the evolution of genes contributing to the sophisticated lactation system of marsupials (Brennan *et al.* 2007; Lefèvre *et al.* 2007; Sharp *et al.* 2008), where the composition of the milk changes over the lactation period to meet the nutritional requirements of the young at different stages of development (Green and Merchant 1988). However, genome sequence data alone are not enough to completely dissect out the genomic features responsible for these interesting marsupial characteristics. We need to understand how the genome sequence is arranged on a chromosome and how a particular chromosome interacts over space and time with other chromosomes, because these factors profoundly affect gene regulation and hence phenotype (Dekker *et al.* 2017). In essence, we need to use a 'chromosomics' approach, where genome sequence data are combined with chromosome biology (Claussen 2005) to understand gene function and evolution.

Although the cost of sequencing a genome has greatly reduced since the first marsupial genome was sequenced, genomes assembled from next-generation sequencing technology are often highly fragmented and not assembled into sequences ordered and oriented on chromosomes. A lack of this knowledge limits the utility of the genome assembly and leaves many important questions regarding genome evolution and the evolution of lineage-specific traits unanswerable. For example, fragmented assemblies make it difficult to select DNA markers associated with particular phenotypes (Andersson and Georges 2004) important for breeding programs (Damas *et al.* 2017). Genome architecture plays a role in genome function and affects phenotype, but without chromosome-level assemblies we will not understand the true extent of the effect genome architecture has played in the evolution of marsupial features. We need to bridge the gap between the generation of genome sequences and

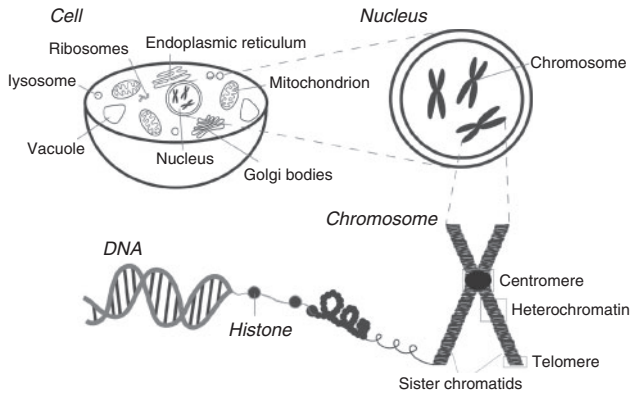

Fig. 1. Schematic of the formation and makeup of chromosomes and their presence in living cells. Here we depict an animal cell that includes the following cellular components: endoplasmic reticulum, Golgi bodies, lysosome, mitochondrion, nucleus, ribosomes and vacuole. The nucleus of each cell contains the chromosomes, which can vary in number between species and carry the genetic information of an individual. The chromosome is made up of sister chromatids, which are linked at the centromere, heterochromatin, which is involved in gene expression, and telomeres at the distal ends of the chromosome. Chromosomes are made up of DNA bound tightly around histones and proteins that condense to form the sister chromatids. The genetic information carried in chromosomes is replicated during cell division and is passed on between cells and from generation to generation, and controls cellular function and development.

understanding how this sequence is packaged into chromosomes (Fig. 1). We also need to remember that chromosomes consist of DNA wrapped around histone proteins. How tightly wound the DNA is contributes to gene regulation. The factors controlling this accessibility of DNA to transcriptional machinery (i.e. the layer of epigenetic control) also need to be considered for a complete view of the genome and its function. As such, we need to embark on the 'chromosomics' era, where we move from sequencing genomes, and thinking of a genome in a linear manner, to thinking of the genome in a multidimensional state to allow us to gain a deeper understanding of the organisation and interaction of chromosomes in the nucleus and how such organisation and interactions change over time. Basically, we need to consider the dynamic nature of chromosomes. Here, we highlight the cases where a chromosomics approach has revealed some important and interesting findings from marsupial genomes and what the future holds for this area of research.

Comparisons of marsupial chromosomes

Although marsupial genomes are generally of a similar size (~3 Gb) to the human genome, marsupials typically package their genomes into fewer and much larger chromosomes than most other mammals (Eldridge and Metcalfe 2006). Diploid chromosome numbers range from as few as 10 in female and 11 in male swamp wallabies (*Wallabia bicolor*) to 32 in the rufous bettong (*Aepyprymnus rufescens*), but common diploid numbers across the marsupial phylogeny are 14 and 22 (Sharman 1974; Hayman 1989). Different approaches, including flow karyotyping and bioinformatic calculations from genome assemblies, have been used to determine the size of marsupial

Table 1. Comparison of marsupial chromosome sizes (Mb) based on flow karyotyping (Renfree *et al.* 2011; Murchison *et al.* 2012) and bioinformatic estimates from the genome sequence (Mikkelsen *et al.* 2007; Renfree *et al.* 2011; Murchison *et al.* 2012)

Chromosome	Devil		Opossum		Tammar wallaby
	Flow	Sequence	Flow	Sequence	Flow
1	610	740	615	748	486
2	571	684	472	538	367
3	556	641	472	528	355
4	450	487	406	435	340
5	341	300	297	304	340
6	277	263	272	292	286
7			243	260	133
8			297	312	
X	122	87	97	79	150
Total	2927	3201	3172	3496	2457

chromosomes. Known marsupial chromosome sizes vary from the smallest at 79 Mb for the grey short-tailed opossum (*Monodelphis domestica*) X chromosome to the largest of 748 Mb for opossum chromosome 1. The size of Tasmanian devil (*Sarcophilus harrisii*) and tammar wallaby (*Notamacropus eugenii*) chromosomes fall between these two extremes, with most marsupial chromosomes being larger than the 248 Mb of the largest human chromosome (Table 1; Mikkelsen *et al.* 2007; Wang *et al.* 2011*a*; Murchison *et al.* 2012).

Prior to the genomics era, the molecular cytogenetic technique of chromosome painting was used to compare genome arrangement on a broad scale between different marsupial species. Based on chromosome painting results, marsupial genomes consisted of 19 conserved segments, varying in arrangement between species (Rens *et al.* 2003). This is far fewer than eutherians, which have experienced extensive genome reshuffling throughout their evolution, resulting in 30–40 conserved segments among eutherians recognised by chromosome painting (Ferguson-Smith and Trifonov 2007). However, there are differences in the level of genome reshuffling between different marsupial families. One family, the Dasyuridae, of which the Tasmanian devil is a member, shows remarkable karyotype conservation, with all species studied to date (~40/70) possessing a 2n = 14 karyotype (Hayman and Martin 1974; Young *et al.* 1982; Baverstock *et al.* 1983; Rofe and Hayman 1985; Westerman and Woolley 1990, 1993). In contrast, the Macropodidae family (~60 species) has experienced the most genomic reshuffling of any marsupial family, displaying more karyotypic diversity than other marsupials (Hayman 1989).

A limitation of chromosome painting is that it is unable to detect rearrangements that may have occurred within the large conserved blocks identified by chromosome painting, such as inversions. Comparisons of gene order on chromosomes using information from cytogenetic maps for the tammar wallaby (Deakin *et al.* 2008, 2013), the Tasmanian devil (Deakin *et al.* 2012) and opossum (Duke *et al.* 2007) have revealed extensive rearrangement of gene order within some of the conserved

Table 2. Comparison of marsupial genome assemblies
N50, genome assembly quality statistic indicating the length of the scaffold at which 50% of scaffolds in the assembly are longer

	Grey short-tailed opossum	Tasmanian devil	Tammar wallaby	Thylacine	Koala
Species name	*Monodelphis domestica*	*Sarcophilus harrisii*	*Notamacropus eugenii*	*Thylacinus cynocephalus*	*Phascolarctos cinereus*
Sequence method	Sanger	Illumina	Sanger, Roche 454, SOLiD, Illumina	Illumina	PacBio (polished with Illumina)
No. scaffolds	5180	35 974	277 711	1 318 979[A]	1906[A]
Scaffold N50 (kb)	59 810	1847	34	9[A]	11 589[A]
Reference	Mikkelsen *et al.* (2007)	Murchison *et al.* (2012)	Renfree *et al.* (2011)	Feigin *et al.* (2018)	Johnson *et al.* (2018)

[A]Contigs rather than scaffolds.

segments identified by chromosome painting (Deakin *et al.* 2013). It is these intrachromosomal rearrangements that could prove important for understanding the evolution of different phenotypic traits, particularly among species with virtually the same karyotype based on banding techniques, and emphasise the need for chromosome-level assemblies for a more in-depth comparison of genome arrangements between species.

Role of cytogenetics in marsupial genome projects

Marsupials have been part of the genomics era from an early stage, with one species (opossum) being among some of the first non-traditional model mammals to be sequenced (Mikkelsen *et al.* 2007). Several other species have since been sequenced using various approaches, from a combination of low coverage, traditional Sanger sequencing and next-generation sequencing, as for the tammar wallaby genome (Renfree *et al.* 2011), to being completely sequenced by one (e.g. Tasmanian devil (Murchison *et al.* 2012) and thylacine (Feigin *et al.* 2018)) or a combination (koala) of next-generation sequencing approaches (Table 2). The sequencing of many more marsupial genomes is planned as part of the Oz Mammal Genomics Initiative (http://www.bioplatforms.com/oz-mammals/, accessed 12 December 2018) and Genome 10K (Koepfli *et al.* 2015), truly bringing marsupials into the genomics era and providing the volume of genomes required to dissect out the genomic features leading to the evolution of marsupial-specific attributes.

Of the five marsupial genomes that have been sequenced to date, only the opossum genome assembly has incorporated cytogenetics to enable the genome to be visualised at a chromosome level (Duke *et al.* 2007; Mikkelsen *et al.* 2007). The opossum genome was sequenced to a depth of 7× coverage by Sanger sequencing to produce an assembly consisting of 5180 scaffolds with a scaffold N50 of 59.8 Mb (where the N50 is a genome assembly quality statistic indicating the length of the scaffold at which 50% of scaffolds in the assembly are longer; Mikkelsen *et al.* 2007). To anchor and orient sequences on chromosomes, two bacterial artificial chromosome (BAC) clones were mapped for every scaffold ≥1 Mb and one BAC clone for scaffolds between 500 and 1 Mb, resulting in a cytogenetic map consisting of 381 BAC clones (Duke *et al.* 2007).

Even for this relatively well-assembled genome, there is approximately 103 Mb of sequence that has not been given a chromosomal assignment (Duke *et al.* 2007) and some genes are missing from the assembly (Deakin 2010). Are genes missing from the assembly actually absent from the genome or have they simply not been sequenced or assembled because of problematic genomic features? Proving a gene or region is absent from a genome can be challenging. Targeted investigations of some genes have been required. For example, the imprinted insulin-like growth factor 2 (*IGF2*) gene is absent from the opossum genome assembly, but isolation and mapping of a BAC clone containing the gene localised this gene to the long arm of opossum chromosome 5 (5q3; Lawton *et al.* 2007). Comparison of the location of *IGF2* in the tammar wallaby demonstrated homology of opossum 5q3 with the short arm of tammar wallaby chromosome 2, a region for which chromosome painting had failed to identify homology between these two genomes (Rens *et al.* 2003).

For sequencing the Tasmanian devil genome, cytogenetics was included as part of the sequencing process. A female Tasmanian devil genome was sequenced using short and large insert libraries sequenced on the Illumina platform and assembled into approximately 35 000 scaffolds with a scaffold N50 of 1.8 Mb (Murchison *et al.* 2012). Chromosomes were flow sorted to generate pools of each of the six autosomes and the X chromosome for sequencing (Murchison *et al.* 2012). Sequence reads from these chromosome pools where then used to assign sequence scaffolds to each of the chromosomes, and the assignments were validated by a cytogenetic map consisting of 105 BAC clones (Deakin *et al.* 2012; Murchison *et al.* 2012). Although the sequencing approach gave an indication of which chromosome a scaffold was located on, it did not provide information on scaffold position on a chromosome, making it necessary to refer to the cytogenetic map for such information (Deakin *et al.* 2012).

The tammar wallaby genome was sequenced to 2× coverage by Sanger sequencing and combined with low coverage ABI SOLiD (Applied Biosystems), 454 GS-FLX Titanium (Roche) and Illumina sequencing (Renfree *et al.* 2011). The resulting assembly consisted of 324 751 scaffolds with a scaffold N50 of only 34.3 kb. Of the large number of sequence scaffolds, only 6979 (163 Mb) were assigned to autosomes by markers on a virtual map (Renfree *et al.* 2011; Wang *et al.* 2011*a*). The virtual map was generated from an integrated linkage map (148 loci)

and cytogenetic map (492 loci), with interpolation of conserved segments from comparison of the opossum and human genome assemblies used to virtually assign genes within these segments to chromosomes (Wang *et al.* 2011*a*, 2011*b*; Deakin *et al.* 2013).

Cytogenetics has not been incorporated into the genome assemblies for the remaining two marsupial genomes. The koala (*Phascolarctos cinereus*) genome was recently sequenced to a depth of 57× coverage by PacBio (Pacific Biosciences) long read technology and polished with 30× Illumina coverage to produce an assembly of 1906 contigs with an N50 of 11.6 Mb. Virtual koala chromosomes have been constructed using Bionano optical maps (Bionano Genomics) for scaffolding contigs and conserved synteny analysis (Johnson *et al.* 2018). Optical mapping identifies the location of restriction sites (fingerprints) using light microscopes and fluorescent labels to generate genome-wide maps that guide contig order and orientation (Lam *et al.* 2012). There is potential to incorporate cytogenetic mapping data into the koala genome in the future. The thylacine (*Thylacinus cynocephalus*) represents the first extinct marsupial to have its genome sequenced. Thylacine DNA was extracted from a 108-year-old museum specimen and sequenced on the Illumina platform (Feigin *et al.* 2018). Being an extinct marsupial, it is obviously impossible to prepare chromosomes for cytogenetic work. In the future, we need to aim to have a combined cytogenetic and genomic approach for achieving chromosome-level assemblies for all genomes where it is possible to obtain the appropriate samples for cytogenetic techniques.

Marsupial chromosomics: past and future

The combination of cytogenetics and genome sequence data for marsupial genomes has proven to be extremely valuable for answering questions of genome evolution. Some of the examples where this chromosomics approach has been applied in the past have focused on particular regions of the genome, such as the major histocompatibility complex (MHC) and the sex chromosomes. Other examples involve a whole-genome approach, such as reconstructing the ancestral marsupial karyotype or tracking the origin and evolution of devil facial tumours. These examples required considerable resources, including the construction of BAC libraries and costly genome sequencing. However, the technological advances in sequencing and cytogenetics are making it possible for a chromosomics approach to be more commonly used on marsupials.

Technological advances in genomic sequencing, particularly long-read sequencing (e.g. PacBio (English *et al.* 2012; Rhoads and Au 2015) and Oxford NanoPore Technologies (Jain *et al.* 2016)) are providing exceptional improvement in scaffold sizes of genome assemblies. However, despite the improvement long-read sequencing has over short reads in genome assembly, new genomes often fail to produce 'chromosome-level' assemblies, where contigs represent a whole chromosome. Even though long-read technology was used for the koala genome (Johnson *et al.* 2018), further work is required to achieve chromosome-level assemblies, and this is where advances in cytogenetics and innovations in sequencing technology are assisting in providing higher-resolution genome assemblies and will be important for implementation in marsupial research moving forward. Without chromosome-level assemblies, our ability to critically examine evolutionary questions, including basic questions surrounding genome evolution and function, as well as adaptation and speciation, is limited (Lewin *et al.* 2009; Seehausen *et al.* 2014). Cytogenetic approaches, such as fluorescent *in situ* hybridisation (FISH), where fluorescent probes are bound to DNA to identify regions on chromosomes with high sequence similarity to probes, will help generate physical maps for anchoring genomes to chromosomes. This will enable the analysis of breakpoints and gene order, positioning of centromeric and telomeric sequences, and structural and genic variation. In the past, we have relied on the costly construction of BAC libraries to generate the probes needed for the construction of cytogenetic maps, but this requirement has now been overcome. A novel approach was recently developed in birds, where a set of chicken BAC clones was bioinformatically identified and empirically validated as a set of universal avian probes to rapidly anchor sequence scaffolds to chromosomes of sequenced species (Damas *et al.* 2017). Developing a similar universal probe set for marsupials would make it possible to anchor the increasing number of marsupial genomes being sequenced for which appropriate samples can be obtained for the preparation of chromosomes. These probe sets, along with optical mapping (Teague *et al.* 2010), will provide high-resolution arrangement of sequence on chromosomes. We can take this a step further by using techniques to explore chromatin interaction and three-dimensional (3D) organisation of the genome, such as chromosome conformation capture (3C) and newer developments based on this approach (e.g. chromosome conformation capture-on-chip (4C), carbon copy chromosome conformation capture (5C), Hi-C (combines 3C with next generation sequencing), Chicago approach; Dekker *et al.* 2002; Dostie *et al.* 2006; Simonis *et al.* 2006; Lieberman-Aiden *et al.* 2009; Putnam *et al.* 2016). Chromosome conformation capture and derivatives assess the interactions between genomic loci across the genome to evaluate 3D nuclear space (Dekker *et al.* 2002). The Hi-C approach (Lieberman-Aiden *et al.* 2009) also gives more information about interactions (both *cis* and *trans*) and gene expression. It provides information on stable structural contacts and gene regulatory contacts (Sati and Cavalli 2017). High-resolution data produced from combinations of these approaches will provide further insight into chromosome evolution (see Kim *et al.* 2017). Combined with epigenetic chromatin information, as well as RNA and protein components, such approaches will advance our knowledge of genome folding and 3D structure. With many more marsupial genomes being sequenced, we have an opportunity to gain a much deeper understanding of the driving forces of marsupial genome evolution. Below we highlight several examples of where a chromosomics approach has been necessary and where building on these findings could lead.

Major histocompatibility complex

The MHC is a gene-dense region of the genome containing genes with an essential role in the immune response of vertebrates. Comparisons among vertebrates have shown the MHC to be a dynamically evolving region in response to different

pathogenic and environmental pressures (Kulski *et al.* 2002; Kelley *et al.* 2005). In most species, MHC genes have remained as a single complex, supposedly for a functional purpose (Trowsdale 2002). MHC Class I and II genes have evolved through gene duplication, making this region difficult to assemble from shotgun sequence data. To overcome this issue, MHC regions have often been assembled by first isolating large insert clones, typically BAC clones, and creating a contig of clones across the region for sequencing (e.g. Daza-Vamenta *et al.* 2004). In eutherian mammals, the complex is divided into three different regions: (1) the Class I region, containing predominantly MHC Class I genes; (2) the MHC Class II region, containing Class II genes and antigen-processing genes; and (3) the particularly gene-dense Class III region, which separates the other two regions and contains many genes with an immune-related function (for a review, see Kelley *et al.* 2005). The low-coverage tammar wallaby genome sequence made a combined cytogenetics and genomics approach essential for studying the evolution of the MHC region in this species.

To determine the organisation of the tammar wallaby MHC, BAC clones were isolated and then mapped to see where they were located in the wallaby genome. Surprisingly, Class I MHC genes were spread across the genome (Fig. 2), although there was an MHC region containing Class II and Class III genes located on chromosome 2, an arrangement that had never been observed before (Deakin *et al.* 2007; Siddle *et al.* 2009, 2011). The movement of Class I genes away from the core MHC may have potentially increased the number of Class I genes involved in classical Class I gene function (i.e. the presenting antigens to T cells), which may be an advantage for wallabies, perhaps permitting them to respond to a broader range of pathogens (Siddle *et al.* 2011).

The approach of creating a BAC contig to sequence MHC regions has been overcome by using long-read technologies for genome sequencing. The core koala MHC, consisting of 138 annotated genes, was assembled on a single scaffold of over 3.7 Mb in length from the long read-based assembly (Johnson *et al.* 2018). Included in this core region were eight of the 23 annotated Class I loci (Cheng *et al.* 2018; Johnson *et al.* 2018). However, it is yet to be determined whether the remaining Class I loci are located near the core MHC or on other chromosomes, demonstrating that a chromosomics approach is still required to fully characterise marsupial MHC gene organisation.

Sex chromosome evolution

Marsupial sex chromosome evolution has probably received more attention over the years than marsupial genome evolution as a whole. This interest is because the sex chromosomes function differently from autosomes. The X chromosome is gene rich and present in both sexes, but varies in dosage from one copy in XY males to two in XX females, making X chromosome inactivation, the mechanism of how dosage differences are overcome by silencing one X chromosome in females, of particular interest. Conversely, the Y chromosome is tiny and, in comparison with the X chromosome, the Y chromosome contains very few genes, is only present in males and is required for male development to proceed. For both the X and Y chromosomes, a chromosomics approach has been necessary to uncover important information regarding their evolution that could not be obtained solely from the genome sequence.

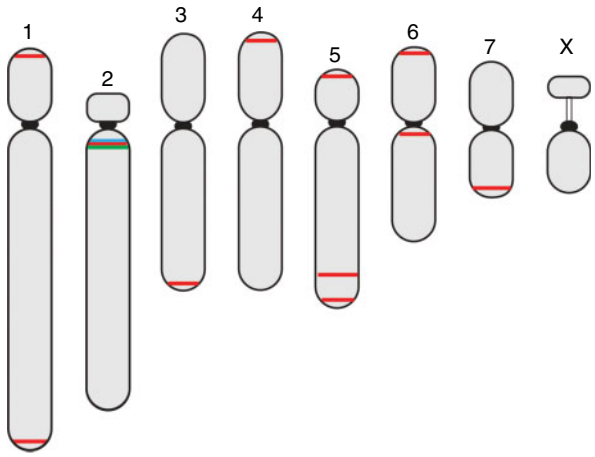

Fig. 2. Distribution of major histocompatibility complex (MHC) genes across the tammar wallaby chromosomes (1–7, X). The core MHC is located on chromosome 2 (Class I is indicated in red, Class II in blue, Class III in green). MHC Class I genes are distributed across each of the other chromosomes, with the exception of the sex chromosomes.

From cytogenetic studies, it is evident that the marsupial X chromosome shares homology with two-thirds of the human X chromosome (Glas *et al.* 1999), with the remaining one-third in marsupials being autosomal (Graves *et al.* 1995). The opossum X chromosome is approximately 97 Mb in size, which is smaller than the X chromosomes of the tammar wallaby (150 Mb; Renfree *et al.* 2011) and Tasmanian devil (122 Mb; Murchison *et al.* 2012). This size difference is attributed to differences in heterochromatin content and not to differences in gene content (Renfree *et al.* 2011). The opossum X chromosome contains 490 annotated protein-coding genes, of which approximately 440 and 330 are annotated in the Tasmanian devil and tammar wallaby assemblies respectively (based on Ensembl 91). Comparisons of eutherian X chromosomes demonstrate a remarkable conservation of gene order with the exception of rodents and cetartiodactyls (Rodríguez Delgado *et al.* 2009; Proskuryakova *et al.* 2017). Rearrangements of the X chromosome of eutherians was proposed as being selected against because it may interfere with the spreading of the non-coding RNA X inactive specific transcript (*XIST*) from the centrally located X chromosome inactivation centre, leading to the disruption of the X chromosome inactivation mechanism (Mikkelsen *et al.* 2007). A comparison of gene order on the opossum X chromosome determined from genome assembly with cytogenetic mapping data for the Tasmanian devil and tammar wallaby demonstrates a remarkable level of rearrangement (Deakin *et al.* 2008, 2012). One gene absent from the opossum assembly was *XIST*. In-depth searches of the opossum assembly failed to find any sequence resembling *XIST*. To determine whether the gene was truly absent from the genome, and not just an assembly issue, a comparative analysis of the region spanning *XIST* in humans was performed. This analysis showed that the genes flanking *XIST* are adjacent in chicken and frog, but these genes are

located at different ends of the opossum chromosome, suggesting that this region was disrupted in marsupials and that *XIST* was acquired after the divergence of marsupials and eutherians (Davidow *et al.* 2007; Hore *et al.* 2007; Shevchenko *et al.* 2007). Furthermore, sequencing revealed that *XIST* evolved partly from the protein-coding gene ligand of numb-protein X 3 (*LNX3*) that lost its function in the eutherian lineage (Duret *et al.* 2006). A marsupial-specific *XIST*-like non-coding RNA was subsequently discovered on the opossum X chromosome, known as RNA-on-the-silent X (*RSX*). *RSX* has features similar to *XIST*, consistent with it having a role in marsupial X chromosome inactivation and pointing to an independent origin of non-coding RNAs involved in X chromosome inactivation in two mammalian lineages (Grant *et al.* 2012).

A chromosome typically neglected by sequencing projects is the Y chromosome. Its highly repetitive nature makes it difficult to sequence and assemble as part of sequencing projects, resulting in female mammals being preferentially sequenced to obtain a better coverage of the X chromosome (for a review, see Tomaszkiewicz *et al.* 2017). Nevertheless, the Y chromosome is an interesting chromosome to study because of its key role in sex determination and differentiation. The Y chromosome varies greatly in size, gene content and gene order among species (Hughes and Page 2015). The Y chromosome of marsupials is remarkable for its tiny size compared with those of most eutherians, estimated to be as small as 10–12 Mb in some species (Toder *et al.* 2000), which is much smaller than the 60 Mb human Y chromosome (Hughes *et al.* 2010).

A novel approach, making use of cytogenetic techniques, was developed in order to obtain BAC clones specific to the tammar wallaby Y chromosome. Probes for the tammar wallaby Y chromosome were generated by flow sorting or microdissection, and these entire-chromosome probes were used to screen the BAC library to generate a sublibrary enriched for Y-specific BAC clones (Sankovic *et al.* 2006; Murtagh *et al.* 2012). BAC clones confirmed to map specifically to the Y chromosome were shotgun sequenced using Sanger sequencing. This sequencing resulted in the discovery of four new Y chromosome genes (ribosomal protein L10 on the Y(*RPL10Y*), methyl-CpG binding protein on the Y (*MECP2Y*), host cell factor C1 on the Y (*HCFC1Y*) and HECT, UBA and WWE domain containing 1, E3 ubiquitin protein ligase on the Y (*HUWE1Y*)) as well as four genes also located on eutherian Y chromosomes (sex determining region of Chr Y(*SRY*), RNA binding motif protein, T chromosome (*RBMY*), ubiquitin-activating enzyme E1, Chr Y (*UBE1Y*) and alpha thalassemia/mental retardation syndrome Y-linked (*ATRY*)). All the genes identified on the tammar wallaby Y chromosome to date also have a partner on the X chromosome (Murtagh *et al.* 2012). Similarly, BAC clones have been sequenced to identify 16 genes on the opossum Y chromosome, including the 10 identified on the tammar wallaby Y (Bellott *et al.* 2014), suggesting that at least these 10 genes were present on the ancestral marsupial Y chromosome.

Technological advances now make sequencing Y chromosomes far more feasible than the labour-intensive BAC sequencing approach. Techniques including chromosome microdissection or flow sorting (Tomaszkiewicz *et al.* 2016; Ma *et al.* 2017) followed by direct sequencing with next-generation technology are providing information on the gene content of Y chromosomes. New long-read technology is also helping by sequencing through highly repetitive regions, making assembly of the Y chromosome more achievable for many more species (e.g. Jain *et al.* 2018). These advances for sequencing sex chromosomes are exciting, because the sex chromosomes provide unique insight into a range of evolutionary questions associated with sexual selection (particularly sexually antagonistic selection; see Kirkpatrick 2017), genome function and sex-specific traits. Given that the Y chromosome has a smaller effective population size (1/4) compared with autosomes (1) and X chromosomes (3/4), this creates a unique selective background (Charlesworth 2009; Meisel and Connallon 2013) for evolution to act upon. In addition, the Y chromosome does not recombine during meiosis, except in the pseudoautosomal region (PAR), which marsupials lack (Toder *et al.* 2000). This makes them an interesting comparison to eutherians to explore the differences in genetic makeup on processes of recombination and selection on the sex chromosomes, as well as sex chromosome versus autosome divergence (see Mank *et al.* 2010). Comparative analyses across a diverse group like the Australian marsupials, with their unique reproductive traits yet varied life history (e.g. generation time and mating systems), will improve our knowledge and understanding of male mutation bias and sex chromosome evolution (Tomaszkiewicz *et al.* 2017).

Reconstruction of the ancestral marsupial chromosomes

We have long known that chromosome rearrangements are associated with speciation (see Coyne and Orr 2004). However, we still lack a good understanding of the effects of structural variation on genic divergence. Structural variation is generated by rearrangement of chromosomes and can include centric shifts, deletions, duplications, inversions, fissions, fusions and translocations (King 1993). We are yet to fully understand the significance of such chromosomal change at a gross level (process of speciation), or at a fine scale (local adaptation). As a starting point to answering these questions, we need to have an idea of how the ancestral marsupial genome may have been arranged and how it has changed over time. Originally, this was attempted simply by comparing marsupial karyotypes, which resulted in two alternative hypothesis based on the common occurrence of two diploid chromosome numbers among marsupials, $2n = 14$ and $2n = 22$ (Sharman 1973; Hayman and Martin 1974; Reig *et al.* 1977; Rofe and Hayman 1985). The $2n = 14$ ancestral karyotype was proposed because it was a chromosome complement among representatives of different marsupial families, with fissions resulting in higher diploid numbers (Rofe and Hayman 1985). Conversely, fusions of chromosomes in a $2n = 22$ ancestor were hypothesised to result in lower diploid numbers (Sharman 1973; Svartman and Vianna-Morgante 1998). Determining which of these hypotheses was more likely required reference to outgroup species, which was not possible using purely a cytogenetics approach.

The anchored opossum genome assembly made it possible to predict the gene content of the 19 conserved marsupial chromosome segments identified by chromosome painting, enabling comparisons of the conserved segment arrangement to be made

to outgroups such as chicken and human (Deakin *et al.* 2013). The fragmented genome assemblies of the Tasmanian devil and tammar wallaby were not appropriate for ancestral chromosome reconstruction, but physical mapping of BAC clones in these species partially compensated for the low quality of the assembly (Deakin *et al.* 2012, 2013).

One chromosome that proved particularly useful for testing the alternative ancestral karyotype hypotheses was Tasmanian devil chromosome 3. This chromosome consists of three conserved segments, referred to as C10, C11 and C12, in a similar arrangement as that predicted if the marsupial ancestor had a $2n = 14$ karyotype, whereas these segments would be distributed across two chromosomes if the ancestor had a $2n = 22$ karyotype. In the chicken genome, genes from all three segments are located on chicken chromosome 1, suggesting they were likely all on one chromosome in the ancestor of all mammals and supporting the $2n = 14$ hypothesis (Deakin *et al.* 2013). This combined cytogenetics and genomics approach is only the first step towards reconstructing ancestral marsupial chromosomes. A much more detailed reconstruction would be possible using an approach developed for reconstruction of ancestral eutherian chromosomes, where a few good-quality chromosome-level genomes in combination with more fragmented genomes could uncover the evolutionary history of marsupial chromosomes using probabilistic algorithms (Kim *et al.* 2017). Identifying chromosomal changes, including evolutionary breakpoint regions, can help determine the role of chromosome changes in relation to chromatin organisation and transcription (O'Connor *et al.* 2018). This has been shown to contribute to phenotype diversity and adaptive responses to climatic changes (O'Connor *et al.* 2018), and will assist in understanding the role of chromosomal change in speciation, adaptation and even disease evolution (Kim *et al.* 2017).

Tracing the evolutionary history of marsupial chromosomes will help understand how genomes evolve. Understanding the processes involved in the evolution of structural variation is also critical to our understanding of how the physical structure of genomes influences gene expression and function. Evolutionary breakpoint regions, sites where chromosomes break and reshuffle, are known to be characterised by repetitive elements, including tandem repeats and transposable elements (Farré *et al.* 2015; Capilla *et al.* 2016). Such regions are also reported to be located in gene-rich regions of the genome, containing functional genes (e.g. Ullastres *et al.* 2014). Early results indicate that the DNA sequence and the epigenetic state of the chromatin and its effect on gene expression are important in determining the location of evolutionary breakpoints (Carbone *et al.* 2009; Farré *et al.* 2015). However, such patterns need to be tested more broadly to examine how universal this relationship is and understand the mechanisms behind genome organisation (see Capilla *et al.* 2016). We also know that structural variation can influence GC levels, transcriptional activity and replication timing (see Federico *et al.* 2017). Recent research on primate genomes indicates that chromosomal rearrangements generally relocate genes into the same functional compartments (Federico *et al.* 2017), implying a strong selective pressure on function and expression. The effects of structural variation on gene expression have been difficult to assess, but recent technical advances have provided insight into the functional role of structural variants. Combining whole-genome sequence data with expression quantitative trait loci (eQTL) in humans identified a disproportionate role of rare structural variants on gene expression outliers (Chiang *et al.* 2017). Combining sequence data with gene expression data from technologies such as chromatin immunoprecipitation (ChIP) sequencing, DNase sequencing and eQTL will advance our understanding of the interactions of genome organisation on gene expression and the adaptive role such changes may have. These data will be important in understanding the evolution of structural variation and associated gene expression. To undertake such analyses, we require high-quality anchored genomes. Such chromosome-level scaffolds will allow for sliding window analyses of DNA divergence, identification of synteny fragments and identification of genomic breakpoints. The chromosomal variation across marsupial groups will provide insight into selective pressures on gene function through evolutionary history, how genomes adapt and evolve and heterogeneity in molecular evolution across the genome (see Vijay *et al.* 2017).

Epigenetic mechanisms have also been indicated in structural changes, with strong evidence emerging for a role of transposable elements in genome instability (see Rebollo *et al.* 2010). A large body of evidence has come from hybrid marsupials (e.g. O'Neill *et al.* 1998, 2004), showing links between heterochromatin and epigenetic modifications as a source for chromosomal change (see Brown and O'Neill 2010). In addition, epigenetic mechanisms have been related to new positioning of centromeres (du Sart *et al.* 1997; Ventura *et al.* 2004; Marshall *et al.* 2008), but not all variations in centromere position affect DNA order (see Brown and O'Neill 2010). Within marsupials, there are abundant opportunities to explore the mechanisms involved in centromere repositioning due to the presence of such variable centromere locations along chromosomes (Marshall *et al.* 2008). It is believed that epigenetics may facilitate changes of centromeres by chemical restructuring (e.g. methylation) of DNA and bound histones, heterochromatisation or deposition of DNA-binding proteins (for a review, see O'Neill *et al.* 2004). Further research is required at intra- and interspecific levels to understand variation in epigenetic regulation and gain important insight into epigenetic mechanisms associated with genomic architectural changes.

Across Australian marsupials, we have groups that are remarkably conserved in chromosome number and morphology (e.g. Dasyurids) and others that have highly varied karyotypes (e.g. Macropods; see O'Neill *et al.* 2004, and references therein). Such variation in stability of chromosomes make this group really exciting for exploration of the underlying causes for this contrasting chromosome evolution and the effects of chromosome variation on molecular evolution, as well as selective pressures on gene function.

The rock wallabies (genus *Petrogale*), with their diverse chromosomes (23 chromosomal races and 17 species), have been extensively studied to examine the role of chromosomal variation in speciation (see Potter *et al.* 2017; Fig. 3). However, it is only now with technological advancements and through integration of cytogenetics and genomics that we can start to truly disentangle the relationship between chromosome

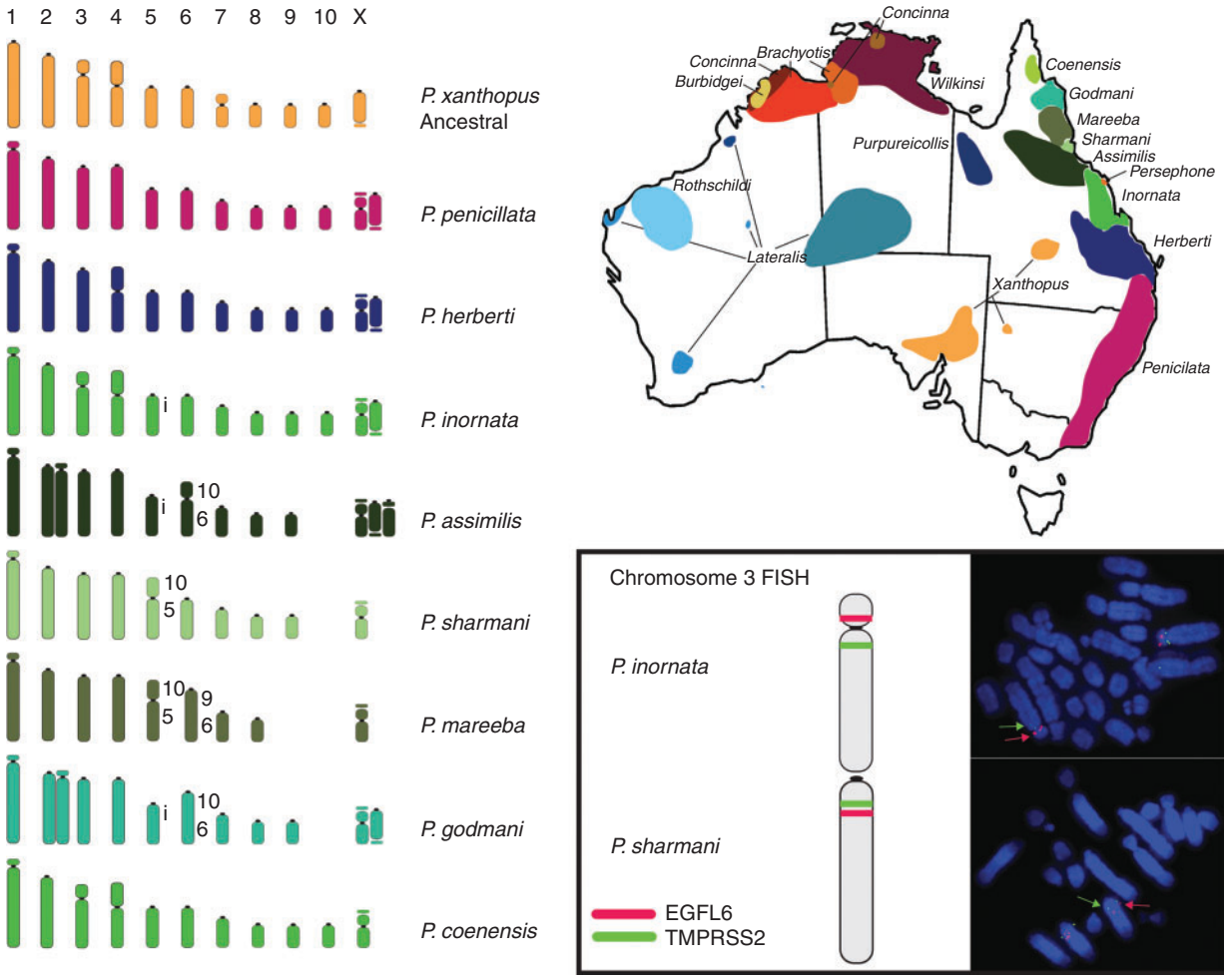

Fig. 3. Chromosome variation across rock wallabies (genus *Petrogale*). The recently diverged and highly variable karyotypes of the east coast of Australia *Petrogale* species are illustrated, including the ancestral karyotype of *P. xanthopus* (yellow-footed rock wallaby). The genus is distributed across Australia and consists of 17 species and 23 chromosomal races and subspecies. Rearrangements include fusions (e.g. chromosomes 5–10, 6–9, 6–10), inversions on chromosome 5 (denoted 'i') and centric shifts that have been used to identify new species. The panel on the bottom right shows recent fluorescence *in situ* hybridisation (FISH) results identifying fine-scale intrachromosomal rearrangement on chromosome 3 between *P. inornata* and *P. sharmani*, in addition to the previously recorded centric shift. Such mapping results will help elucidate the gene order changes associated with centric shifts and the potential role of such structural variation in reproductive isolation. Chromosome variants occurring within a species are indicated (e.g. *P. herberti*, *P. inornata*, *P. assimilis* and *P. godmani* have X chromosome variants).

variation and genic divergence to understand the causes of reproductive isolation in this group. To this end, combining whole-genome sequencing with physical mapping of genes to chromosomes using FISH techniques is providing insight into fine-scale structural variation and genetic consequences at a molecular level. Assessment of fine-scale genome structure is required to assess the variation in structural organisation not only between, but also within species. It was only through cytogenetics work that three new species were identified and described (Eldridge and Close 1992). There is evidence of polymorphism of X chromosomes within species of rock wallaby (e.g. *Petrogale assimilis*, *P. godmani*, *P. inornata*, *P. herberti*; Eldridge and Close 1992) at a gross chromosomal scale, but there could be intrachromosomal variation associated with heterochromatin or repetitive DNA, or even inversions within many more species. Such polymorphisms may lead to reproductive isolation and speciation by destabilising chromosome segregation during meiosis. Such structural changes are still difficult to detect via sequencing and assembly, therefore cytogenetics approaches that complement sequencing will help our understanding of the genomic basis of reproductive isolation and speciation. Analysing DNA alone can be misinformative in this group due to the recent nature of their divergence and the effects of introgression and incomplete lineage sorting (see Potter *et al.* 2015). Detailed examination of intraspecific and interspecific structural diversity will improve our knowledge of how chromosome rearrangements affect molecular evolution and speciation, as well as the functional role of structural changes. Integrative approaches of genomics, cytogenetics and epigenetics are providing exciting results in understanding

the principles of genome function and regulation (Sati and Cavalli 2017).

Tracing the origin and evolution of devil facial tumour disease

A major driver behind sequencing the Tasmanian devil genome was for it to be used as a reference for understanding devil facial tumour disease (DFTD) caused by two transmissible tumours (DFT1 and DFT2; Pearse and Swift 2006; Pye *et al.* 2016) and leading to the decline of the Tasmanian devil population. From cytogenetic analysis, it was evident that there were major chromosome rearrangements present in the tumour (Pearse and Swift 2006). Unfortunately, the fragmented nature of the genome assembly (over 35 000 scaffolds; Murchison *et al.* 2012) made detecting the extent of rearrangement challenging but, by using a combination of cytogenetic and sequencing approaches, the proposed origin of the chromosome rearrangements of these two tumours has been pieced together.

Molecular cytogenetic analysis and genome sequencing of DFT1 established that the animal in which the tumour initially arose was a female because there were two copies of most X chromosome genes and no Y chromosome material detected (Deakin *et al.* 2012; Murchison *et al.* 2012). Physical mapping of genes using FISH demonstrated that marker chromosome 1 (M1), so named because of its distinctive morphology and its unknown origin based on standard chromosome banding (Pearse and Swift 2006), is the most rearranged chromosome in DFT1. This chromosome consists of almost an entire chromosome 1 and a fragment of the X chromosome (Deakin *et al.* 2012). Although comparisons of tumour sequences to the reference have identified many rearrangements of chromosome 1 material (Murchison *et al.* 2012; Stammnitz *et al.* 2018), they are unable to determine to which DFT1 chromosome these rearrangements belong.

Tasmanian devil chromosomes have some features that have helped trace the formation of the M1 chromosome, including an unusual telomere length dimorphism where the paternally derived chromosomes are proposed to have long telomeres and the maternally derived chromosomes have short telomeres (Bender *et al.* 2012; Fig. 4*a*). The telomere length dimorphism has been lost in DFT1, but fortunately the paternal and maternal copies of the X chromosomes in female Tasmanian devils are epigenetically distinguishable because of imprinted X chromosome inactivation in marsupials (Ingles and Deakin 2015). The maternally derived X chromosome is hypermethylated in marsupials (Loebel and Johnston 1993; Rens *et al.* 2010; Ingles and Deakin 2015). Regions derived from the X chromosome and hypermethylated on DFT1 chromosomes would be derived from the maternal X chromosome. Such regions are distributed over several DFT1 chromosomes, including M1, leading to the hypothesis that the telomeres of the maternal copies of chromosomes X and 1 became critically short (Ingles and Deakin 2015), permitting the end-to-end fusion of these two chromosomes. A series of breakage–fusion–bridge cycles during mitosis ensued because the fusion would have resulted in a chromosome with two active centromeres (Fig. 4*b*). The end result was a seemingly shattered X chromosome and a highly rearranged chromosome 1 (Fig. 4*c*; Taylor *et al.* 2017).

Although M1 initially underwent extensive rearrangement, it now appears stable despite other chromosomes continuing to be subject to rearrangement. The stability of M1 suggests that its arrangement is required to drive the tumour and makes it a strong candidate chromosome for the initial mutation leading to DFTD (Deakin *et al.* 2012; Taylor *et al.* 2017). Transcriptome sequencing indicated that DFT1 likely started in a Schwann cell (Murchison *et al.* 2010). The neurofibromin 2 (*NF2*) gene, a tumour suppressor gene, and other genes implicated in schwannomas reside close to the end of Tasmanian devil chromosome 1. This region, being close to the predicted initial mutation site, is consistent with the Schwann cell origin of DFT1 (Taylor *et al.* 2017). Furthermore, sequencing of DFT1 tumours and physical mapping have identified structural rearrangements involving genes from a 10 Mb region surrounding the *NF2* gene (Taylor *et al.* 2017; Stammnitz *et al.* 2018).

A combination of molecular cytogenetics and sequencing has also provided insight into DFT2. Overall, the rearrangements of DFT2 are much simpler than DFT1, with the main rearrangement involving the insertion of chromosome 6 near the centromere of chromosome 1 (Deakin and Kruger-Andrzejewska 2016; Stammnitz *et al.* 2018). In this case, the telomere length dimorphism has been retained in the tumour, making it easy to determine that the copy of chromosome 6 with short telomeres has been translocated (Fig. 4*d*; Stammnitz *et al.* 2018). DFT2 originated in a male devil as a Y chromosome is present (Pye *et al.* 2016; Stammnitz *et al.* 2018), although there is evidence to suggest that the Y chromosome may have been lost from a tumour infecting a female devil, perhaps to overcome the female's immune response to proteins produced by the Y chromosome (Stammnitz *et al.* 2018).

It is interesting that in both transmissible devil facial tumours chromosome 1 is the most rearranged, because chromosome 1 has also experienced the most rearrangement during marsupial evolution (Deakin *et al.* 2012). The higher occurrence of rearrangement is not a factor that can be attributed to its larger size because it is only approximately 40–60 Mb larger than chromosome 2 (Table 1) and chromosome 2 has, in comparison, very few rearrangements, either in DFTs or during marsupial evolution. Is there something about the 3D structure of this chromosome that makes it more susceptible to rearrangement?

Although there is still much to learn about these two transmissible tumours, the combination of molecular cytogenetics and sequencing technologies has rapidly advanced our understanding of these two tumours. More could be learnt about the role of chromosome rearrangements in these tumours by improving the reference genome assembly to be of a chromosomal-level standard. By incorporating chromatin conformation and epigenomic data, we may uncover the chromosome features driving genome reshuffling in these tumours and determine whether there is something about chromosome 1 that has made the most rearranged chromosome in both tumours and during marsupial evolution or merely a coincidence.

Conclusions

Despite the improvement long-read sequencing has over short reads in genome assembly, genome sequencing often fails to

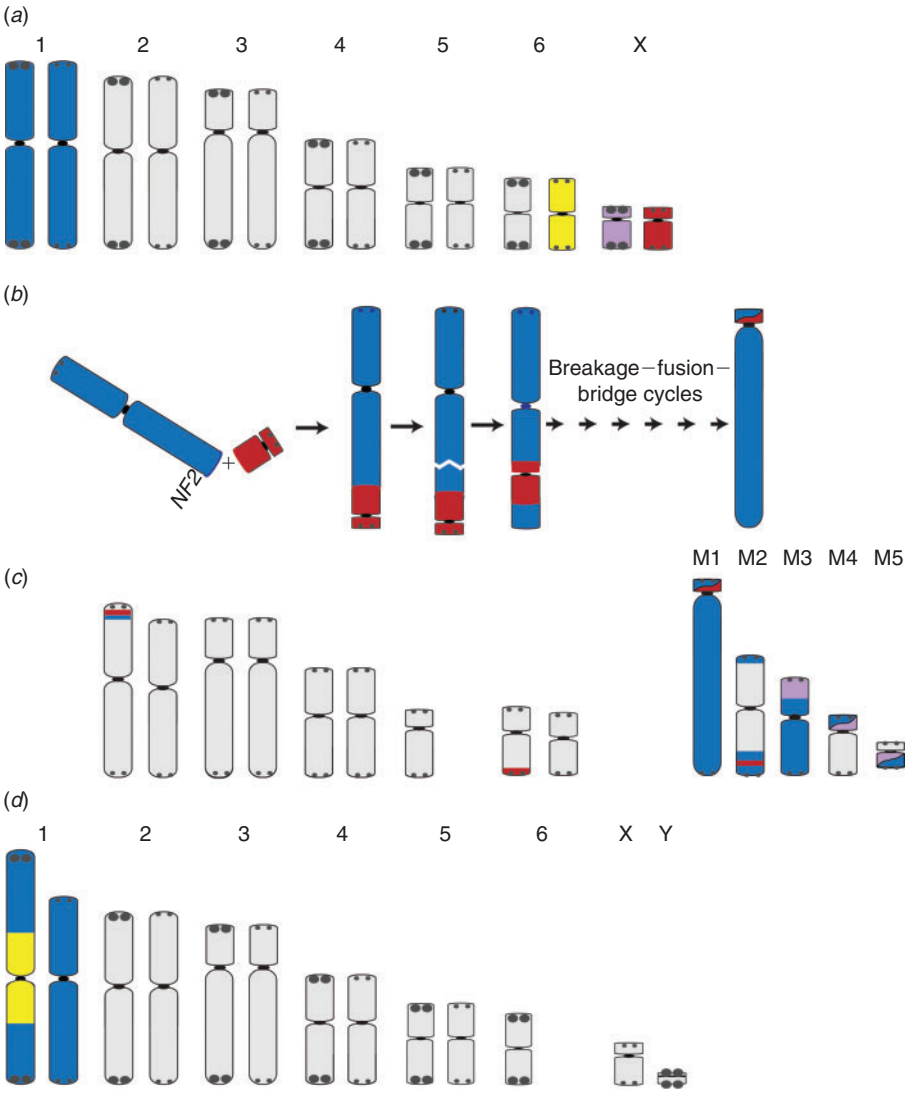

Fig. 4. Chromosome rearrangement in devil facial tumours (DFTs). (*a*) Normal devil chromosomes have a telomere length dimorphism indicated by the size of the dots at the end of each chromosome. The main chromosomes involved in DFT rearrangements are coloured. The X chromosomes are indicated in different colours to correspond with the different epigenetic states of the paternally derived X (pink) and maternally derived X (red). (*b*) The fusion of one copy of chromosome 1 with the maternally derived X chromosome and the series of breakage–fusion–bridge cycles leading to Marker chromosome 1 (M1). *NF2*, neurofibromin 2; (*c*) The DFT1 karyotype with chromosome 1 and X rearrangements highlighted. (*d*) The DFT2 karyotype with the chromosome 1 and 6 fusion being the main rearrangement.

produce 'chromosome-level' assemblies, where contigs represent a whole chromosome. Scaffolding is still required to achieve chromosome-level assemblies, and this is where advances in cytogenetics and innovations across the fields are assisting in providing higher-resolution genome assemblies and will be important for implementation in marsupial research moving forward. As stated recently, cytogenetics has much to offer the postgenomics era (Dion-Côté *et al.* 2017). One important component moving forward is collecting samples for cytogenetics as well as for genomics and building integrative datasets together with transcriptomics, proteomics and epigenetics to understand the mechanisms driving genome evolution and function. Without these data, we limit our ability to examine critically evolutionary questions, including basic questions surrounding genome evolution and function, as well as adaptation and speciation (see Lewin *et al.* 2009; Seehausen *et al.* 2014).

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

The authors acknowledge Marilyn Renfree for her contributions to marsupial genomics and the connection she has provided between marsupial biology and genomics. Janine E. Deakin especially thanks Marilyn for being a role model and mentor over many years. The authors' rock wallaby research is supported by an Australian Research Council Discovery Project (DP160100187) awarded to Janine E. Deakin, Jason Bragg, Craig Moritz, Mark Eldridge and Mark Kirkpatrick.

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