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Perspective

Cytogenetics: an important inclusion in the conservation genetics toolbox

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Abstract. Conservation uses information from genetics to assist in management decisions. However, conservation genetics typically assesses genetic diversity at the DNA level but this alone does not address all the risks associated with managing wild and captive populations. DNA is packaged into chromosomes. Differences in the number and morphology of chromosomes between species or even between populations of the same species can have important implications for management programs for threatened species. Cytogenetics, analysis of the higher molecular chromosome structure, can provide invaluable insight for the management of threatened species, where DNA alone could not address all genetic risks and threats to populations. Here we outline the important and valuable role of cytogenetics in conservation, highlighting two case studies based on threatened Australian marsupials: rock-wallabies and the Tasmanian devil. In conclusion, we summarise how cytogenetics should be better linked to conservation genetics and integrated into our management of threatened species, to ensure they have the best platform from which to persist and adapt into the future.

Additional keywords: genome, marsupials, wildlife management

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Introduction

Brief history of genetics in conservation

The application of genetics in conservation has developed rapidly since the 1970s (Frankel 1970, 1974), as we continue to evaluate the genetic consequences of small population size and its effects on survival and adaptability of individuals facing environmental change (see Frankham *et al.* 2010). Threatened species and associated small populations result from population decline as a result of anthropogenic processes, including introduced species, disease, changed fire regimes, habitat loss and altered ecosystems (Purvis *et al.* 2000; Mace *et al.* 2008; Woinarski *et al.* 2015). These threats have consequences for the fitness of individuals and populations, as a result of population size declines. Such declines cause a reduction in heterozygosity (genetic diversity) and allelic diversity within individuals and across populations, resulting in lower individual fitness. Such genetic changes reduce the ability for individuals and populations to alter their genetic makeup to adapt and survive environmental change or disease and thus can elevate the extinction risk of a population (see Frankham 1995).

The genetic consequences of population decline influence not only individuals in the wild, but also active management from captive breeding programs in zoos, reintroduction programs and on-ground management (e.g. translocations). Genetic considerations are particularly critical in relation to disease and fitness, to ensure the development of the most genetically robust and diverse populations for insurance and population growth. Genetically diverse populations are necessary to provide organisms with the best foundation to respond genetically (adapt) to environmental changes to persist into the future (Frankham 1995). Although assessing the genetic diversity of threatened species is important for the reasons mentioned above, one factor that is generally overlooked is that the DNA is packaged into chromosomes. Determining how this packaging varies within and between species can also have critical implications for conservation.

Some of the earliest genetic work carried out on many species was to determine the number and morphology of their chromosomes (i.e. karyotype) (e.g. Yonenaga 1974; Ryder *et al.* 1978; Baker and Bickham 1980; Patton and Sherwood 1983; see Ferguson-Smith and Trifonov 2007 for mammalian chromosome diversity). Such cytogenetic studies identified chromosomal differences, not only between closely related species but also between populations of the same species, and how these differences could impact management programs. In cases where species are difficult to tell apart phenotypically, breeding programs could end up crossing two chromosomally different species, resulting in several potential issues for the conservation of a threatened species, including reduced fertility of the unbeknownst hybrid offspring and loss of the taxonomically pure species. Similar issues could potentially arise from crossing individuals from chromosomally distinct populations

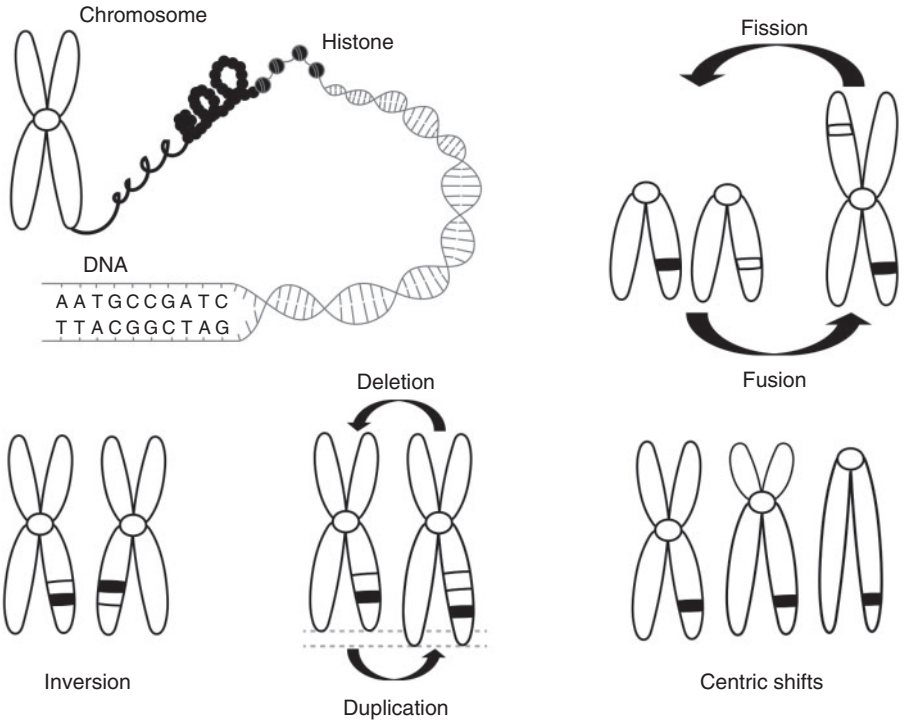

Fig. 1. Chromosome structure and types of chromosome rearrangements.

(Rubes *et al.* 2008). Despite these obvious and serious implications for species management, cytogenetic analysis is seldom carried out on animals in management programs. Instead, advances in molecular genetics and genomics, which have made conservation genetics more broadly accessible, are more commonly used to assess the genetic status of threatened species.

In the past decade, technology has advanced rapidly in genomics, and our ability to evaluate large parts of the genome and across diverse organisms via DNA sequencing is now achievable and affordable. Our ability to extrapolate and learn from a whole-of-genome perspective now provides greater opportunity to understand the role of functional genetic variation (e.g. selection) and the conditions and mechanisms that drive such variation (reviewed in Allendorf *et al.* 2010; Ouborg *et al.* 2010). However, we are only beginning to understand how genome structure (chromosome architecture) influences genome function (Harewood and Fraser 2014). There are cases where understanding the chromosome structure has been pivotal in managing populations for disease (e.g. Tasmanian devil – see below) and for mating chromosomally compatible individuals (Benirschke and Kumamoto 1991). Therefore, both cytogenetics and genomics offer valuable information for species conservation and should be carried out in unison to achieve the best conservation outcomes.

Genome architecture – chromosomes

Each chromosome is made up of DNA that is tightly packaged and coiled around proteins (histones) (Fig. 1). Chromosomes contain all the genetic material of an organism and their structure influences gene function, with varying configurations across diverse organisms. Chromosomal variation, where the genome is packaged differently between individuals, is known to influence transmission of genetic variation within and between populations (Darlington 1958; White 1973). This chromosome variation is generated by rearrangements, including inversions, fusions, fissions, translocations, centric shifts, deletions and duplications (King 1993) (see Fig. 1). Such structural variation can, but not always, result in genetic incompatibilities. For example, overcoming non-homology can depend on the size and type of rearrangement, the gene content affected by the rearrangement, the physical position in relation to centromeres, telomeres and genetic background (see Torgasheva and Borodin 2010). Balanced gametes with no effect on fitness are evident for assorted rearrangements including insertions and Robertsonian fusions (e.g. Kingswood *et al.* 1994; Vozdova *et al.* 2014). However, various rearrangements have been shown to have strong impacts on reproductive compatibilities and fitness, either through disruption of segregation during meiosis, or as the result of coadapted alleles fixing in separate populations and subsequent accumulation of genetic incompatibilities, known as Dobzhansky–Muller incompatibilities (reviewed in Navarro and Barton 2003; Ortiz-Barrientos *et al.* 2016).

Currently, we lack a clear understanding of the fine-scale chromosomal variation within and between populations of diverse organisms and therefore subsequent effects on fitness. Whether structural variation is the cause or consequence of divergence is irrelevant at this level. We purely need to identify whether such structural variation is associated with factors that would impede management of populations in a genetic context. Chromosome variation may not be detectable from examining

DNA sequence data and mutations alone. Therefore, cytogenetics plays an important role in understanding structural variation and the implications of this on gene and protein function.

Cytogenetics and current applications/technology

Cytogenetics, originally used to determine differences in chromosome number and morphology between species, has also experienced technological advances, but not to the same extent as genomics. In the 1970s, banding techniques, such as G-banding, permitted more detailed comparisons of karyotypes between species than traditional karyotyping, which was only able to determine chromosome number and morphology (e.g. Baker *et al.* 1983; Nash and O'Brien 1987; Romanenko *et al.* 2007). Molecular cytogenetic approaches such as fluorescent *in situ* hybridisation (FISH), where either a small segment of the genome (perhaps even a single gene) or an entire chromosome (chromosome painting) are fluorescently labelled and hydridised to chromosomes, has given more precise information on the extent of chromosome rearrangement between species (e.g. Heng *et al.* 1992; Matsuda and Chapman 1995; Wienberg and Stanyon 1997). Genomics can also be directly combined with cytogenetics by isolating particular chromosomes for sequencing, either by flow-sorting chromosomes based on size and DNA content or microdissecting a chromosome or portion thereof, followed by next-generation sequencing (Dolezel *et al.* 2012; Seifertova *et al.* 2013).

Despite the benefits to be gained from combining cytogenetics and genomics, the uptake of combined approaches, particularly for species conservation, has been limited. While cytogenetics was once one of the few tools available to researchers for comparing genomes between species, the technological advances in genomics and the rapid and affordable generation of data possible with current sequencing technologies have displaced cytogenetics from the genetics toolbox and resulted in a shortage in trained cytogeneticists and in an understanding of the requirements for sample collection for conservation projects.

Case studies

Below we outline two exemplars from Australia's marsupials where cytogenetics has made major contributions to conservation management. Both scenarios would have completely different outcomes if only DNA variation had been evaluated. Genetics alone could not provide the necessary information nor understanding to effectively manage threatened species.

Rock-wallabies

Rock-wallabies (genus *Petrogale*) are iconic Australian marsupials that form part of the kangaroo and kin family. There are currently 17 described species and 23 chromosomal races/subspecies, including five Endangered and seven Vulnerable taxa (under the *Environment Protection Biodiversity Conservation Act 1999*). Of these, six species have had or have ongoing conservation management, including captive breeding programs, translocations and reintroductions (M. Eldridge, pers. comm.). Their declines across Australia have been the result of introduced cats and foxes, competition from introduced herbivores, changed fire regimes, habitat destruction and degradation, and reduced genetic diversity (e.g. Dovey *et al.* 1997; Pearson 2013). To date, genetic researchers have helped guide conservation management of rock-wallabies by providing valuable insight into taxonomy, population structure, genetic diversity, captive management and reintroduction (e.g. Close *et al.* 1994; Moritz *et al.* 1996; Browning *et al.* 2001; Hazlitt *et al.* 2006; Piggott *et al.* 2006; West *et al.* 2018).

Rock-wallabies have the greatest chromosomal diversity amongst Australian marsupials (Superorder Australidelphida). Chromosome rearrangements range from simple to complex, including fusions, fissions, centric shifts and inversions. The most recently diverged species from north-east Australia have the most complex rearrangements and form the *penicillata* complex (including the threatened Sharman's rock-wallaby (see Fig. 2), and the Cape York rock-wallaby: Eldridge *et al.* 1988, 1989, 1990; Eldridge and Close 1992; Potter *et al.* 2015, 2017). In fact, it was only through cytogenetics approaches that three of these species were identified and described (Eldridge and Close 1992). Despite reproductive isolation among these species identified by hybrid crosses and infertility (Eldridge and Close 1992; Eldridge and Close 1997), they show extreme levels of DNA sequence homoplasy (introgression or incomplete lineage sorting: Briscoe *et al.* 1982; Bee and Close 1993; Eldridge and Close 1993; Potter *et al.* 2015, 2017). High levels of gene flow have been estimated from studies of neutral genetic markers, which under normal circumstances would imply they are one single species. These species are morphologically similar (Eldridge 2008) and therefore without an understanding of their genetics, or if DNA mutations were examined alone, there could be disastrous consequences for conservation management.

In addition, the black-footed rock-wallaby (*Petrogale lateralis*) consists of five chromosomal races and subspecies, which form the *lateralis* complex. This complex has simpler chromosomal rearrangements than the *penicillata* group, including some fusions and a centric shift (Eldridge *et al.* 1991). Three of these races and subspecies are currently listed under the *Environment Protection Biodiversity Conservation Act* and have ongoing conservation management and recovery teams (Pearson 2013). The full extent of fine-scale chromosome variation is unknown, as is how this influences diversity within and between these groups. Previous hybrid crosses between *P. lateralis pearsoni* and *P. lateralis* MacDonnell Ranges race produced fertile male and female offspring (R. L. Close, unpubl. data); however, the long-term viability of the hybrid animals is unknown, as is the long-term effect of such crosses on a population from a genomics context. These different races and subspecies inhabit different geographic regions (range from temperate to arid to tropical) and thus could exhibit localised adaptations. Further research is required to establish the cytogenetics and genomics differences in the *lateralis* complex.

We are currently undertaking research linking cytogenetics and genomics of the *penicillata* complex to understand how genome architecture and divergence at the DNA level create reproductive isolation. It is crucial that we have detailed physical maps of chromosomes to identify fine-scale rearrangements, to understand what parts of the genome are responsible or are influenced by incompatibilities, and to link the genes and their functions (if this is a consequence) to reproductive isolation.

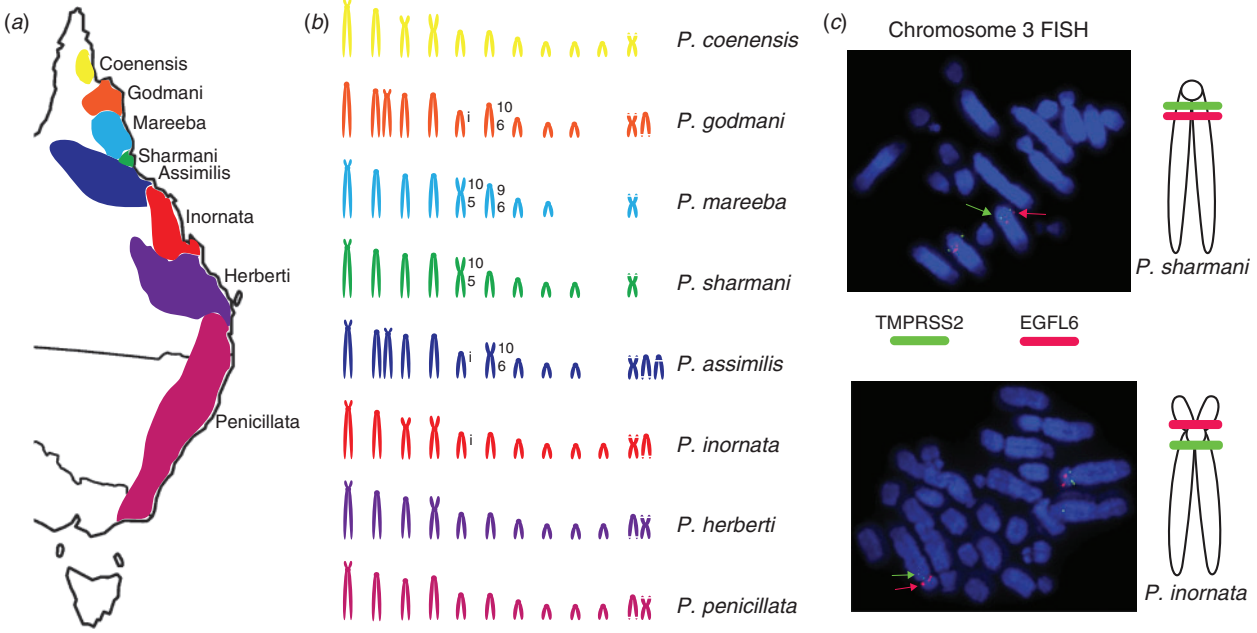

Fig. 2. Illustration of chromosomally diverse Australian east coast rock-wallabies (*Petrogale*). (*a*) Distribution map of the species. (*b*) Karyotypes of eight of the east coast species, highlighting rearrangements, including fusions (6–10, 5–10, 6–9), inversions (i) and variable karyotype morphology of Chromosome 2 and the X chromosome. The nucleolus organiser region is highlighted on the X chromosomes by white lines. (*c*) Mapping of Chromosome 3 genes *EGFL6* (red) and *TMPRSS2* (green) on *P. inornata* and *P. sharmani* chromosomes using FISH. The location of these genes demonstrates that a rearrangement of Chromosome 3 has occurred between these two species, highlighting the value of cytogenetics in determining gene order.

Our work will provide insight for management of *P. sharmani* and a broader understanding in general as to where in the genome the incompatibilities may lie. We already have evidence from gene mapping using FISH, that there are more fine-scale chromosome rearrangements (see Fig. 2) that were not previously detected from G-banding (Eldridge and Close 1992). Using the rock-wallaby system we will start to gain a better understanding of variation within species and between species. This will result in ongoing transfer of knowledge into conservation management of this genus.

Tasmanian devil and devil facial tumour disease

The iconic Tasmanian devil (*Sarcophilus harrisii*) has experienced massive population declines because of a deadly facial tumour disease. The disease was first reported in 1996 and has caused population declines of as much as 90% for some populations (McCallum *et al.* 2007). As a consequence, the devil was enlisted as an endangered species in 2008, with predictions of devils facing extinction in the wild within decades (McCallum *et al.* 2009). The devil provides a case study for how genomics and cytogenetics can be integrated into conservation management, starting from understanding the disease devils are fighting, to ensuring a viable insurance population was established for the future of the species.

The first step towards saving devils from extinction has been to gain an understanding of the enemy, the disease-causing agent, because the nature of the infectious agent would impact on the approach taken for establishing an insurance population and the disease management strategies employed. In this case, cytogenetic analysis of tumours from different individuals revealed an unusual infectious agent. All tumours had the same, highly rearranged karyotype, suggesting that the tumour cells themselves were the infectious agent, being transmitted from one individual to another when devils bite each other during social interactions (Pearse and Swift 2006). This hypothesis was supported when tumours from matched host samples were genotyped for microsatellite markers, major histocompatibility complex alleles and mitochondrial sequence where tumour samples from different individuals showed identical or almost identical genotypes but did not match the genotypes of their host (Siddle *et al.* 2007; Murchison *et al.* 2010, 2012). Devil facial tumour disease was, therefore, a transmissible tumour, requiring direct contact for transmission, meaning an insurance population could be established in Tasmania and on mainland Australia.

Further understanding of the disease came from a combination of genomic and molecular cytogenetic approaches. Transcriptome sequencing, where all the genes expressed in the tumour were sequenced and compared with the genes expressed in other devil tissues, uncovered the tissue origin of the tumour. Devil facial tumour cells had an expression profile most closely matching that of peripheral nerve and expressed a gene specific to Schwann cells, the cells that wrap around the axon to form the myelin sheath (Murchison *et al.* 2010). A combination of molecular cytogenetic, genomic and epigenetic analyses provided insight into the original chromosome rearrangements involved in tumour formation (Fig. 3). The founder animal was a female as there were two copies of the X chromosome and no Y chromosome material present (Deakin *et al.* 2012; Murchison *et al.* 2012). The tumour is thought to have started by

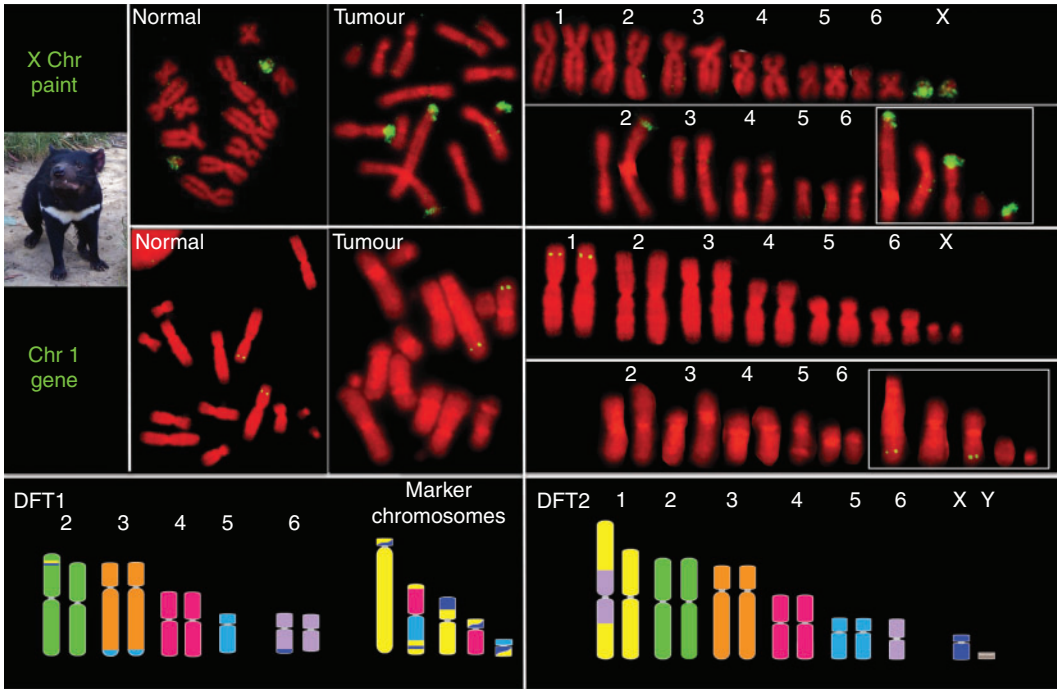

Fig. 3. Chromosome rearrangements in DFT1 identified by chromosome painting or gene mapping. In each case, the chromosome spreads are shown for normal and tumour chromosomes as well as an arranged karyotype. A comparison of chromosome painting with an X chromosome probe between normal female and DFT1 chromosomes shows the location of X chromosome material on Chromosome 2 and several marker chromosomes (boxed in karyotype). The mapping of a Chromosome 1 gene demonstrates the rearrangement of Chromosome 1 material in the tumour with this gene located on two marker chromosomes (boxed in karyotype). The complexity of DFT1 and DFT2 karyotypes are schematically depicted, with chromosomes colour-coded on the basis of their homology to normal devil chromosomes.

the loss of telomeres (the caps on the ends of the chromosome that prevent chromosomes from fusing to one another), permitting the fusion of one X chromosome to one homolgue of Chromosome 1, which then led to a series of rearrangements of this newly fused chromosome (Deakin *et al.* 2012; Ingles and Deakin 2015; Taylor *et al.* 2017). The tumour is continuing to evolve, with different chromosomal lineages arising from the original tumour (Deakin *et al.* 2012; Pearse *et al.* 2012).

More recently, a second facial tumour (DFT2) has been discovered. In this case, the tumour appears to have originated in a male as a Y chromosome is present and the rearrangements are less complex than in the first facial tumour (DFT1) (Pye *et al.* 2016; Deakin and Kruger-Andrezjewska 2016). As with DFT1, tumour genotypes from different individuals were identical to each other but did not match those of their host (Pye *et al.* 2016). DFT2 involves fusion, once again, of Chromosome 1 to another chromosome – Chromosome 6 (Deakin and Kruger-Andrezjewska 2016). Two independently derived transmissible tumours are a major concern for devil conservation. Devils appear to be susceptible to developing such tumours, which may even be a risk for the insurance population if such a tumour were to arise independently in an insurance animal. Continued cytogenetic monitoring of tumours is essential to ascertain whether we are dealing with multiple, independently derived tumours as well as tracking tumour evolution. Tumour evolution has also been shown to impact management strategies, with a culling trial actually appearing to result in more rapid tumour evolution (Murchison *et al.* 2012; Ujvari *et al.* 2014), perhaps giving the tumour the upper hand in the arms race between tumour and devils.

The other side of devil conservation is establishing an insurance population that adequately captures the genetic diversity of the wild population. Several genomic studies have demonstrated the population structure across Tasmania, with the requirement that insurance population founders be selected from different areas of the island (Miller *et al.* 2011; Hendricks *et al.* 2017) and continued genetic monitoring of insurance animals (Hogg *et al.* 2015). However, one aspect not captured by a purely genomics approach is variation at the chromosome level. Basic chromosome morphology analysis has demonstrated a polymorphic Chromosome 5 in the population caused by an inversion around the centromere (Pyecroft *et al.* 2007). Molecular cytogenetics has demonstrated a far greater level of Chromosome 5 polymorphism than a simple inversion (Deakin and Kruger-Andrezjewska 2016). Hence, a population study of chromosome polymorphisms and the impact on breeding could be useful for ensuring the maintenance of a genetically healthy insurance population.

The future – cytogenetics in conservation management

There are three main areas to address in order for cytogenetics to contribute meaningfully to conservation (Fig. 4). These include: (1) Sampling – which encompasses screening all individuals in

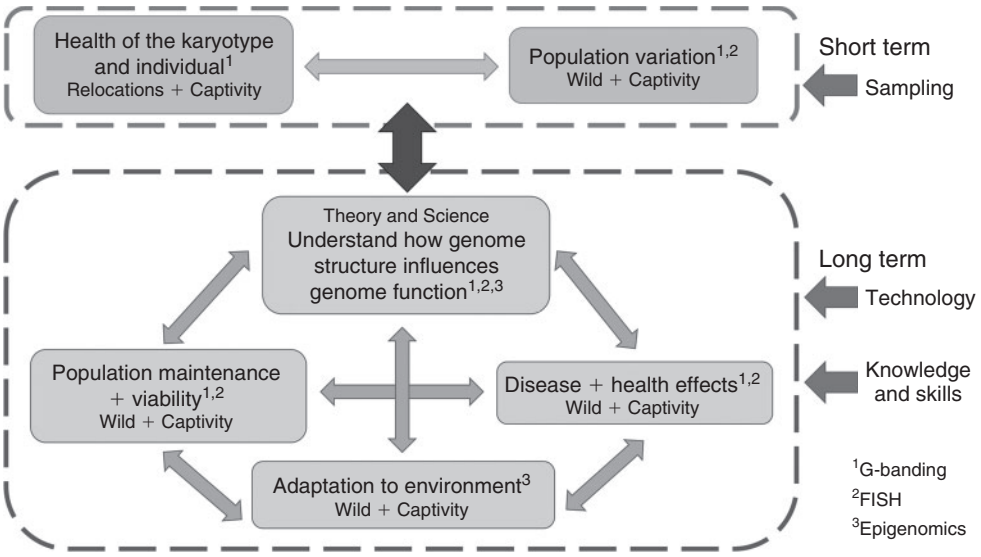

Fig. 4. Areas needing to be addressed in the short term and long term for cytogenetics and the contribution that these areas (sampling, technology, and knowledge and skills) could make to conservation.

captivity (blood samples) to ensure they have compatible karyotypes for mating, as well as representatives from wild populations to ensure the long-term success of reintroductions and animal translocations. General screening could start at a coarse level (karyotyping) and more detailed screening techniques could be applied if necessary to understand fine-scale questions in relation to genome function. Human patients are screened routinely for chromosome abnormalities as part of modern medical practice. It would be ideal to establish screening applications for organisms that have so much more at stake than us, and that often have such small numbers that risks should not be taken. To facilitate this process longer term, establishing cell banks of captive organisms (e.g. San Diego Zoo Institute for Conservation Research) and wild populations is crucial, and money needs to be invested in long-term storage and laboratory set-up. If this essential screening is not performed, conservation managers may augment populations with unfit or unviable individuals and thus create problems with long-term conservation implications. (2) Technology – applying suitable techniques to the necessary level of enquiry, as well as development in screening techniques will enable broader screening and inference of chromosome variation. Higher resolution of genome structure (e.g. super-resolution microscopy) will provide greater understanding of the variation of chromosome structure that exists as well as the impacts of changes in genome structure. As technology improves, the cost and capability of screening individuals will allow routine evaluation to become practical. Lastly, (3) skills and knowledge transfer – creating laboratories prepared to undertake routine screening and research of non-model organisms, as well as training staff and students in current technologies will enable progression from short- to long-term benefits of cytogenetics in conservation. This will not only help active management, but also have broader implications in resolving taxonomic uncertainties and understanding mechanisms of adaptation. An important part to this process is sharing knowledge with managers, researchers and the public through open-access software and websites (e.g. Atlas of Living Australia). We realise that cytogenetic screening of large numbers of animals is currently not achievable, so prioritising threatened species for translocation and captive management are the first targets. Such research and applications need to commence in parallel with fundamental research on diverse organisms (not just threatened species) to continue to understand the role of genome structure on gene expression and function, as well as continue to understand how chromosome rearrangements influence disease, adaptation and speciation. Ultimately, as we learn about these interacting mechanisms, we will understand and be able to anticipate the outcomes of what will or will not segregate normally before decisions need to be made for conservation management.

What led to our research in cytogenetics and marsupial conservation?

Sally Potter

Growing up in Australia and spending a large amount of my childhood camping, I had an appreciation for nature from an early age and this led me to study zoology and molecular biology at the University of Adelaide. After my undergraduate degree, I followed a few different research paths in the beginning, from trying to understand the evolution of a particular gene in relation to temperature and function in lungs, to trying to understand the role of selection across the genome in relation to milk production in cows! However, my passion for Australian wildlife was not being addressed. My continued volunteering on fieldwork and projects associated with Australian marsupials made me realise I really wanted to focus on questions associated with understanding the biology, evolution and conservation of our country's unique biota. It was through this volunteering that I developed my Ph.D. project examining a range of evolutionary questions about a group of rock-wallabies in northern Australia.

I have always been enthralled by Australian marsupials, with such varied adaptations – environmentally, physiologically and genetically. I have continued my research on this group and have always tried to influence the way we manage species for conservation through my work connected with museums, indigenous ranger groups and conservation agencies. However, my passion for Australia's fauna is not restricted to mammals! For the past six years I have been studying reptiles in northern Australia with Professor Craig Moritz, whose research interests overlap closely with my own. I started out as his laboratory manager but developed the role into a postdoctoral research position. This research was focussed at understanding the true diversity of skinks and geckos across the monsoonal tropics and how populations have persisted, adapted and diverged through past climatic events.

My research interests are broad and in general I aim to understand processes from the population level through to how different species evolve and persist. Currently, I am doing a postdoc evaluating the role of chromosomes in rock-wallaby speciation. This project emerged from my early postdoctoral research and continued fascination with this unique and interesting genus. It is during this process that I have really started to appreciate that DNA mutations are not the only factor to create differences amongst individuals and populations, but also how those strands are packaged and interact in chromosomes. This is where I met Professor Janine Deakin and she has been an important mentor ever since. I am an early-to-mid-career academic still trying to pave my way in research. The biggest reward for me is to make discoveries that impact on Australian biodiversity and conservation.

Janine Deakin

From the age of ~6 years old, I knew I wanted a career in research. I remember having a blood test for the first time and being totally fascinated with everything from the blood collection process (I was never scared of needles) to what they would do with the blood afterwards. Throughout my school years and even during my undergraduate degree, where one of my favourite courses was 'Mammals of Australia', I was fascinated by Australia's unique animals – marsupials and monotremes. I never envisaged that I would be fortunate enough to study the ins and outs of their unique biology as part of my career. I also thoroughly enjoyed all three genetics courses I completed during my degree and was inspired by lectures from Professor Dick Frankham, Dr Peter Johnston and Professor Des Cooper. During my Honours year, my interests collided when I completed a project with Peter Johnston on X chromosomes in wallaroos and later on a Ph.D. with Des Cooper on the flip side of the coin to conservation – controlling pest species like the brushtail possum in New Zealand.

The topics of my studies have varied since my first foray into marsupial research, ranging from identifying bacteria in the pouch of brushtail possums where the immunologically immature pouch young develop, to reconstructing the events that have shaped marsupial genomes. Over the course of these different projects, I not only developed a deeper fascination for all things marsupial but, thanks to Professor Jenny Graves, fell in love with their beautiful chromosomes. This combination of passions has driven my research into its current direction of a closer union between genomics and cytogenetics for understanding biological mechanisms important for species conservation, whether that be the role of chromosome rearrangements in the formation of new species (rock-wallabies) or in understanding the origin and evolution of a devastating transmissible tumour (Tasmanian devils).

I may have made following my dream of becoming a researcher sound easy. Indeed, I was fortunate to have a supportive family growing up who encouraged me to follow my passion, teachers who helped my interest in biology to grow and mentors who showed me the way. Of course, my journey has also involved a lot of hard work and 14 years of working on two-to-four-year contracts before finally securing a dream job with a continuing position as a research-focussed academic. However, with this new position came the realisation that there were far too few women in the higher levels of academia. Rather than dwelling on the negative of this realisation, I decided to positively influence the younger generation of women coming through. I established a support network for women in STEM in our university, and I mentor and provide opportunities for early-career researchers to help their careers. As much as I hope I'm helping these women to reach their dreams, I am being mentored by them as well. I'm encouraged to see a bold new generation of women embarking on careers in sciences and am confident that, in time, we will see many more women leading the way in conservation research.

Conflicts of interest

The authors declare no conflicts of interest.

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