The multiple sex chromosomes of platypus and echidna are not completely identical and several share homology with the avian Z

Willem Rens*, Patricia CM O'Brien*, Frank Grützner†, Oliver Clarke*, Daria Graphodatskaya*, Enkhjargal Tsend-Ayush†, Vladimir A Trifonov**, Helen Skelton*, Mary C Wallis*, Steve Johnston§, Frederic Veyrunes*, Jennifer AM Graves¶ and Malcolm A Ferguson-Smith*

Addresses: *Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 OES, UK. †School of Molecular and Biomedical Science, The University of Adelaide, Adelaide, 5005 SA, Australia. ‡Institute of Cytology and Genetics, Russian Academy of Sciences, Siberian Department, 630090, Novosibirsk, Russia. §School of Animal Sciences, Currumbin Sanctuary, Queensland University, Brisbane, QLD 4072, Australia. ¶Research School of Biological Sciences, The Australian National University, Canberra, ACT 2601, Australia.

Correspondence: Willem Rens. Email: wr203@mole.bio.cam.ac.uk

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Abstract

Background: Sex-determining systems have evolved independently in vertebrates. Placental mammals and marsupials have an XY system, birds have a ZW system. Reptiles and amphibians have different systems, including temperature-dependent sex determination, and XY and ZW systems that differ in origin from birds and placental mammals. Monotremes diverged early in mammalian evolution, just after the mammalian clade diverged from the sauropsid clade. Our previous studies showed that male platypus has five X and five Y chromosomes, no SRY, and DMRT1 on an X chromosome. In order to investigate monotreme sex chromosome evolution, we performed a comparative study of platypus and echidna by chromosome painting and comparative gene mapping.

Results: Chromosome painting reveals a meiotic chain of nine sex chromosomes in the male echidna and establishes their order in the chain. Two of those differ from those in the platypus, three of the platypus sex chromosomes differ from those of the echidna and the order of several chromosomes is rearranged. Comparative gene mapping shows that, in addition to bird autosome regions, regions of bird Z chromosomes are homologous to regions in four platypus X chromosomes, that is, X1, X2, X3, X5, and in chromosome Y1.

Conclusion: Monotreme sex chromosomes are easiest to explain on the hypothesis that autosomes were added sequentially to the translocation chain, with the final additions after platypus and echidna divergence. Genome sequencing and contig anchoring show no homology yet between platypus and therian Xs; thus, monotremes have a unique XY sex chromosome system that shares some homology with the avian Z.
Background
Monotreme mammals are receiving increasing attention in genomic research, with interests varying from karyotype evolution and gene mapping, to comparative sequencing. This should not come as a surprise, as monotremes (mammalian Subclass Proototheria) occupy a unique branch at the base of the mammalian phylogenetic tree, and serve as an evolutionary outgroup for marsupial and eutherian species (that together comprise Subclass Theria). The time of divergence of Proototheria and Theria is estimated to be in the Early Jurassic (166 million years ago (MYA)), while marsupials and eutherians diverged in the Late Jurassic (148 MYA) [1]. Five extant monotreme species are recognized; platypus (*Ornithorhynchus anatinus*), short-beaked echidna (*Tachyglossus aculeatus*) and three long-beaked echidnas (*Zaglossus brunneiji, Zaglossus attenboroughi, Zaglossus bartoni*). *Zaglossus bartoni* is divided into three subspecies *Z. b. smeenki, Z. b. diamondi*, and *Z. b. clunius* [2].

A full karyotype characterization is essential for genomic research in any species. It is particularly important for monotremes because of their exceptional sex chromosome complement. The inclusion of a set of tiny chromosomes was recognized early and thought to be a reptilian feature [3], but this suggestion was later refuted [4]. A surprise was the discovery of several unpaired chromosomes [5]. A final identification and description of the platypus unpaired chromosomes was achieved only recently by our chromosome painting studies [6,7]. The 21 autosome pairs were assigned by chromosome paints. Ten paints identified ten unpaired mitotic chromosomes as well as the ten members of the meiotic chain and the homologous regions between them. Five paints identified X chromosomes present in single copy in males but two copies in females, and five paints identified Y chromosomes that were present only in males. It was, therefore, concluded that the ten male unpaired chromosomes consisted of five X and five Y sex chromosomes. The ten sex chromosomes form a multivalent chain at meiosis held together by chiasmata within homologous pairing regions. Alternate segregation of these chromosomes into X,X,X,X,X,Y and Y,Y,Y,Y,Y sperm was proposed and must be very efficient as shown by meiotic analysis of spermatids and sperm using the paint probes [6]. Remarkably, X5 shows some homology with the chicken Z, as demonstrated by its inclusion of the *DMRT-1, DMRT-2* and *DMRT-3* orthologues [6,8]. Chicken Z is largely homologous to parts of human chromosomes 5 and 9, with some genes represented on 8 and 18 [9]. A region containing *ATRX, RBMX* and genes flanking *XIST*, present on Xq in human and other therians, maps to chromosome 6 in platypus [10], as does SOX3, the gene from which the sex-determining *SRY* gene evolved (M Wallis, personal communication), and this is consistent with the absence of a platypus homologue of the Y-linked *SRY*. Other genes involved in the eutherian sex determining pathway have recently been mapped to platypus autosomes, so do not qualify as candidate primary sex determining genes [11]. There is no platypus homologue of the human X-borne *XIST* in platypus [12] and marsupials [13]. In addition, platypus Ensembl release 44 and separate mapping work (F Veyrunes, personal communication) show an absence of human X-linked orthologues from platypus X chromosomes, contradicting original localizations using radioactive fluorescent *in situ* hybridization (FISH) with heterologous cDNA probes [14-18]. It follows that *SRY* and the therian XY sex determining system have evolved between 166 and 148 MYA after the divergence of monotremes and before the divergence of marsupials, which is being explored further (F Veyrunes, personal communication).

To provide new clues to the organization, function and evolution of the platypus multiple sex chromosomes, we defined the sex chromosomes of the distantly related short-beaked echidna, *T. aculeatus*, and established the sex chromosome order in the echidna multivalent chain. Our genome-wide comparison using chromosome painting between echidna and platypus (called Tac (for *T. aculeatus*) and Oan (for *O. anatinus*) in this report) showed, surprisingly, that one member of the *Oan* chain is replaced by an autosome in *Tac*, and the X homologous to *Oan* X5 occupies a central position in the Tac chain rather than a position at the end as seen in *Oan*. To investigate the participation of the ancestral avian Z in the evolution of the monotreme sex chromosome system and to map genes to the members of the sex chromosomes, we also localized the platypus homologues of genes on chicken autosomes and Z. We conclude that the ancestral monotreme sex chromosome system bears considerable homology to the sex chromosomes of birds.

Results
Characterization of the short-beaked echidna karyotype

The male short-beaked echidna has 63 chromosomes and the female 64 [5]. We characterized and classified the male karyotype by flow cytometric analysis, flow sorting, and chromosome painting. The chromosomes produced 36 peaks in the flow karyotype (Figure 1); 17 peaks represent single chromosome pairs and 4 peaks represent 2 chromosome pairs each. The homologues of chromosomes 1, 6, 16, and 27 (which are frequently heteromorphic) each sort in two different peaks. The nine remaining peaks represent single chromosomes, which we show to be the nine unpaired sex-chromosomes that constitute the meiotic chain in the male echidna. Thus, the male echidna has 27 autosome pairs and 9 sex chromosomes.

The chromosome paints were used to identify autosome pairs and sex chromosomes in male and female echidnas. Figure 2 shows a G-banded karyotype of male (upper) and female (lower) echidna chromosomes arranged in size and identified by the chromosome painting results (see below). The upper part of both the male and female karyotypes show 27 pairs of...
Autosomes that should form bivalents at meiosis, and the lower parts show the nine unpaired sex-chromosomes in male and five paired sex-chromosomes in female. The paired autosomes can be divided into a group that contains submetacentric chromosomes (chromosomes 1 to 8) and a group of smaller metacentric and submetacentric chromosomes. Chromosomes 3, 6 and X5 contain nucleolus organizer regions (Figure 2) determined by Ag-NOR (nucleolar organizing region) staining and by FISH using a probe specific for 28S rDNA (Figure 3).

Special attention was given to chromosome pair 27, as it shows complete homology with platypus X4 and partial homology with platypus Y3 and Y4 (see below), and might, therefore, be an unrecognized sex chromosome. However, both the paints, produced from the two peaks representing the heteromorphic chromosome pair 27, painted both chromosomes 27 totally in both male and female metaphases (Figure 4a). Based on this analysis, the pair was defined as an autosome pair and this was confirmed by analysis of meiotic preparations, which revealed that 27 is not part of the chain.

Chromosome painting in echidna reveals non-specific signals that are present on more than one chromosome pair, indicated by coloured bars next to chromosomes in Figure 2. For instance, when the paint specific for chromosome 1 was hybridized to metaphases, stronger signals were visible on the region indicated on chromosome 1 and a region on chromosome 2. A hybridized chromosome 4 paint gave strong signals on chromosomes 4, 7, and 16 (and others as indicated), weaker signals on X1 and Y1, and even weaker signals on chromosomes 1 and 2.

The chromosome specificity of these regions was apparent using paints produced by microdissection of these regions. Hybridization to these regions (which hamper identification of chromosomes, and especially pairing regions of the sex chromosomes) was not blocked by pre-hybridization with echidna Cot-1 DNA. To facilitate chromosome identification, an image enhancement procedure was developed to remove these non-specific signals from the image [19]. Molecular characterization of these regions is not considered in this paper.

Sex chromosomes in the echidna male

The nine chromosome paints that identified unpaired chromosomes (denoted by X1, Y1, X2, Y2, and so on) were used to identify the sex chromosomes of the echidna and predict their order in the meiotic chain. Paint X1 hybridized to the whole of chromosome X1 and the long arm of Y1 (Figure 4b), whereas paint Y1 hybridizes to the single chromosome Y1 and the short arm of X1 (Figure 4c). X1 is known to be the first chromosome in the chain [20], and its short arm pairs with the acrocentric Y1q (second chromosome). The paint X2 shows a complete coverage of a single chromosome X2 as well as signals on the pairing regions on Y1p and Y2p (Figure 4d). This result shows that X2 is the third and Y2 the fourth in the chain. Paint Y2 covers Y1 and paints the pairing regions on X2 and X3 (Figure 4e). Paint X3 paints the entire chromosome X3, as well as the long arm of Y2 (Figure 4f), so is the fifth chromosome.
Figure 2 (see legend on next page)
in the chain. The order of the last four elements is less certain at this stage. Paint Y3 covers the tiny chromosome Y3 with no signal denoting pairing regions on X3 and X4 (Figure 4g). Paint X4 hybridized to chromosome X4 and to Y3 and the heterochromatic centromeric regions of chromosome 22 or 23 (Figure 4h). Y3 is also painted by chromosome paints 25 and 26, suggesting that it contains shared large non-specific sequences. Paint Y4 hybridized to chromosome Y4 and to X5p (Figure 4i). Paint X5 paints the whole X5 and the long arm of Y4 (Figure 4j); it also showed hybridization to a heterochromatic centromeric region on an autosome.

Sex chromosomes in the echidna female
The same set of nine sex chromosome paints was hybridized to female metaphases to verify which element is an X-chromosome (defined as having one copy in the male and two copies in the female) and which element is a Y-chromosome (one copy in the male and absent in the female). Figure 5 shows some examples of these hybridizations. For instance, chromosome paint Y3 hybridized to the pairing regions of X3 and X9, but identified no copy of the male-specific Y3 (Figure 5b).

The results show that indeed X1-X5 are X chromosomes and Y1-Y4 are Y chromosomes. These results also clarified the order of the alternating X and Y chromosomes.

Homologous regions between adjacent X and Y chromosomes are, therefore, demonstrated for all members of the chain of nine except between the small X3, Y3 and X4. These pairing regions all include the distal end of one chromosome arm and do not cross the centromere of the unpaired chromosome. The order of the first five chromosomes of the chain can be deduced from the homology relationships as X1,Y1,X2,Y2,X3. However, the order of the last two X and two Y chromosomes is uncertain by chromosome painting on echidna mitoses, as the pairing regions are too small to detect. However, the results of cross-species painting (see below) and our analyses of chromosome painting of meiotic chains (Figure 5e–g) revealed that X4 is the seventh element in the chain (Figure 5g), confirming the order shown in Figure 2.

Genome wide comparison between echidna and platypus
Cross-species chromosome painting was used to define chromosome regions conserved between Oan and Tac and to identify rearrangements that differentiate the karyotypes of the two species. Figures 2a and 6 show the Oan and Tac homology maps, with the homologous regions indicated on the right of each chromosome.

Comparison of platypus-echidna autosomes
Cross-species painting showed that entire Oan chromosomes 1, 4, 5, 9, 11, 14, 16, and 19 are conserved in the Tac karyotype as chromosomes 1, 4, 3, 10, 11, 14, 19, and 22, respectively (Figures 2a, 6 and 7b). Oan chromosomes 15 and 20 are conserved on either Tac 25 or 26, which are similar in size and could not be separated by flow sorting.

Several platypus chromosomes (Oan 2, 3, 7, 8, 10, 12, 13) paint two echidna chromosomes or chromosome arms, representing a centric fusion in the platypus lineage, or a fission in the echidna lineage (Figures 2a and 7a,d). Vice versa, there are also four echidna chromosomes (Tac 2, 7, 9, 12) each of whose arms are homologous to two platypus chromosomes, implying either fissions in the platypus lineage or fusions in the echidna lineage (Figures 6 and 7c). It is not possible to distinguish centric fusions from centric fissions without reference to outgroup species.
Figure 4 (see legend on next page)
Tac homologous regions for *Oan* 18, 17p and 21 could not be determined, probably because these regions are homologous to *Tac* chromosomes with large amounts of 'non-chromosome-specific' repetitive DNA (see first section of results). Similarly, homologous regions were not determined for short regions on *Tac* 3p, 4p, 5p, 6p, 8p, 13p, 15q, 16p, 17q, 18, 20, 21, 23, 24p and the large regions on 1q and 2q. These blocks correspond to specific *Tac* regions identified by paints made from the equivalent regions by microdissection (depicted in different colors at the left in Figure 2 top).

The NOR-bearing regions are not on homologous chromosomes in the platypus and echidna. In platypus, *Oan* 6 contains the NOR region; this chromosome is homologous to *Tac* 16. In echidna, *Tac* chromosomes 3, 6, and X3 are the NOR bearing chromosomes (Figure 3); these chromosomes are homologous to *Oan* 5, 7, and 12p.

**Comparison of platypus and echidna sex chromosomes**

Cross-species painting with *Oan* and *Tac* X-Y probes shows that *Oan* X1, Y1, X2, Y2, X3, and Y3, respectively (Figures 8a–d and 9a–c), but one X chromosome in each and homologous regions of the flanking Y chromosomes are non-homologous. Reciprocal chromosome painting shows that *Oan* X2 and *Tac* X3 are homologous. The *Oan* X1 paint hybridized to *Tac* X4 (Figure 8h), the *Tac* X4 paint hybridized to *Oan* X3 (Figure 9f); neither hybridization detected pairing regions in adjacent Y chromosomes. Confirmation that the large *Tac* X4 chromosome is the genetic homologue of the *Oan* X4 is provided by the assignment of the *DMRT1* gene complex to *Tac* X4q (Figure 10a).

An important result was that *Oan* Y1 and *Tac* Y1p paints hybridized to a *Tac* autosome, and that *Tac* Xp paint hybridized to an *Oan* autosome. *Oan* paint X4 hybridized to the whole *Tac* 27 (Figure 8f). *Oan* paint Y4 hybridized to a small region of *Tac* chromosome 27 (Figure 8e), confirmed as an autosome (Figure 4a). *Oan* paint Y2 hybridized to *Tac* Y4 and the distal end of *Tac* X3p (Figure 8g). Reciprocally, *Tac* Xp paint hybridized to platypus chromosome 12 (Figure 9d). *Tac* paint Y4 hybridized to four regions: the two homologues *Oan* 12p, *Oan* Xp, and *Oan* Yp and *Tac* 27 identified *Oan* Yq, *Oan* Xq, and *Oan* Y4q (Figure 9e). Many metaphases were observed to confirm these signals. We conclude that *Tac* Y4q and Xq represent autosomes in platypus and parts of *Oan* Yq, Xq, and Y4q are homologous to autosomal regions in echidna.

In the echidna, there are only four Y chromosomes, compared to five in the platypus, suggesting that the small Y5 in platypus has been completely lost in the echidna lineage [21]. A surprising result, therefore, was the hybridization of the *Oan* Y5 paint to *Tac* Y5, a strong indication that *Oan* Y5 is represented in the echidna chromosome chain of nine (Figure 8i). No reliable signals on chromosomes other than *Tac* Y3 were observed. Attempts to hybridize the chromosome paint of *Tac* Y3 paint onto *Oan* male metaphases produced no reliable signal.

**Mapping chicken-human homologous genes**

In order to test the hypothesis that the bird Z chromosome is represented in the monotreme sex chromosome chain, platypus homologues of nineteen chicken-Z genes (together with chicken 2, 3, and 13 genes) were mapped to platypus chromosomes by two independent methods, PCR amplification of chromosome specific DNA and FISH localization of platypus bacterial artificial chromosome (BACs). Gene mapping results are summarized in Figures 11 and 12, and see Additional data file 1. This file also shows platypus contigs that contain the mapped and predicted genes extending the regions of homology.

Gene loci in chicken are designated in the following section according to their established conserved syntenies with human chromosomes. For instance, chicken Z is homologous with regions of human chromosomes 5, 9, 8 and 18 and chicken chromosome 2, 3 and 13 also share homology with human chromosome 5 [9]. Figure 12 presents a standard idiogram, based on G-banding and chromosome painting, on which the present and previous [10,11,22-25] gene assignments are shown. Consideration of the contigs in Ensembl to which these genes belong greatly expands the size of the syntenic regions (Additional data file 1). Most of the conserved syntenies that we have observed between platypus chromosomes and chicken Z are in five groups.

The first group includes platypus homologues of chicken Z and human 9 (*Gallus gallus* (GGA)-Z/*Homo sapiens* (HSA)-9) genes. Three genes mapped to platypus Xp, one mapped to Xq and two mapped to X3 (Figure 12). Consideration of the contigs to which these genes belong greatly expands the size of the homologous regions (Additional data file 1).

The second group includes platypus homologues of chicken Z and human 5 (GGA-Z/HSA-5) genes. Ten genes are distributed over platypus chromosomes 1, 2, 3 and the sex chromosomes. Only one GGA-Z/HSA-5 gene (PDE6A) mapped to platypus X, by both PCR and BAC-clone mapping. This gene mapped on the pairing region of Xp and Yq, distal to the...
Figure 5 (see legend on next page)
centromere on each (Figure 10b,c), and is contained in platypus contig 269 with nine other genes, four of which are homologous to GGA-13/HSA-5q (Additional data file 1). Two platypus genes (LMNB1 and DMXL1) homologous to chicken Z and human 5 (GGA-Z/HSA-5) mapped to platypus Xs by both PCR and BAC-cloning. The homologue of the chicken gene LMNB1 PCR-mapped also to X1 but not to Y1, suggesting that the PCR product of X1 may represent a section of the Xs gene that has a copy (paralogue) on X. Likewise, DMXL1 was PCR-mapped also to Oan 10 as well as X1 (but not Y1), again indicating that a section of the DMXL1-Xs gene has a copy (paralogue) on both Oan 10 and Xs. The seven other GGA-Z/HSA 5 genes map to Oan 1, 2 and 3 by PCR or BAC-FISH.

The third group includes platypus homologues of chicken 13 and human 5 (GGA-13/HSA-5), and chicken 3 and human 2 (GGA-3/HSA-2) genes. Five GGA-13/HSA-5 genes and one GGA-3/HSA-2 gene mapped to the pairing regions of X1 and Y1 (Figures 11 and 12). PANK3 also PCR-mapped to Oan 8, which might be a paralogue. Thus, the three GGA-Z/HSA-5 homologues on X1 are accompanied by GGA-13/HSA-5 genes
and one GGA-3/HSA-2 gene. The majority of genes in the respective contigs (10, 269, 847, 127) are GGA-13/HSA-5 genes, one is a GGA-3/HSA-8 gene and three are GGA-3/HSA-2 genes.

The fourth group includes platypus homologues of chicken 2 and human 5 (GGA-2/HSA-5), and chicken 2 and human 18 (GGA-2/HSA-18) genes. Three GGA-2/HSA-5 genes mapped to Oan Y2 and X3. These three genes are accompanied by the GGA-2/HSA-18 gene P15RS. This gene is in contig 29, which

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**Figure 7**
Examples of cross-species chromosome painting to autosomes. (a) Paint Oan 7 and (b) paint Oan 16 hybridized to Tac 6q and 9p, and Tac 19, respectively. (c) Paint Tac 7 and (d) paint Tac 6 hybridized to Oan 2p and Oan 7q (reverse of 6a), respectively. Note that Tac 6 is a NOR-bearing chromosome but Oan 7 is not.
contains 7 GGA-2/HSA-5 genes, 3 GGA-2/HSA-9 genes and 3 GGA-2/HSA-18 genes.

The fifth group includes platypus homologues of chicken Z and human 8 (GGA-Z/HSA-8), and chicken Z and human 18 (GGA-Z/HSA-18) genes. Two GGA-Z/HSA-8 genes mapped to platypus chromosome 5, and one GGA-Z/HSA-18 gene mapped to platypus chromosome 3.

Thus, mapping platypus homologues of chicken Z genes revealed homology not only with platypus X, but also with platypus X, Y, X, and X. Other genes from human chromosomes 5 and 9, which are not homologous to the chicken Z, also mapped to platypus sex chromosomes. A set of GGA-Z genes homologous to HSA-5, 8 or 18 (see above), but so far not HSA-9, mapped to platypus autosomes. So far, no chicken Z genes have been mapped to platypus X, but this may not be surprising as X is homologous to an echidna autosome.

A selection of these genes was PCR-mapped to echidna chromosome-specific DNA using the same primers. GPR98 (Oan 1) mapped to Tac 1, GRHL1 (Oan X1, Y1) mapped to Tac X, but not Y, RAPGEF6 (Oan X, Y) mapped to Tac X and Y, MLLT3 (Oan X) mapped to Tac X, TRIO (Oan Y, X) mapped to Tac Y and X, TSCOT (Oan X) mapped to Tac X, and LMNB1 (Oan X, Y) mapped to Tac X, DMR1 (Oan X) mapped to Tac X, by BAC-FISH (Figure 10a). These results confirm homologies between the platypus and echidna chromosomes determined by chromosome painting.

Discussion

We confirm previous cytological studies of echidna mitotic and meiotic chromosomes that showed that the male short-beaked echidna has 63 chromosomes - 27 pairs of autosomes and 9 sex chromosomes [20,21,26] - and establish that the sex chromosome constitution is 5 Xs and 4 Ys. These chromosomes form a chain of nine at meiosis, expected (by analogy to platypus) to be in an alternating X-Y order. Adjacent members are expected to be held together by pairing within 16 pseudoautosomal regions (one per chromosome arm except for X and Y, which terminate the chain). Each sex chromosome and 13 of the expected 16 pairing regions of the 5 Xs and 4 Ys (Figure 2, middle) were identified in this study. The alternating order in the chain is directly confirmed by painting in meiosis I metaphases (Figure 5e–g).

Homology between platypus and echidna chromosomes

Ten chromosomes are completely conserved between echidna and platypus. The non-conserved chromosomes differ between the two species by centric rearrangements only, which are charted in Figures 2 and 6. Without comparative data from an outgroup it is not possible to distinguish fusions in one lineage from fissions in the other.

Both platypus and echidna have prominent NORs on the short arm of chromosome 6, which was thought to be homologous, because of their similar size and morphology [21]. However, chromosome painting shows that platypus chromosome 6 is homologous to echidna chromosome 16. In addition, echidna has NORs on X and chromosome 3. Non-homology of NOR-bearing chromosomes is not surprising in view of their rearrangement in many closely related species.

Differences between platypus and echidna sex chromosome chains

The constitution of the sex chromosome system in Tac differs from that in Oan in their number, order and in the identity of one XY pair. Firstly, chromosome painting shows that there are five Xs and five Ys in platypus but five Xs and only four Ys in the echidna. The missing platypus Y is a very small chromosome, and it had been previously supposed to have been lost from the echidna lineage [21,27]. However, the presence of a strongly hybridizing region on the larger echidna Y using the platypus Y5 paint suggests that the content of this platypus Y is incorporated into echidna Y5. The surprising finding is that these two Y-chromosomes are at different locations in the chain. Secondly, Oan X and Tac X are shown to be homologous chromosomes by chromosome painting, and by sharing the DMR1 gene cluster (Figure 10a). However, they occupy different positions in the chain. Thirdly, the most telling result we obtained was the finding that the platypus and echidna sex chromosome chains contain elements (Oan chromosomes Y, X, and Y, and Tac and Y) that are not homologous; platypus X paints an autosome (chromosome 27) in echidna and echidna X paints an autosome (chromosome 12) in platypus.

Thus, the chain in these monotreme species differs in both order and constitution, indicating that the chain continued to evolve after the divergence of platypus and echidna approximately 25 MYA [28]. One can speculate that the pri-
Figure 8 (see legend on previous page)
mary sex determining locus is more likely to be found on the non-pairing regions of the X or Y chromosomes that are shared between platypus and echidna rather than on those that are autosomal in either.

Although the comparative painting reported here is a genome-wide comparison between the two monotreme species, it cannot, of course, reveal the gene content of the chromosomes, which requires comparative gene mapping.

**Mapping chicken-human homologous genes**

Comparative gene mapping was used to establish that homologous genes are together in the same contiguous region in both species indicating chromosome homology. This does not exclude the possibility of non-orthology for some of the contiguous genes, but the likelihood of multiple, independent exceptional events is reduced as more homologues are discovered in the region. As the chance of independent evolution of syntenic regions is reduced, the likelihood of shared descent from the same chromosomal region in a common ancestor is increased.

The comparative mapping results show that monotreme sex chromosomes contain genes homologous to the chicken Z chromosome and chicken autosomes, with the implication chromosomes contain genes homologous to the chicken Z chromosome. The early finding of the **DMRT1** gene on platypus X5 prompted our search for other chicken Z genes on this chromosome by mapping homologues of GGA-Z/HSA-9 genes followed by homologues of GGA-Z/HSA-5 genes. This led to the observation of chicken Z and autosomal homologues on other platypus sex chromosomes.

With regard to platypus X1, the preliminary results of the draft genome sequence of the female platypus (Ensembl release 44) and a recent comparative mapping study of the therian X-linked genes (F Veyrunes, personal communication) show that platypus X1 does not share homology with the therian X as previously reported [14-18]. X-linked genes on human Xq assigned by FISH localization of BACs so far all map to platypus chromosome 6 [10]. These on human Xp map to platypus chromosome 15 and 18 [23] (F Veyrunes, personal communication). The results presented here reveal instead that platypus X1 shares homology with chicken chromosomes 3, 13, and Z and the corresponding human chromosomes 2, 5, 8, and 9 (Additional data file 1). Thus, our mapping data show that X1 shares homology with those chicken chromosomes that are homologous to human autosome.

The implications of some of the platypus gene assignments require further discussion. For example, the motivation to map **RAPGEF6** was that this gene is close to the chicken Z **HINT1** homologue in human (HSA-5q), although on a different chromosome in chicken (GGA-13). **HINT1** might be involved in the chicken sex determining pathway, so may be a candidate for the primary sex determining locus in platypus; mapping a **HINT1** homologue was unsuccessful. However, the localization of **RAPGEF6** to Oan X, Y makes it unlikely that this region contains the primary sex determinant. Note that platypus Xp and Yq contain homologues of genes on chicken 3, 13 and Z (mapped in this report). The chicken 13 and the chicken Z regions were presumably syntenic before the divergence of Prototheria and Theria as these regions are syntenic in both monotremes and human.

The platypus homologues that map to X2 (**MLLT3**), X3 (**TSCOT, PALM2**), and both X3 and Y2 (**CDH12, DNAH5, TRIO, P15RS**) assign their respective contigs to these sex chromosomes (Figure 12 and Additional data file 1), indicating that parts of chicken 2 and chicken Z were fused before the divergence of Prototheria and Theria, and that the fission of chicken chromosome 2 into the regions on human chromosome 5, 9, and 18 occurred after the divergence of Prototheria and Theria.

Previous work showed that platypus chromosome X5 contains the **DMRT1-2-3** complex, whose homologue maps to the Z chromosome in chicken, and to human chromosome 9 [6,8]. We show here that several other genes with homologues on the chicken Z also map to the platypus X1. Identification of the contigs that include these genes maps a large fraction of the chicken Z to platypus X5, indicating that a large region of human 9 homologous to chicken Z is conserved on platypus chromosome X5. Platypus major histocompatibility complex (MHC) class III genes have been mapped recently to the pairing segments of X4 and Y4 and MHC class I and II genes have been mapped to the pairing regions of X4 and Y4 [29]. We note that platypus X5 (homologous to the echidna autosome **Tac** 27) separates these two clusters, while in echidna the homologues of platypus X4 and Y4 (that is, **Tac** X4 and Y4) that carry the MHC genes are adjacent to X4 and Y4, suggesting that the insertion of X4 in platypus was a later event in the evolution of the monotreme meiotic chain. So far, no homologues of

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**Figure 9 (see following page)**

**Tac** chromosome paints hybridized to male **Oan** metaphases. (**a**) Paint **Tac X1** (red) hybridized to **Oan X1** and **Oan Y1q**. Paint **Tac X2** (green) hybridized to **Oan X2** and the pairing region on **Oan Y2** and **Y1p** (arrow head). (**b**) Paint **Tac Y1** covers **Oan Y2** and hybridized to **Oan X2** and **Oan X1**. (**c**) Paint **Tac X3** hybridized to **Oan X1** and the pairing region on **Oan Y2**, the two additional signals are centromeric heterochromatin. (**d**) **Tac** paint **X3** hybridized to **Oan 12p**. (**e**) Paint **Tac Y4** (red) hybridizes to **Oan 12p**, and to **Oan X3p** and **Oan Y3p**. Paint** Tac 27** identified **Oan Y2**, **Oan Xp**, and **Oan Y4q**. (**f**) Paint **Tac X4** covers **Oan X5**.
Figure 9 (see legend on previous page)
chicken Z map to platypus X4, possibly because it is homolo-
gous to an echidna autosome.

The gene mapping results described here assign several genes
to the pairing regions of platypus Y chromosomes. The
sequence reported in Ensembl does not provide this informa-
tion as it is based on the female genome, so Y-linked genes are
not included.

Not all chicken Z homologous genes are located on the
monotreme sex chromosomes. Seven additional GGA-Z/
HSA-5 genes map to platypus autosomes 1, 2, or 3 (Figures 11
and 12). The GGA-Z/HSA-8 genes LPL and CHRNA3 map to
platypus chromosome 5 and the GGA-Z/HSA-18 gene
ATP5A1 maps to platypus chromosome 3. Platypus chromo-
some 3 contains homologues of genes localized on chicken Z
and human 5 and 18. This means that these two human
regions were syntenic before monotreme-therian divergence
and became separated only in the mammalian lineage after
this divergence.

The monotreme regions homologous to chicken Z are consid-
erably rearranged and distributed over autosomes and at
least four X chromosomes and the corresponding Ys. The
(single) Z homologues on Xp, Y, and X4 are accompanied by
other non-Z homologues in their respective contigs. X4 seems
to have a large region homologous to chicken Z as it contains
contig 278 with a size around 0.8 Mb. This contig has chicken
Z genes that are homologous only to human chromosome 9
and 5. In Ensembl release 45, contig 278 is part of ultracontig
84 with a size of around 8 Mb containing more chicken Z
(human 5 and 9) homologous genes. Only chicken Z genes
have so far been mapped to platypus X1, and most of these are
homologous to human 9 and a few to human 5. As both X5 and
X6 contain human 5 and 9 genes (that are homologous to
chicken Z), the separation into these human 5 and human 9
regions must have occurred later in the therian lineage.
Finally, the platypus autosomes 1, 2 and 3 also contain
chicken Z genes, suggesting that these chromosomes may
represent the partners in original translocations involving a
putative ancestral Z chromosome.

The results shown in Figure 12 all confirmed the homologies
found by chromosome painting in this report and in our
previous paper [7]. In particular, a number of FISH assign-
ments confirmed the pairing regions between X and Y chro-
mosomes. These early mapping results should help in the
identification of the primary sex-determining locus, in the
investigation of mechanisms of dosage compensation and in understanding the evolution of vertebrate sex chromosomes.

An exchange mechanism for the evolution of the multiple sex chromosomes of the platypus was postulated previously [7,21]. The chain development was suggested to start with an ancestral pair of differentiated sex chromosomes, one of which was repeatedly involved in exchanges with autosomes. An alternative view [27,30] suggests that the chain arose as the result of hybridization between two ancestral monotreme populations, each with a different set of Robertsonian translocations resulting in a male heterozygous for unpaired sex chromosomes. Common to all models is that the rearranged autosomes in the chain evolved into Y chromosomes. Our finding that different rearrangements occurred in the two monotreme lineages after the platypus-echidna divergence (25 MYA [28]) is easier to reconcile with a model of successive translocation, rather than the unlikely alternative of additional hybridizations between populations differing in other Robertsonian rearrangements.

**Conclusion**

Our cross-species painting studies of the monotreme sex chromosome complements shows that the platypus and echidna translocation chains share homology over four of the
five X chromosomes, but one in each species is entirely non-homologous. This means that the chains continued to evolve after the divergence of platypus and echidna.

Our comparative mapping studies show chicken Z homologous genes in the sex chromosome system with the main clusters on platypus X3 and X5 and echidna X3 and X4. Other Z homologous genes map to autosomes, indicating many rearrangements between the monotreme and avian lineages.

In combination with the mapping data available in current Ensembl release 44, our results also reveal homology of platypus X to chicken 3, 13, Z, 11, and 12, which are homologous to human autosomes. This suggests that the monotreme’s XY chromosome system is unrelated to the therian XY system. This is further explored by F Veyrunes (personal communication) in comparative studies with therian X-linked genes, and it may mean that the therian XY system evolved after the pro-therian and therian divergence, but before the divergence of marsupials, and is, therefore, younger than previously anticipated [31].

It is important to note that three monotreme X chromosomes have large differential regions. The differential region on platypus X5 (echidna X4) is homologous to chicken Z, that of X3 is homologous to chicken 2 and Z, and the large differential region on X1 seems mostly homologous to chicken 3 and 12. These differential regions are completely different from those of the therian X and Y chromosomes, indicating again that the monotreme and therian sex chromosome systems have different origins. We believe that the comparative mapping results reported here will be useful in the continuing search for the monotreme sex determining switch, and in future studies on sex chromosome evolution and dosage compensation mechanisms.

It will be instructive to extend the genome comparison between birds and monotremes to other amniotes, such as snakes and lizards. These comparisons will enable the construction of the ancestral karyotype of sauropsids and mammals and reveal the chromosome evolutionary events that occurred at the origin of the sauropsid and mammalian lineages.
Materials and methods

Chromosome paint generation

Primary fibroblast cultures from the short-beaked echidna (*T. aculeatus*, 2n = 63 male, 64 female) were established routinely in standard medium at 32°C (AEEC permit no. R.CG.07.03 and AEC permit no S-049-2006, NSW P&W permit S10443). Flow sorting, chromosome paint production and FISH were performed according to the protocol described previously [7,32]. Platypus (2n = 52) chromosome paints and metaphases were generated as previously described for the characterization of the platypus sex chromosome complement [7].

Cot-1 preparation

Cot-1 DNA was prepared as described [33], except that the renatured (double-stranded) Cot-1 DNA was separated from the digested (single-stranded) DNA using a phenol-chloroform-extraction method. Purified echidna Cot-1 DNA (30 μg) was used for FISH.

Preparation of meiotic cells

Meiotic cells were obtained from animals captured at the upper Barnard river, New South Wales, Australia during breeding season (AEEC and AEC permits to FG as above). The captured animals were euthanased with an intraperitoneal injection of pentobarbitone sodium (Nembutal, Boehringer Ingelheim, NSW, Australia) at a dose of 0.1 mg/g body weight. Meiotic cells were obtained by disaggregating the testis. The material was either directly fixed in methanol/acetic acid (3:1) or incubated in 0.075 M KCl M at 37°C as hypotonic treatment to improve spreading of metaphase cells and then fixed.

Chromosome microdissection

Microdissection-derived chromosome specific paints of specific blocks were generated as described previously [34]. In short, short-beaked echidna metaphases were dropped onto wet cover slips and subsequently digested with 0.015% trypsin for 1 minute and stained with Giemsa. Microdissected material was collected in a drop containing 10 mM NaCl, 10 mM Tris-HCL, 1 mM EDTA, 0.1% SDS, 0.1% Triton ×100, 1.44 mg/ml Proteinase K (Sigma-Aldrich, Gillingham, Dorset, UK), 30% glycerol. After 1 hour incubation at 60°C this drop was transferred to 5 μl of 1× sequenase buffer (USB, Staufen, Germany), 0.2 mM dNTPs, 5 μM 6 m w primer. A PCR protocol for low temp cycles was next: 5 minutes at 92°C followed by 8 cycles of 2 minutes 20 s at 25°C, 2 minutes at 34°C, 1 minute at 90°C. At the start of each cycle 0.4 units of sequenase (USB) was added. For the high temp cycles 45 μl 1× Buffer D (Invitrogen, Paisley, UK), 0.2 mM dNTP, 5 μM 6 mW primer, 0.3 u SuperTaq polymerase (HT Biotechnology, Cambridge, UK), 0.05% W1 (Sigma) were added. This solution was subjected to 33 cycles of 1 minute at 92°C, 2 minutes at 56°C, 2 minutes at 72°C with a final extension for 5 minutes at 72°C. The DNA was labeled using a standard protocol [32].

Fluorescence microscopy

Images were captured using the Leica QFISH software (Leica Microsystems, Milton Keynes, UK) and a cooled CCD camera (Photometrics Sensys, Photometrics, Tucson, AZ, USA) mounted on a Leica DMRXA microscope equipped with a 63×, 1.3 NA objective. Cy3, FITC 9 (fluorescein isothiocyanate) and DAPI (4′,6-diamidino-2-phenylindole) signals were captured separately as 16 bit black and white images, and merged to a color image. The DAPI image was enhanced with a spatial filter to obtain enhanced chromosome bands. All image processing was performed with Leica CW4000 software.

Characterization of the short-beaked echidna karyotype

The Tac paints produced were hybridized to male (three individuals) and female (one individual) Tac metaphase preparations. Multicolor chromosome painting was used to ensure that different peaks represent different chromosomes, to define the order of the unpaired chromosomes and to determine the homologous parts that link these chromosomes in the meiotic chain.

Mapping chicken-human chromosome 5, 9, 8, 18 homologous genes on platypus and echidna chromosomes

Twenty-nine genes were mapped to platypus chromosomes (Table 1), and eight of these to echidna chromosomes. Two methods, PCR and BAC-clone mapping (indicated by ‘a’ and ‘b’ in Table 1) were used to localize the platypus homologues.

PCR

A human or chicken exon of the specific gene was blasted to find alignments with the NCBI trace archives of platypus using discontinuous megablast. The alignment was used to design platypus specific primers using PrimerQuest [35]. The primers were used first to amplify pools of chromosome specific DNA, and second to amplify chromosome specific DNAs of the positive pool. The exon was considered to be mapped to a single chromosome if only one pool was positive and only one ‘chromosome’ in that pool was positive. The size of the PCR product was checked to verify that it was as expected from the primer design section, and the product was sequenced for confirmation.

The sequence of the PCR products was blasted to find alignments with the Ensembl Platypus *Ornithorhynchus anatinus* database release 5. The platypus contigs in the database contain several predicted genes, which were identified by blasting to find alignments with the NCBI human genome database. Homologues of these genes were subsequently localized in chicken by BLAST alignment in Ensembl Chicken.
BAC-clone mapping

The above PCR product was used to screen a platypus BAC-clone library (Oa-Bb, Clemson University, South Carolina, USA). Positive clones were labeled by nick translation and positioned on platypus chromosomes by FISH. The presence of the target gene in the BAC clones above was confirmed by sequencing using the same gene specific primers.

Chromosome homology by comparative gene mapping

Comparative gene mapping was used for the assessment of chromosome homology. It is important to consider whether the homologous genes are likely to be true orthologues. The definition of orthologues is two genes from two different species that derive from a single gene in the last common ancestor of the species [36]. Absolute proof of orthology is difficult on this criterion and was not pursued in this report. Instead, support for chromosome homology was provided when homologous genes were together within the same contiguous region in both species. Instances of non-orthology in the syntenies may exist but the likelihood of multiple, independent exceptional events is reduced when several gene homologues are found in one region.

Abbreviations

BAC, bacterial artificial chromosome; FISH, fluorescent in situ hybridization; GGA, Gallus gallus. HSA, Homo sapiens. MHC, major histocompatibility complex; MYA, million years ago; NOR, nucleolar organizing region; Oan, Ornithorhynchus anatinus; Tac, Tachyglossus aculeatus.

Authors' contributions

WR designed and performed most of the experiments and analyzed the data. PCMOB sorted the platypus and echidna chromosomes, FG and ETA undertook the meiotic analysis, OC, DG, VAT, HS, MCW, and FV performed other experi-
ments. JAMG and SJ provided material and JAMG contributed to the writing of the paper. WR and MAFS conceived and supervised the research and wrote the paper.

Additional data files
The following additional data are available with the on-line version of this paper. Additional data file 1 is a table listing gene assignments to platypus contigs and platypus chromosomes, together with human and chicken locations.

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