PROPOSAL TO SEQUENCE THE GENOME OF THE MODEL MARSUPIAL *MACROPUS EUGENII* (TAMMAR WALLABY)

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I. Overview

Marsupials were regarded as curiosities by their early European discoverers, animals to be wondered at. Nevertheless, it was recognised very early that they could make a major contribution to our understanding of reproductive processes, and indeed marsupial research led the field of reproduction during the first half of the 20th century. Marsupials are mammals, but represent a 100+ million year isolate from eutherian ("placental") mammals. This makes them particularly valuable "alternative mammals" for comparative studies, and their inclusion has dealt many blows to basic genetic and reproductive hypotheses. They are equally valuable for enlarging and enhancing our understanding of the mammalian genome, being distant enough from mice and man, but closer to them than chicken or fugu.

The tammar wallaby, a small kangaroo species, is a thoroughly typical Australian marsupial. Its size, availability and ease of handling make it the most intensively studied model marsupial for anatomical and physiological studies, especially of reproduction and development. Considerable genetic and cytological data, as well as genomic resources, are available for this species.

The tammar genome comprises about 3.6 Gb, with a relatively short genetic map length of 1172 cM. It is arranged as 8 large chromosomes in a 2n=16 karyotype that is every cytologist's dream. It is easily related to the conserved 2n=14 marsupial karyotype that is thought to be ancestral to marsupials. Its genome represents a rich and untapped unique resource for comparative genomics, offering power to identify genes and regulatory sequences by phylogenetic footprinting, dissecting complex pathways of genetic control, and to answer "how?" and even "why?" questions of mammalian genome function and evolution.

The approach that we would propose for sequencing the tammar genome combines a BAC clone-based physical mapping effort with a whole genome shotgun phase utilizing read pairs from both small and large insert clones. BAC fingerprinting and physical map construction would progress simultaneously with generation of ~8-fold sequence coverage from plasmid, fosmid and BAC clones. In this approach, high-quality plasmid reads would provide the bulk of the sequence data while fosmid and BAC-based reads would provide anchors to the physical map, order and orientation information for the assembled sequence contigs, and the substrate for either targeted or complete finishing of the tammar genome. Following genome sequence assembly, one or two rounds of computer-directed finishing could be performed to reduce the number of sequence contigs and resolve most assembly ambiguities.

II. Biological Rationale for sequencing the tammar genome

An alternative mammal for comparative studies

Marsupials are unquestionably mammals, since they bear fur and suckle their young with milk. However, fundamental differences in modes of reproduction and development provide opportunities for understanding mammalian anatomy and physiology, as well as to seek answers to some of the

deepest questions of mammalian evolution. For this reason, many groups throughout the world have studied marsupial genes and chromosomes, marsupial sex and reproduction, as well as marsupial physiology and biochemistry. The unique pattern of mammalian organization and development will be reflected by differences in their genomes.

Marsupials are justly famous for their alternative mode of reproduction (Renfree 1981), much envied by the mothers of large human babies. Different groups of mammals have evolved different strategies for achieving the transfer of energy from mother to young first via the uterus and placenta, and subsequently via the milk. In this equation, marsupials have traded the umbilical cord for the teat. However, marsupials do have a fully functional placenta that elaborates all the key hormones essential for mammalian pregnancy and parturition, controls the growth of the young and provides the signals for the maternal recognition of pregnancy (Renfree, 2000; Renfree and Shaw 2000).

The neonate of the kangaroos and wallabies range from 0.4-1.0g, and the honey possum neonate is less than 5mg (the smallest mammal at birth). The marsupial embryo develops in utero for only a short time, and is born before even the hindlimbs – or the gonads – have developed (Renfree, 1995). The tammar wallaby embryo develops for only 26 days *in utero*, and is born when only 16 mm long and weighing about 400mg, at a developmental stage roughly equivalent to a 40 day human or 15 day mouse embryo (Tyndale-Biscoe and Renfree, 1987). The bean-sized newborn has forelimbs to climb up to the mother's pouch, where it attaches to a nipple. It has functional respiratory, circulatory and digestive systems, but it is born with an embryonic kidney and undifferentiated immune, thermoregulatory and reproductive systems, all of which become functionally differentiated during the lengthy pouch life. Most major structures and organs, including the hindlimbs, eyes, gonads and a significant portion of the brain, develop while the young is in the pouch (Renfree et al; 1997; Mark and Marotte, 1992; Renfree et al 1996; Pask and Renfree, 2001; Reynolds et al., 1985; Tyndale-Biscoe and Janssens, 1988).

Marsupials therefore provide unparalleled opportunities to observe, and manipulate mammal development. The early postnatal development provides a unique experimental model, with ready access to developmental stages that are much more difficult to manipulate in eutherian mammals because they develop within the uterus. For instance, differentiation of the gonad in marsupials occurs postnatally, so it is comparatively simple to correlate morphological changes with gene expression. It has been possible to administer hormones to neonatal young using a feeding tube alongside the nipple, to clarify the role of androgens and estrogens, and even to induce gonadal sex reversal (Lucas et al 1997; Ryorchuk et al, 1997; Coveney et al 2001; Renfree et al 2001).

Lactation is comparatively prolonged in marsupials, and much more sophisticated. The composition of the milk changes throughout the lengthy pouch life, being perfectly matched for each stage of development. Different teats in a pouch can deliver milk appropriate for a pouch young, and a joey at heel. Understanding milk composition and control is important both for our understanding of human nutrition and our ability to manipulate milk production in domestic animals (Nicholas, 1997)

In most species of kangaroo and wallaby, the mother immediately conceives again, but development of the embryo is delayed during a period of suspended animation (embryonic diapause) so long as the pouch young provides a sucking stimulus. This allows early stage embryos to be accurately timed through the removal of the pouch young (Tyndale-Biscoe and Renfree, 1987). Understanding how diapause is controlled is of intense interest because it will present new opportunities for control of mammalian fertility and development.

The marsupial mode of reproduction does not imply inferiority, nor does it represent a transitory evolutionary stage, as was originally thought. It is a successful and adaptable lifestyle. In a bad season in a harsh land, a pouch young is disposable, and there is another ready to replace it. The maternal investment is minimal during the relatively brief pregnancy and in early lactation, allowing the mother to respond to altered environmental conditions (Renfree, 1993; Tyndale-Biscoe, 2001; Tyndale-Biscoe and Janssens, 1988).

Thus marsupials are of special value in studying mammalian reproduction and the mechanisms that control fertility, seasonal breeding, pregnancy, parturition, lactation, and in particular, sex determination and sexual differentiation.

Informing the human sequence

Marsupials occupy a phylogenetic position that provides unique opportunities to explore mammalian genome organization and function (Figure 1).

Comparison with other genomes is now an integral part of the analysis of the human genome sequence and is one of the most effective methods for identifying genes (Batzoglou et al., 2000; Roest Crollius et al., 2000, Guigó et al., 2003). Phylogenetic footprinting is also an efficient method for identifying conserved regulatory signals within non-coding regions. In fact, the comparison between the mouse and human genomes identified ~5% of the genome that is clearly conserved between the species (MGSC, 2002). Less than half of this is coding, leaving several per cent of the genome as good candidates for regulatory elements.

The power of this analysis depends on the richness and evolutionary depth of the species being compared. A huge international effort has been put into sequencing the human genome. The genomes of chimpanzee, rodents, chicken, fish and domestic mammals also are being sequenced. These vertebrates will all provide unique and valuable comparative information. However, a quick glance at their phylogenetic relationships (Figure 1) reveals a gaping hole in the phylogeny between the divergence of birds and mammals ~350 million years ago, and the radiation of eutherian mammals ~80 million years ago (Benton, 1990; Murphy et al 2001)! For many important comparisons with human, other eutherians are too close and chicken is too far away.

Marsupials will fill this gap. Comparisons between marsupials and eutherians allow relatively straightforward alignment, and display a high ratio of conservation signal to random noise. This reduces the extent and degree of homology required to infer functionally conserved sequences (Wakefield & Graves, 2003). Marsupial/Eutherian comparisons are particularly valuable within non-coding regions, which are now thought to include more than 90% of the human genome (Wong et al., 2000). Proof of principle recently has been supplied by the first large-scale sequencing of a marsupial genome region: a BAC clone that encompasses four human (and 5 mouse and marsupial!) genes including LYL1 (Chapman et al, 2003). Mouse and human sequences were highly conserved over the whole region, but marsupial-human and marsupial-mouse comparisons showed clear conservation signals in exons and promoters, against a background of reduced overall homology. Conserved sites included putative transcription factor binding sites, which were confirmed by ChIP assays and reporter constructs.

Comparisons between human and tammar genomes have already been responsible for the discovery of new human genes including RBMX, a candidate for mental retardation (Delbridge et al, 1999) and several related genes (Lingenfelter et al, 2001).

Perhaps even more important than phylogenetic footprinting is the unique usefulness of the marsupial genome to test hypotheses of mammal gene organization and function. There have already been many surprises from the tammar genome that have necessitated fundamental re-thinks of mammalian sex determination, as well as the formulation of more general rules of basic processes like recombination and X chromosome inactivation. For instance, just in the fields of sex chromosome and sex determination, data from the tammar genome showed that

- *ZFY* is not the mammalian sex-determining gene, as was originally proposed (Sinclair et al 1988)
- A gene(s) on the X chromosome controls development of the scrotum, mammary glands and pouch (O et al 1988; Renfree and Short; 1988: Cooper et al 1993)
- Male to female sex reversal can be induced by estrogen (Coveney et al 2001)
- Virilisation during early male development is due to actions of a testosterone metabolite, 5α androstanediol (Shaw et al 2000; Renfree et al 2001; Wilson et al 2002)

• *SRY* is not Y-specific but has an X-borne brain-expressed partner SOX3 from which it evolved (Foster et al 1994), and with which it may interact (Graves, 1998)
• A *de novo* intron was recently inserted into *SRY* in one marsupial lineage (O'Neill et al, 1998)
• The human X-borne sex reversing gene (ATRX) has a Y-borne partner (*ATRY*) that may represent the original sex determining gene (Pask, 2000)
• The human X chromosome consists of an ancient and a recently added region, still only partly inactivated (Graves 1995)
• The human Y chromosome consists of a tiny ancient region (nearly all degraded) and an added region (Waters et al 2001)
• DNA methylation is not basic to X inactivation, but histone deacetylation is (Cooper et al, 1993; Wakefield et al 1997)

Unique features of the marsupial genome
Marsupial chromosomes are every cytologist's dream. Marsupials have a low diploid number (tammar is 2n=16) and very large chromosomes that are easily distinguished by size and morphology. These have been favourite subjects for many classic studies of mitosis, cell cycles (Scheneider 1977), DNA replication (Graves, 1967), radiation sensitivity (Yao, 1971) and genome stability, as well as B chromosomes (McQuade 1985), chromosome elimination (Hayman and Martin, 1965; Watson et al, 1998) and chromosome evolution (Hayman and Martin, 1974, Rofe and Hayman, 1985).

The sex chromosomes are particularly valuable. They are small; the X chromosome constituting only 3% of the haploid genome (c.f. 5% in eutherians), and the Y is a tiny dot. Comparative studies show that they represent the original mammalian X and Y chromosomes (Graves 1995). The tiny marsupial Y chromosome will facilitate identification of the minimal set of male-specific genes (Toder et al., 2000). Sequence conservation of these genes across all mammals will highlight genes and functional domains that are particularly important for male development and function.

Fundamental differences in marsupial chromosome behaviour have necessitated rewriting some of the genetic rules for mammals. For instance, the sex difference in recombination rate is reversed in marsupials, which show a lower recombination frequency in females (Bennett et al., 1986; van Oorschot et al., 1992; Zenger et al., 2002). This is not consistent with the classic theory that sex-related differences in recombination rates are due to heterogamy (Haldane, 1922). Marsupials provide a novel system by which to dissect the factors that affect recombination, and such investigations would be made possible by genome scale data.

Evolution of genome organization.
Classic studies of chromosome evolution in marsupials established important general principles, including astonishing karyotype conservation, now apparent for eutherians, the role of Robertsonian fusions (Eldridge et al, 1988), the effect of translocations between sex chromosomes and autosomes (Martin and Hayman, 1966; Toder et al 1997), the evolution of B chromosomes (McQuade et al, 1985), and the rapid remodelling of chromosomes in species hybrids (O'Neill et al, 1998, 1999).

Seminal work showed that marsupial karyotypes are very stable across diverse lineages (Sharman 1961, Hayman and Martin, 1974; Rofe and Hayman 1985). Chromosome painting between marsupial groups has confirmed the original deduction of an ancestral 2n=14 marsupial karyotype (De Leo et al., 1999; Glas, 1998; O'Neill et al 1999; Rens et al., 1999; 2001; Toder et al., 1997), and gene mapping confirms this conservation (Wilcox 1996, Sinclair et al 1991: Maccarone et al 1992; reviewed Samollow and Graves, 1998). The extreme conservation allows extrapolation of a cytogenetic map from a model marsupial to any other marsupial. The ancestral 2n=14 marsupial karyotype is a good basis on which to reconstruct the karyotype of an ancient therian, and ultimately the common mammalian ancestor. Given the number of mammalian species now being sequenced, it might also be possible to examine the minimal number and order of rearrangement that have taken place within the eutherian mammals using the tammar genome as an outgroup (Pevzner, 2003).

Marsupials have been particularly valuable in deducing the origins and evolution of human sex chromosomes. The small marsupial X chromosome has been shown to consist of a region conserved on the X in all mammals (Spencer et al, 2000), which therefore represents the ancient mammalian proto-sex chromosome pair (Graves, 1995: Glas et al, 1999), and provides a base-line for assessing changes in gene content, activity and function on the human X and Y (Graves et al, 2003). The demonstration of a recent addition to the human X explained why many genes on the short arm of the human X escape inactivation (Graves 1995, Carrel et al., 1999). Comparative mapping of human pseudoautosomal genes showed that human PAR1 is part of the same recent addition (Toder et a1, 1998), but human PAR2 has a complex evolutionary history (not revealed by comparing eutherians) that suggests that unpaired terminal chromosome regions are particularly unstable (Charchar et al, in press). The demonstration by comparative mapping that most of the genes on the original mammal Y were progressively lost (e.g. Mitchell et al, 1998) has provided new models of Y chromosome degeneration (Graves 2000; Waters et al, 2001).

Evolution of complex multigene families

Many genes are members of complex multigene families that have evolved through multiple duplications and divergence in functions. In order to compare these genes in model systems to enhance our understanding of their function it is essential to know if these genes are orthologous (i.e. the same gene in different species) or paralogues that have diverged in function within the same species. Marsupial sequence can be critical in determining these relationships. An example of the key role of marsupial data in determining orthology is the globin genes. It was initially believed that the beta globin genes in birds and eutherian mammals were orthologous, and the absence of conserved regulatory elements was puzzling. However, analysis of marsupial globins showed that avian globins are probably paralogues derived from an ancestral globin that was lost from eutherian mammals (Wheeler et al. 2001). In the comparison of mouse and humans approximately 80% of genes have a 1:1 ortholog, leaving 20% of genes with a 1:many relationship between the species. Comparison with a marsupial gene set should clarify whether certain gene families have expanded (as seems more likely) within mouse or human or whether deletions have occurred in one lineage.

Evolution of regulatory systems and complex pathways

Marsupials are particularly valuable because they share with eutherians mammal-specific regulatory systems, but show many genetic differences that can be exploited to analyze how pathways and complex regulatory systems evolved, and indirectly, how they work. Recent investigations of the sex-determination pathway, X-chromosome inactivation and genomic imprinting exemplify the insights to be gained.

Several genes involved in eutherian sex determination have been isolated and characterized in marsupials. In fact, marsupials first achieved genetic fame when they unexpectedly provided a critical test of the credentials of early candidates for the Y-borne testis-determining gene. This test decisively eliminated the first contender ZFY, which was discovered to be autosomal in marsupials (Sinclair et al, 1988), but it later endorsed SRY, which was Y-borne in marsupials (Foster et al 1992). Further studies in marsupials unexpectedly revealed that SRY has a brain-expressed partner SOX3 on the X from which it evolved (Foster et al 1994), and with which it may interact in sex determination (Graves, 1998). How genes on the Y chromosome may acquire male-specific functions has been addressed by wide comparisons of the SRY and SOX genes (Graves, 2002), and changes in SRY have been studied in detail in the particularly favorable marsupial material (O'Neill et al, 1997, 1999). Cloning, mapping and expression analysis of other genes implicated in eutherian sex determination (DAX1, SOX9, WT1, SF1, AMH) has also delivered some surprises (Pask and Graves, 1998; Pask et al, 2002; Whitworth et al, 2001).

Both the X inactivation control region and imprinted domains contain complex and enigmatic controls of gene expression, and involve unknown interactions of functional RNA molecules and chromatin. Comparative analysis of such complex regions will identify conserved features for direct experimental investigation. X-chromosome inactivation demonstrates the part that marsupial genetics can play in sorting out complex control systems. X inactivation seems to be simpler in marsupials than in

eutherians, and could be ancestral (reviewed Cooper et al, 1993). Some molecular mechanisms are shared, such as delayed replication (Graves, 1967) and histone deacetylation (Wakefield et al, 1997), so are likely to be ancestral. Others, such as sex chromatin formation (McKay et al, 1988) and DNA methylation (Piper et al, 1993) are specific to eutherians and may therefore be responsible for eutherian-specific features like randomness and hyperstability.

In the same way, analysis of genomic imprinting in marsupials (O'Neill et al, 2000) promises to disentangle the mechanism by which some autosomal genes are expressed only if they come from the mother, or only from the father. Because of the minimal maternal investment in the marsupial embryo, marsupials may provide an answer to the real question about imprinting – *why* did it evolve?

Mammalian phylogeny and choice of a model marsupial

Mammals evolved from a branch of reptiles that left no other descendants; so marsupials, monotremes and eutherians are all equally related to birds and reptiles.

Marsupial phylogeny and taxonomy were reviewed by Graves and Westerman (2002). Marsupials comprise a single taxonomic unit (mammalian Infraclass Metatheria), traditionally considered the sister group of Eutheria, from which they diverged 100-130Myr ago (Hope et al, 1990) (Fig. 1). Eutherians and marsupials together constitute the mammalian Subclass Theria, which diverged 170-200 Myr ago from Subclass Prototheria (the egg-laying monotremes). Comparisons of nuclear genes, as well as fossil evidence and much anatomical data, favor this Therian grouping (Killian et al, 2001), although mitochondrial DNA sequence comparisons suggest closer relationship between marsupials and monotremes (Marsupionta) (Janke et al,1997; 2002).

Marsupials originated in North America, radiated into South America more than 65 Myr ago, then colonized Antarctica and Australia while these lands were still united with South America as the super-continent Gondwana (Woodburne et al, 1996; Goin et al, 1999). Australian marsupials were subsequently cut off from their American relatives when Australia and Antarctica separated 84–38 Myr ago (Veevers et al, 1991). The oldest Australian fossils are dated at 55 Myr. The ancestral marsupials were small, mouse-like animals that had many teeth and probably produced many young.

There are 270 species of marsupials (compared with ~4500 eutherians). They are found only in Australasia (200 species) and the Americas (69 South American and a single North American species). Kangaroos, the large hopping mammals of children's storybooks, must be considered the archetypal marsupial, but other marsupials are rodent-like, arboreal, burrowing and even swimming animals. The relationships between marsupial groups are comparable to those between familiar eutherian models (Kirsch et al, 1997). American and Australian marsupials are equivalent to a human–mouse comparison, (~60Myr), whereas the dasyurid and macropodid relationship is equivalent to a human–monkey comparison (~45 Myr).

Several species have provided models for marsupial physiology and anatomy, and others, such as the brushtailed possum (*Trichosurus vulpecula*) have been investigated because they have become a pest. However, there is significant genetics for only three marsupial species, and at a conference of marsupial geneticists in 1988, the advantages and disadvantages of each as a laboratory marsupial were debated (Graves et al, 1990):

The fat-tailed dunnart *Sminthopsis crassicaudata* (a widespread Australian desert species of the Family Dasyuridae) is a rodent-like marsupial that can be kept in mouse-scale cages. A colony was established in Adelaide in 1964, survived inbreeding depression and was managed for many years as a genetic resource, though one not readily accessible to other workers. The first genetic mapping was accomplished with this species (Bennett et al, 1986). The species is not very easy or cheap to maintain because the animals are carnivorous and quite fierce. Captive breeding is reasonably reliable, and there appeared to be abundant genetic variability, although the colony had relatively few founders and variation was constantly compromised because a few males were responsible for most of the successful matings. Unfortunately, genetic work on the colony has now been discontinued.

The Brazilian grey short-tailed opossum *Monodelphis domestica* is a small arboreal animal that breeds well in captivity, and has been used as the model didelphid marsupial. A colony has been maintained since 1979 at the Southwestern Biomedical Foundation at San Antonio, and animals were generously provided for research in many laboratories. Although the colony has a narrow genetic base (9 founder animals, supplemented recently), there is adequate genetic variation. Progress has been made with genetic mapping in this species (Samollow, in prep), but few groups now do genetic work on this species. The species has been valuable for embryological work because litters of 10-14 young are available. However, the adults are small and have not been favoured for reproductive research because it is difficult to collect blood and tissue samples or to perform invasive surgery, it is difficult to treat the young (which are only 80-100mg at birth), they lose young when handled frequently, and they can fit only a limited number of pregnancies in their relatively short reproductive life span. It is difficult to determine the exact time of conception and therefore the gestation time, and almost nothing is known of its lactational physiology (Renfree et al 1990; Robinson et al 1991), its seasonality, its behaviour, its ecology or its life history strategies in the wild.

The tammar wallaby *Macropus eugenii* is the ideal model kangaroo, typical of the largest marsupial family, Macropodidae. It is small enough to be amenable to handling and husbandry, yet big enough to take repeated tissue or blood samples. Female tammars are around 5 Kg; males around 7-9 Kg. Breeding is reliable and highly manipulable (Hinds et al, 1990). It breeds seasonally, and in the wild all animals deliver young on or about 22 January (one gestation period after the longest day in the Southern hemisphere, Dec 21/22). It has a gestation period of 26.4±0.5 days, and a 9-10 month lactation. By removing pouch young, up to 5 pregnancies can be achieved each year. The anatomy, physiology, embryology, endocrinology and genetics of the tammar are described in detail throughout development (Tyndale-Biscoe and Renfree, 1987; Tyndale-Biscoe and Janssens 1988).

Tammars are readily held in captivity in open grassy enclosures with pasture and water ad libitum, supplemented by alfalfa, hay and oats. The species is free of major parasites; it breeds well, and can be caught, sampled, treated and handled at will. For all these reasons, it is by far the most intensively studied marsupial. It is extensively used in Australia for a wide variety of genetic, physiological, biochemical, neurobiological and ecological studies (Tyndale-Biscoe and Renfree, 1987; Hume, 2002; Lee and Cockburn 1985; Tyndale-Biscoe and Janssens 1988; Richardson et al 2002) and increasingly is used in genomic comparisons.

One of the great advantages of the tammar for genetic research is the genetic variation available. There are several colonies in Australia (the largest now at the University of Melbourne), all established with many founders. In addition, fixed differences at many loci are available in sub-species living on different offshore islands. These animals have been added to breeding colonies (McKenzie and Cooper, 1997), and exploited in constructing a genetic map (McKenzie et al 1993; Zenger et al, 2002). Tammar is highly abundant in its habitat on the offshore islands of Southern Australia and the species was exported to New Zealand, where it has become a pest, so supply internationally is not a problem.

III. Strategic Issues

The tammar genetics community

There are few research groups internationally that specialize in marsupial genetics and genomics, but there are large and growing numbers of investigators who wish to add marsupial material to comparative studies.

Practically all the significant work directly on marsupial genetics and genomics is, not surprisingly, done in Australia. Leaders in the field (authors of this proposal Graves, Cooper, Renfree) run major research groups with many staff and students doing research on tammar genetics and reproduction. They have recently recruited other Australian scientists, including co-author of this proposal Prof.

Terry Speed, to build an Australian consortium to consolidate genetic and genomic work on the tammar and to seek major collaboration with genomics and comparative genetics groups overseas. This consortium includes well-known bioinformatics and genomics experts Prof. John Mattick (University of Queensland), Dr. Simon Easteal and Prof. Susan Wilson (Centre for Bioinformation Sciences, Australian National University, Canberra).

There are few other groups worldwide that specialize in marsupial genetics. The most significant work (on *Monodelphis domestica*) is done by Dr. Paul Samollow (Southwestern Biomedical Foundation, San Antonio, TX), and research on genomic imprinting in this species is also ongoing in the laboratories of Drs. Michael and Rachael O'Neill (University of Connecticut).

The current trend towards the tammar wallaby as the major model marsupial for genetic research is best evidenced by sequence deposited in GenBank. In the last 12 months there have been 49 new tammar wallaby nuclear sequences submitted to GenBank, compared with 11 sequences from *M. domestica* and 7 from *S. crassicaudata.*

International use of tammar wallaby

The major use of marsupial genome data will not be by specialist marsupial geneticists but by investigators seeking appropriate model systems to provide information on the their favorite gene or genomic region through comparative genomics. Already, marsupial tissue, DNA, clones and sequences are in such demand by numerous investigators worldwide that, unfortunately, Australian scientists simply cannot fulfil them all. However, many enquiries have already grown into active international and Australian collaborations, for example:

• Global genome comparisons; for instance exploring the function of conserved non-coding regions on human chromosome 21 (Geneva), X chromosome organization, function, evolution and role in speciation (Cambridge, Ulm).
• Mechanism and evolution of genomic imprinting (Cambridge, Uppsala, Salzburg, Philadelphia, Storrs, Yokohama).
• Function and evolution of visual pigment genes (Seattle)
• Organization and evolution of the MHC locus and immunoglobulins (Phoenix, Osaka)
• Phylogenetic footprinting around individual genes or clusters (Cambridge, Seattle, Paris)
• Discovery and function of genes in the mammalian sex-determining pathway (London, Houston, Dallas, Duke)

Current state of knowledge of tammar genetics and genomics

Tammar genetic maps will greatly enhance our ability to generate, interpret, annotate and utilise kangaroo genome data. The following mapping resources are available:

A linkage map with complete genome coverage has recently been developed by matings between the Kangaroo Island and Garden Island subspecies of tammar wallaby at Macquarie University (Zenger et al., 2002).

Physical mapping data, especially for sex chromosomes. The small number of large, readily identifiable chromosomes in the tammar karyotype has facilitated the development of a skeleton cytogenetic map, which currently has a higher coverage of the sex chromosomes (Graves 1995; Wilcox et al 1996; reviewed Samollow et al 1998). A project to integrate the linkage map with the cytogenetic map is in development.

Comparative tammar-human map, especially for sex chromosomes. Comparative gene mapping data have allowed alignment of large parts of the tammar genome to the human genome (Wilcox et al 1996, Spencer 1991,1991a; Sinclair et al, 1991; Maccarone et al, 1992: Hawken et al 1999, Miller et al, 1994). Comparative painting has directly revealed homologies between the tammar and human X (Glas et al, 1999).

Well-established relationships between genomes of tammar and other marsupials

Chromosome painting between tammar wallaby and other marsupial groups shows the extensive homologies that characterize the extremely conserved marsupial karyotype. Other kangaroo species differ from tammar by one or a few Robertsonian fusions or fissions (Glas 1998). Dasyurids and other species with the ancestral 2n=14 differ by 3 or 4 fusions, and even American marsupials by only a few rearrangements (De Leo et al., 1999; Rens et al., 1999, 2001; Toder et al., 1997, Svartman, 1998; O'Neill 1999). The extreme conservation allows extrapolation of a cytogenetic map from a model marsupial to any other marsupial.

Current status of resources for assembling tammar maps

Tammar cell lines. Many tammar wallaby fibroblast cell lines have been established in the Graves lab, and primary lines are being routinely produced. These remain diploid and grow well for at least 30 passages. They have been shipped far and wide.

Tammar-rodent cell hybrids. Kangaroo-rodent cell hybrids are a valuable mapping resource. Most rodent-marsupial cell hybrids contain only fragments of the marsupial genome, and can be used to order genes in the same way as for radiation hybrids (in fact, their analysis pre-dated radiation hybrid mapping; Dobrovic and Graves, 1986; Donald and Hope, 1981). A bank of hybrids would allow rapid mapping of EST and other markers onto the existing linkage map, and integration of the physical, cytogenetic and genetic maps.

Micro-dissected chromosome and regional libraries. Tammar chromosomes have been individually sorted in collaboration with Prof Malcolm Ferguson-Smith (Cambridge). Individual chromosomes, and chromosome arms have been micro-dissected. DOP-PCR amplified chromosome paints have been used in many laboratories.

cDNA libraries and sequence arrays for expression analysis. Tammar EST libraries have been prepared from mammary gland and ovary, and are available through collaborations at Melbourne University. Other cDNA libraries are being prepared for reproductive and developmental studies.

Genomic libraries. Several tammar genomic libraries have been prepared. A 2x BAC library (average insert size 120kb) was constructed in 2000, and a NHGRI funded 10x BAC library is nearing completion at the University of Arizona.

Sequencing strategy and cost

Experience and insights gained while recently mapping and sequencing the mouse genome lead us to propose a combined strategy for the tammar genome project. An ~8-fold whole genome shotgun component consisting of both small and large insert clones (see Table 1.) will provide the community of users with rapid access to most of the tammar genome sequence. A BAC clone-based physical map, along with paired end sequences from fosmids and the mapped BAC clones, will provide a framework by which the genome sequence can be accurately assembled, and ordered and oriented on the chromosomes. In addition, we wish to confirm the anchoring to the genome by doing FISH with fosmids from major supercontigs. We expect that this project will cost approximately $50M. As with the mouse genome sequence, we would expect that the proposed approach would result in anchored "supercontigs" (sequence contigs connected by at least two read-pair links) of greater than 10 Mb in average length (MGSC, 2002).

Table 1. Proposed whole genome shotgun sequencing of the *M. eugenii* genome.

Clone type	Insert size	No. of reads	Seq coverage	Phys coverage
plasmid	4 kb	33M	6.0x	18x
plasmid	10 kb	8M	1.5x	11x
fosmid	40 kb	1.6M	0.3x	9x
BAC	150 kb	0.7M	0.15x	14x
TOTALS	---	43.3M	7.95x	52x

Note: based on a genome size of 3.6Gb and an average sequence read length of 650 bp; Phys coverage calculation assumes that all clones contribute read pairs.

The resulting genome sequence will be of sufficient contiguity and quality for preliminary analyses and gene discovery by comparative methods. To improve on the utility of the sequence for these and other types of analyses, we advocate a round of computer-directed "automated finishing" in which oligonucleotides are algorithmically selected to extend sequence contigs into gap regions. BAC and fosmid clones would serve as the templates for the necessary sequencing reactions. The methods, computational tools and laboratory pipelines for automated finishing are already in place at the Washington University and Whitehead Institute genome centers.

At this point, manual finishing could be employed for targeted regions (or the whole genome, if deemed necessary) to further improve contiguity and sequence accuracy. Our experience with finishing the mouse genome from a combined approach and initial finishing of the *C. briggsae* genome from a whole genome shotgun approach suggests that most of this work would involve using PCR to sequence and/or size regions that were missing, ambiguous or repetitive in content.

SNP discovery for genetic map

To facilitate linkage and positional cloning of genetic traits in tammar, we propose generating a low coverage single nucleotide polymorphism (SNP) map of the tammar genome. To achieve this we would generate 100,000 whole genome shotgun reads from one or two different tammar individuals, align the reads to the whole genome shotgun assembly and identify SNPs using SSAHA-SNP (Ning, 2001). Assuming a repeat content and polymorphism rate in tammar similar to that of humans and other mammals, we would expect to discover ~ 25,000 uniquely placed SNPs (The SNP Consortium 2000; MGSC, 2002; Wade, 2002). This SNP density should suffice to pick a well-spaced set of SNPs with high heterozygosity, constituting a mapping set of ~ 1,000-3,000 SNP markers.

cDNA/EST sequencing

To further enhance the utility of a tammar genome project, we would propose the inclusion of a small EST/cDNA sequencing component. Sequence analysis of up to 100,000 ESTs would permit detection of a significant number of expressed genes that could be compared to genes expressed in eutherian mammals, as well as used to seed analysis of the genome sequence. Complete sequencing a small number (e.g. 10,000) of "full length" cDNA clones would further shed light on the differences and similarities between marsupials and eutherian mammals with respect to alternative splicing patterns. Since several cDNA libraries already exist and additional libraries could easily be constructed from additional tissues, we expect that this component would greatly enhance the value of the project at a minimal cost.

Conclusion

The kangaroo genome represents a treasure trove of comparative genomics data. Analysis of individual genes, and of gene arrangement, has already contributed significantly to our understanding of human biology and genetics. Analysis on a genome-wide scale will provide sequences for identifying conserved genes, functional domains and regulatory elements. The unique value of marsupial sequences for such comparisons will motivate the complete sequencing the kangaroo genome.

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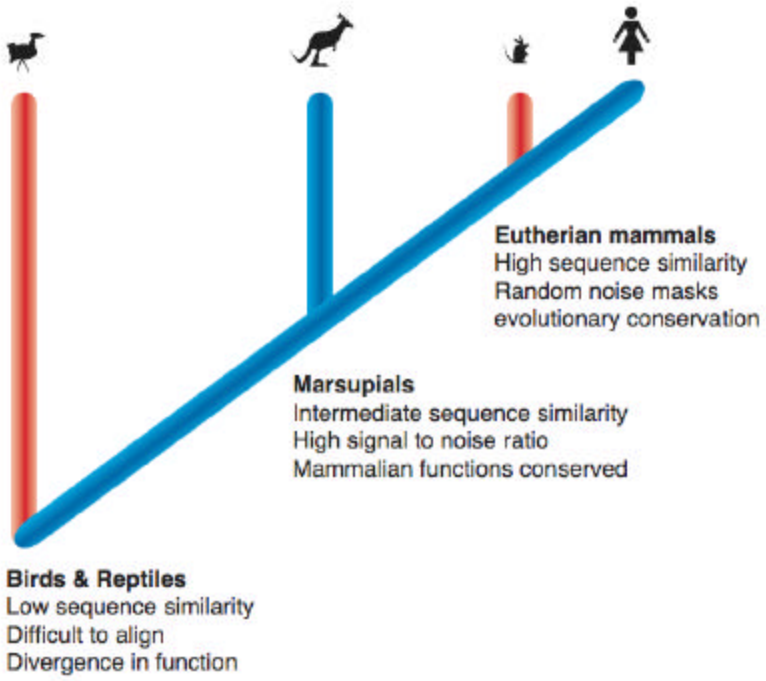

Figure 1. The kangaroo fills a large gap in the vertebrate phylogeny and provides a middle ground between birds and eutherian mammals. Most eutherian mammals diverged less than 80 million years ago while birds diverged approximately 350 million years ago (Benton, 1990; Murphy, 2001). Marsupials diverged from eutherian mammals 100-130million years ago and fill the phylogenetic gap.

Response to the Interim report on the proposal to sequence the genome of the model marsupial *Macropus eugenii* (tammar wallaby)

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We here respond to the Interim Report by the GRASPP Committee on our proposal to sequence the genome of the tammar wallaby *Macropus eugenii* and advise the committee of an exciting new development in kangaroo genomics since we submitted the proposal. The establishment of a new Centre for Kangaroo Genomics will put us in a strong position to advance tammar wallaby genome sequencing by providing expertise and resources internationally.

We are encouraged by the committee's enthusiastic endorsement of our case for sequencing the genome of a marsupial, but disappointed that the decision on which marsupial species to sequence may not be based entirely on the biological suitability of the species or the existence of a community to back up the sequencing project and utilize its results.

Here we will apprise the Committeee of the recent dramatic developments in kangaroo genomics, answer the specific questions posed by the committee and contrast the attibutes of the tammar wallaby to the Brazilian grey short-tailed opossum, *Monodelphis domestica*, the only other marsupial seriously considered by the committee.

Establishment of the Centre for Kangaroo Genomics
Since the submission of our proposal to sequence the tammar wallaby genome, a Centre for Kangaroo Genomics has been established. The Australian co-authors of the proposal (Graves, Renfree, Cooper and Speed) have just accepted an offer of major support from the Australian Research Council (Australia's peak basic research body) to set up an ARC Centre for Kangaroo Genomics in Australia (Director Jenny Graves, ANU), focusing on the genome of the tammar wallaby. This will enormously boost the support available to back up sequencing of the tammar wallaby genome, and to use the sequence to investigate gene function, regulation and conservation.

The aims of the Centre for Kangaroo Genomics are:
- to back up sequencing work on the tammar wallaby genome by genetic and physical mapping and BAC binning and contigging
- to produce libraries of expressed sequences in the tammar wallaby

- to produce arrays of RNA from different tissues and stages
- to provide resources such as tissue, cell lines, DNA, RNA, arrays, sets of DNA from mapping families to the international genetics and genomics communities

In particular, the Centre for Kangaroo Genomics will produce, as top priority, a normalized tammar EST library containing expressed sequences from a wide range of tissues and developmental stages. We hope to have 60,000 tammar ESTs sequenced within a year. This will provide a major resource for mapping and function studies unmatched for any other marsupial species.

The Centre for Kangaroo Genomics will be a world-class centre that gathers all the experts in marsupial genetics and genomics in Australia. There will be nothing anywhere else in the world to match the concentration of expertise and resources to explore the marsupial genome and relate marsupial to eutherian sequence.

Availability of marsupial species in the USA and internationally

The Interim report from GRASPP understandably considered that choice of a marsupial species to be sequenced should be influenced by the availability of the species to researchers in the USA.

The marsupial species most available within the USA is the ubiquitous Virginia opossum, *Didelphis virginiana*, which is little favoured for genetic, physiological or ecological work. The Brazilian grey short-tailed opossum (*Monodelphis domestica*), although no more native to the USA than the tammar wallaby, is (as the committee notes) available in the USA, although in only two laboratory colonies, whose continued survival is by no means guaranteed. The larger colony was established at the Southwestern Biomedical Foundation in San Antonio and its use encouraged by generous provision of animals and establishment of collaborations by Dr John VandeBerg (now working on primates). It has supported fine research into X inactivation (Drs VandeBerg and John McCarrey, now working on baboons and mice) and embryogenesis (Dr Ted Robinson, now retired). It is now managed by Dr Paul Samollow, with whom we collaborate on physical mapping in this species. Paul is now the sole researcher at Southwestern who works with the colony, and has single-handedly supported its maintenance, despite chronic difficulty in funding. A small derivative colony has recently been set up by Drs Mike and Rachael O'Neill (ex-student of Prof Graves, and who also studies Australian wallabies) at the University of Connecticut to study genomic imprinting.

The tammar wallaby is not presently being captive bred in the USA for research purposes, although the species is listed at several US zoos. If some US researchers specifically require access to colonies of the tammar wallaby, breeding colonies could readily be established in US centres. The tammar is readily available. The species was exported to New Zealand, where it has become a pest, so supply internationally is not a problem. The New Zealand population represents an extinct South Australian mainland population very closely related to the Kangaroo Island population which is the main source of animals used in Australia. Export of tammars from NZ is permitted almost without restriction and animals would be available to any research institution in the world. The cost of setting up and maintaining colonies would be low and would compare favourably with Monodelphis.

This will probably be unnecessary, however, since the establishment of the ARC Centre for Kangaroo Genomics will ensure that tammar material is available for the US genomics and genetics communities. The stated aims of the centre are to supply resources internationally, including tammar wallaby tissues and cell lines, RNA arrays, ESTs and cDNAs, DNA sets from the mapping families and mapped BACs. We are confident that the availability of these resources will more than compensate for any perceived difficulty in obtaining the raw material needed to adopt this species to study marsupial biology and genetics in the USA.

The Centre for Kangaroo Genomics will also be a hub for initiating many new collaborations with US scientists on gene expression in this model marsupial. Many collaborations are already in place between the co-authors and scientists in the USA (e.g. Dr S.J. O'Brien, Frederick; Dr R. Behringer, Houston; Drs S Deeb and C. Disteche, Seattle).

Advantages of the tammar wallaby as a model species

We submit that, if only one marsupial is to be sequenced, it should be a species that represents the entire Infraclass Metatheria. The tammar wallaby is the ideal model marsupial, representing the largest, most typical and most widespread marsupial family, the Macropodidae (literally "big-feet", that is kangaroos and wallabies). The grey short-tailed opossum, in contrast, is a specialized and restricted rainforest dweller.

For some decades, the tammar wallaby has been the most widely used marsupial model worldwide for research on marsupial genetics, genomics and evolution. All the classic work was done on this species, and research on tammar is still extremely active. More than 100 papers have been published in the last 10 years describing genetic and physical mapping in the tammar, gene cloning, sequencing and expression studies. To our knowledge the grey short-tailed opossum is now used for limited genetic studies in only two laboratories in the USA. No physical mapping is yet available for this species – indeed, one of the authors of the tammar proposal (Prof Graves) is performing the cytogenetic mapping in the Brazilian grey short-tailed opossum.

The most intriguing aspect of masupial biology is the unique reproductive system. The tammar was the subject of classic studies, and has been, for some decades, by far the most widely studied marsupial for reproduction and development. There are many hundreds of papers published on this species. The only book entirely devoted to marsupial reproductive biology rests largely on the advances made from study of the tammar. Basic reproduction studies of the grey short-tailed opossum are more recent, and to our knowledge, are the major focus of only two or three laboratories in the USA, one in UK, one in Europe and one in Australia.

The reasons for the wide use of the tammar wallaby are:

<u>Husbandry and breeding.</u> Tammars are readily (and inexpensively) held in captivity in open grassy enclosures with pasture and water, supplemented by alfalfa, hay and oats. Animals are long-lived and can continue breeding for 16 years. The species is free of major parasites, it breeds well, and can be caught, sampled, treated and handled at will. In contrast, the Brazilian

grey short-tailed opossum has a short lifespan and breeds only into its second year.

The tammar wallaby is small enough to be amenable to handling and husbandry, yet big enough to take repeated tissue or blood samples. Female tammars are around 5 Kg; males around 7-9 Kg. Breeding is reliable and highly manipulable. It breeds seasonally in the wild on or about 22 January (exactly one cycle after midsummer). It has a gestation period of 26 days, and a 9-10 month lactation. The tammar can be manipulated to breed all year round by removing pouch young, up to 5 pregnancies can be achieved each year. The anatomy, physiology, embryology and endocrinology of the tammar are described in detail throughout development, and there are precise growth curves for fetal and pouch young stages (Tyndale-Biscoe and Renfree, 1987; Tyndale-Biscoe and Janssens 1988). Isolation and study of the tammar homologues of genes involved in eutherian sex determination and reproduction has already proved highly informative. There is a major study on the genes that control the changing milk composition of the tammar throughout lactation which promises to yield information about the genes that control milk composition and yield in all mammals, as well as unique opportunities to manipulate these characteristics.

The Brazilian grey short-tailed opossum has been useful because it litters 10-14 young, but the young are very small (100mg) and difficult to sample, especially during the critical early stages of development when mothers lose their litters as a result of handling. The adults, too, are small and have not been favoured for reproductive research because it is difficult to collect blood and tissue samples or to perform invasive surgery. It is difficult to determine the exact time of conception and therefore the gestation time. Almost nothing is known of its lactational physiology (Renfree et al 1990; Robinson et al 1991). They do not undergo embryonic diapause.

Relevance to wild marsupial populations. The tammar wallaby also offers unmatched advantages of ready availability of wild populations, and in-depth studies of the species in the wild and in captivity. The results of tammar genome sequencing will be of immediate use to the very considerable conservation and ecological genetics groups in Australia and elsewhere who work on this and closely related kangaroo species, several of which pose the contrasting problems of either being overabundant or critically endangered.

In contrast, the Brazilian grey short-tailed opossum is difficult to access in the wild. Almost nothing is known of its seasonality, behaviour, ecology or life history strategies in the wild. Unfortunately, funding for marsupial research is almost non-existent in South America.

Genetic variability

One of the great advantages of the tammar for full genome sequencing is the availability of populations with reduced heterozygosity and allele number (Taylor, 1999). This will improve shotgun sequence assembly and reduce reliance on BAC sequencing. A population resulting from the introduction of a few tammar wallabies to Kawau Island (New Zealand) in 1870 and the subsequent introduction of a few from this population to Rotorua in the 1920s has only a third the number of microsatellite alleles, and an observed heterozygosity of 0.56 compared to 0.79 for the Kangaroo Island population (Taylor, 1999).

As well as populations with limited genetic variability useful for shotgun sequencing, there are tammar subspecies with phenotypic and genetic differences that facilitate genetic mapping and offer the prospect of discovering genes unique to marsupials. Breeding colonies have been established from four long-isolated (though conspecific) island populations that diverged up to 250,000 years ago (McKenzie and Cooper, 1997). Animals from different subspecies will be hybridized as part of the mission of the Centre for Kangaoo Genomics to provide mapping families parallel to the *Mus musculus* x *M. spretus* crosses. The fixed differences at many loci have already been exploited to construct a genetic map of the tammar (McKenzie et al 1993; Zenger et al, 2002).

These distantly related tammar populations differ in phenotypic traits of considerable interest (e.g. the onset of embryonic diapause and blastocyst reactivation, degree of pesticide and parasite resistance). The hybrids and backcrosses of mapping families will be phenotyped extensively for reproductive parameters, disease susceptibility and growth patterns. These traits can be mapped, and ultimately the genes responsible isolated.

Summary

The tammar wallaby is a more typical representative of the Infraclass Metatheria than is the Brazilian grey short-tailed opossum, and has been more widely and intensively studied worldwide. We submit that it should therefore receive priority for full sequencing.

However, the Infraclass Metatheria is a large and varied group, consisting of 260 extant species that diverged as long ago as 80 million years. We would therefore urge the committee to recommend sequencing the Brazilian grey short-tailed opossum as a second, highly divergent, marsupial species. Comparison of the genomes of two marsupials that are as distantly related (about 80 million years) as mouse and human would greatly enhance the value of marsupial sequence.

We believe that it would be tragic to waste the very considerable resources, expertise and enthusiasm available to back up and to utilize a tammar wallaby genome initiative for reasons unrelated to the biological suitability of the species. Even though these resources and expertise are presently concentrated in Australia, the establishment of the new Centre for Kangaroo Genomics will ensure their readily availability to the world.

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