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Gene discovery and comparative analysis of X-degenerate genes from the domestic cat Y chromosome ☆

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ABSTRACT

Mammalian sex chromosomes are the remnants of an ancient autosomal pair present in the ancestral mammalian karyotype. As a consequence of random decay and chromosome rearrangements over evolutionary time, Y chromosome gene repertoires differ between eutherian lineages. To investigate the gene repertoire and transcriptional analysis of the domestic cat Y chromosome, and their potential roles in spermatogenesis, we obtained full-length cDNA sequences for all known Y genes and their X chromosome gametologues and used those sequences to create a BAC-based physical map of the X-degenerate region. Our results indicate the domestic cat Y chromosome has retained most X-degenerate genes that were present on the ancestral eutherian Y chromosome. Transcriptional analysis revealed that most feline X-degenerate genes have retained housekeeping functions shared by their X chromosome partners and have not been specialized for testis-specific functions. Physical mapping data indicate that the cat *SRY* gene is present as multiple functional copies and that very little of the felid Y chromosome may be single copy. X-Y gene divergence time estimates obtained using Bayesian methods confirm an early origin of Stratum 1 genes prior to the origin of therian mammals. We observed no statistical difference in the ages of Stratum 2 and Stratum 3 gene pairs, suggesting that eutherian and marsupial Stratum 2 genes may have been independently retained in each lineage.

The Y chromosome is the most distinctive and structurally repetitive chromosome in a mammalian genome. Derived from half of an ancient autosomal pair present in the ancestor of mammals, the Y chromosome evolved by subsequent selection for a male-determining factor, *SRY,* and then suppression of recombination with the X chromosome, resulting in the gradual divergence of similarity between the Y chromosome and its former partner [1]. As a result of 230–300 million years of independent, nonrecombining evolution, the Y chromosome has lost more than 95% of its approximately 1100 original genes, which still survive today on the X chromosome of placental mammals [2]. Despite this ongoing decaying trend, many Y chromosome genes with X chromosome homologues (termed X-degenerate genes) are widely expressed, encode housekeeping proteins that are likely required for viability, and have persisted for over 100 million years in various mammalian lineages [1,3,4].

Lahn and Page [5] calculated the synonymous substitution rate per synonymous site (K_S) between X and Y gene pairs and observed that the human X chromosome appeared to be organized into four evolutionary strata, each representing a distinct portion that ceased recombination with the Y chromosome at various periods in evolutionary time. The authors hypothesized that inversions of the Y chromosome led to the cessation of recombination with the X chromosome. A similar study of mouse X and Y chromosome genes confirmed the presence of X chromosome evolutionary strata, despite the fact that chromosomal rearrangements have shuffled the contents and order of genes in the mouse relative to the original four strata on the human X [6]. However, these authors found that the boundary between Stratum 1 and Stratum 2 was not statistically significant in mice. More recent analysis of the finished human Y chromosome sequence indicated that the boundaries between Strata 2 and 3, and between Strata 3 and 4, were not clearly defined [4]. This may be due to several factors, including gene conversion artificially lowering the whole gene K_S values or the use of K_S values in principle to estimate divergence times under the assumption of a molecular clock, which rarely holds across gene orthologues from different mammalian orders [2,4,7]. Therefore a more detailed test of the proposed X chromosome strata is needed that includes a greater diversity of mammalian species and their X-and Y-borne gene sequences and utilizes methods that account for differential divergence rates between X and Y genes as well as across species.

☆ Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. EU879967-EU879988.

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As a consequence of random decay and chromosome rearrangements over evolutionary time, Y chromosome gene repertoires differ between eutherian mammal lineages [1]. For example, *RPS4Y* is found in human and some primates, and is also Y linked in marsupials, yet it has not been detected or isolated from other eutherian orders [8]. Similarly, most eutherians and marsupials examined share *UBE1Y*, whereas this gene is found only as a pseudogene in some primate lineages and is absent in human [9,10]. Further, the mouse Y chromosome lacks *AMELY* and *EIF1AY* genes, which are present on most other eutherian Y chromosomes [11–13]. Copy number changes are also prevalent: *SRY* is present as a single copy in humans, yet has been found as multiple copies in the rabbit, rat, and other rodent genomes [14–19]. Similarly, *Zfy* is duplicated on the mouse Y chromosome.

In addition to changes in copy number, expression profiles can also change between species. Most X-degenerate genes on the Y chromosome have ubiquitous tissue expression profiles, but occasionally these genes acquire testis-limited expression, such as the mouse *Zfy, Ube1y1,* and *Usp9y* genes [3]. These changes in gene repertoires and expression patterns may have consequences for reproductive isolation and speciation. The human and mouse Y chromosomes are notably enriched for genes involved in spermatogenesis, which when deleted or reduced in number impair sperm development, resulting in extensive structural abnormalities or lack of spermatogenesis altogether [20–22]. Sperm defects are prevalent among many free-ranging mammalian species and are well described in the domestic cat and its wild relatives, including cheetahs, Gir forest lions, and Florida panthers [23,24]. It is therefore of interest to characterize the functional roles of Y chromosome genes in felids, as they may influence normal spermatogenesis.

Previous studies have identified short fragments of X-degenerate genes in the domestic cat and mapped these in radiation hybrid panels [12,25]. Subsequent studies isolated novel gene transcripts that were found to be multicopy and expressed exclusively in the testes [12]. Here we describe the isolation and transcriptional analysis of domestic cat full-length Y-linked, putatively single-copy, X-degenerate cDNA sequences and their X chromosome gametologues. We use these sequences to create an initial physical map of the X-degenerate region of the domestic cat Y chromosome and identify additional genes that were previously unknown. Comparative analyses of these sequences to other eutherian orthologues using Bayesian relaxed-clock dating methods are used to estimate divergence times between X and Y gene pairs/sets and refine the age of the evolutionary strata within eutherian X chromosomes.

Results

Generation of full-length feline X and Y gene cDNAs and expression patterns

Using a combination of rapid-amplification of cDNA ends (RACE) and RT-PCR approaches we generated full-length, or nearly full-length, cDNA sequences from the following gene pairs in the domestic cat: *AMELX/AMELY, CXorf15/CYorf15, DDX3X/DDX3Y, JARID1C/JARID1D, UBE1X/UBE1Y, USP9X/USP9Y, UTX/UTY, EIF1AX/EIF1AY, EIF2S3X/EIF2S3Y,* and *ZFX/ZFY.* Feline *UBE1Y* and *CYorf15* sequences were previously published [12]. For other genes, we used preexisting cDNA or genomic fragments from public databases [12,25] as templates for designing

Table 1
Length of cDNA and inferred amino acid sequences for eight feline X-degenerate Y chromosome genes generated in this study and comparison to human (or mouse when human is absent) orthologues

Gene	Cat cDNA	Cat protein	Human cDNA[a]	Human protein[a]
AMELY	567[b]	188[b]	579	192
EIF1AY	435	144	435	144
EIF2S3Y	1269	422	1419—mouse	473—mouse
JARID1D	4653	1550	4620	1539
USP9Y	7674	2557	7668	2555
DDX3Y	2001	666	1983	660
UTY	3840	1279	4044	1347
ZFY	2406	801	2406	801

[a] [54].
[b] Nearly complete—generated by genomic PCR.

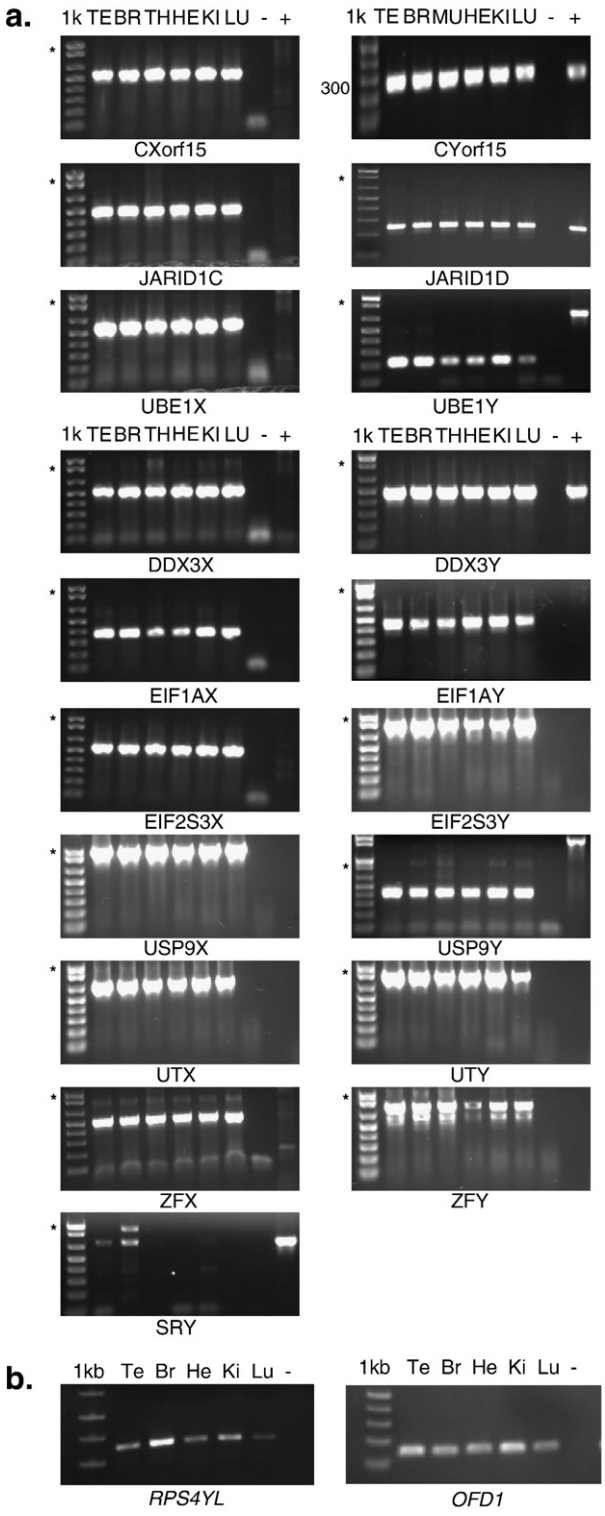


Fig. 1. (a) Gene expression profiles of cat X and Y gene pairs. Amplification results for each feline gene are shown in a panel of adult male domestic cat mRNA samples: TE, testis; BR, brain; MU, muscle, TH, thymus, HE, heart, KI, kidney; LU, lung; –, no mRNA control; +, male domestic cat genomic DNA control. Each reaction contains 10 ng of mRNA. Negative RT reactions (data not shown) were also run for each STS marker and showed no amplification. (b) Gene expression profiles for two cDNAs isolated by BAC-based cDNA selection: *RPS4Y*-like (*RPS4YL*) and *OFD1.*

PCR primers for RT-PCR and RACE reactions using testis cDNA. Additional cDNA fragments were obtained from cDNA selection experiments (see below).

In general the overall structure and sequence length of feline X-Y gene pairs are conserved with human or mouse orthologues (Table 1). Little change was observed in start or stop sites and coding sequence length. The expression profiles of nearly all X chromosome genes and their degenerate Y chromosome gametologues were also similar: most X and Y genes are broadly expressed in all adult male tissues examined (Fig. 1a). These results are similar to patterns observed for human X-degenerate Y chromosome genes [4,26]. Few exceptions to this rule were observed: *SRY* is expressed in testis and brain, and more weakly in kidney, while *UBE1Y* is expressed at higher levels in brain, testes, and kidney relative to other tissues.

Physical mapping of the domestic cat X-degenerate region of the Y chromosome and identification of novel transcripts using cDNA selection with BAC clones

Overgo probes derived from the cDNA sequences were used to identify 92 BAC clones from the domestic cat male 10× BAC library. PCR primers derived from nonrepetitive BAC-end sequences (BES) and from throughout the Y chromosome cDNAs were used to identify gene content and putative overlaps by STS-content mapping. Approximately 50% of all bidirectional BESs were found to be mostly or completely repetitive, limiting the utility of STS-content mapping for clone overlap and assembly. In addition, many BES-derived STS markers determined to be lacking repeats by RepeatMasker mapped to multiple contigs, suggesting they were low-copy duplicated sequences. Therefore an independent fingerprinting analysis of the BAC clones was used to verify clone overlaps and construct contigs. A physical map derived from both sources of information is shown in Figs. 2 and 3. Most of the single-copy X-degenerate genes are resolved into three larger contigs (Figs. 2 and 3) separated by two gaps that were not able to be filled, three smaller contigs (Fig. 3), and 14 singleton BACs (not shown). Genes tend to cluster as defined by the published Y chromosome radiation hybrid (RH) map [12], although the order between gene clusters is not resolved in the physical map. For example, *USP9Y, UTY,* and *DDX3Y* form a single contig; *JARID1D* and *UBE1Y* form a second; and *AMELY, EIF1AY, EIF2S3Y,* and *ZFY* comprise a third. Most of these genes appear to be single copy, though STS-content mapping suggests that *UBE1Y* may be present in two copies (Fig. 2a). However, our data cannot discriminate between two functional genes or the presence elsewhere of a pseudogene. Fluorescence in situ hybridization (FISH) analysis assigned two BAC clones that contain putative single-copy genes to the short arm of the cat Y chromosome (Fig. 2b). Interphase FISH results

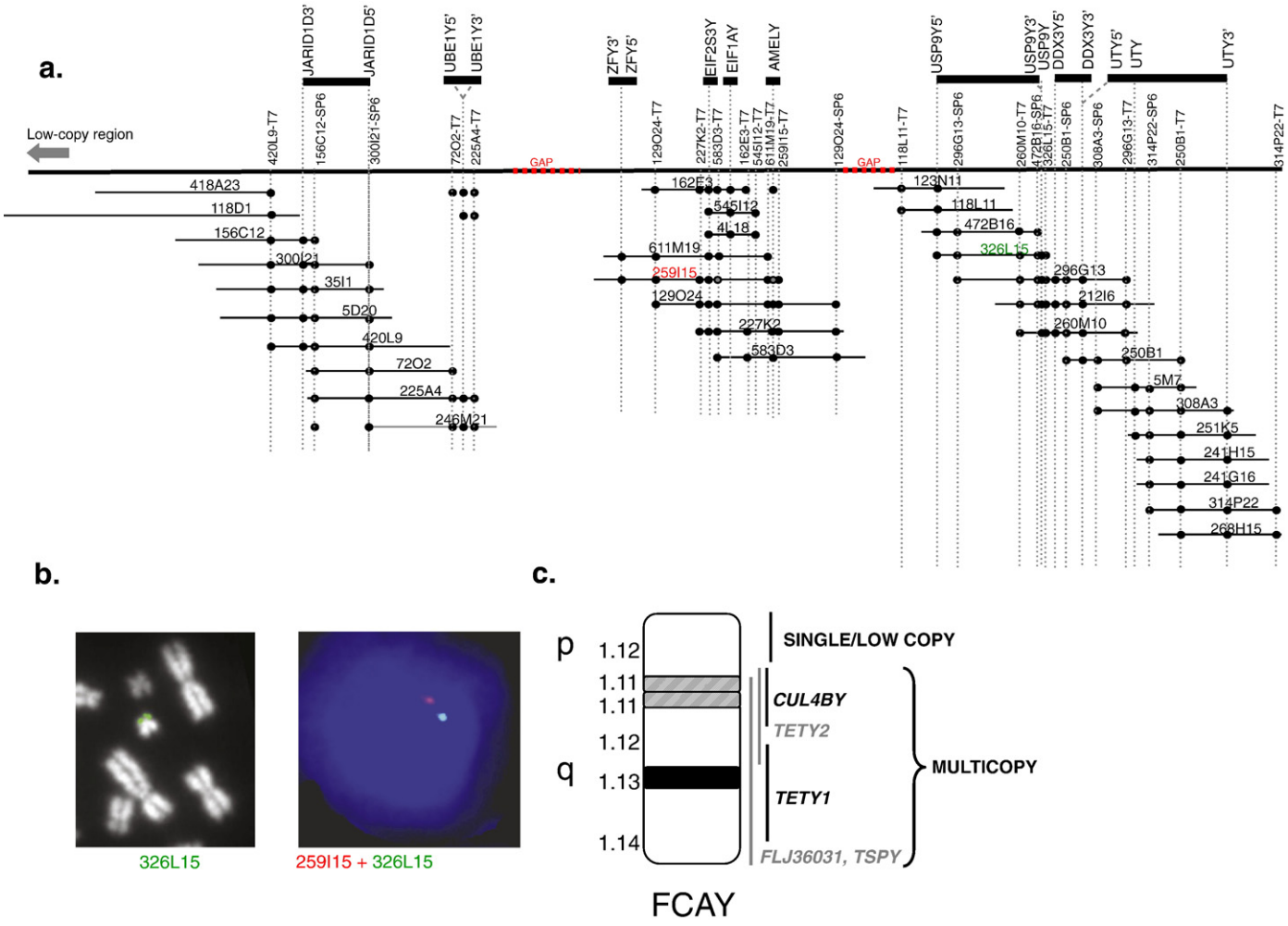


Fig. 2. (a) Physical map for the single-copy X-degenerate region of the domestic cat Y chromosome. BAC clones (represented by horizontal lines, labeled with their library address) are ordered, and overlaps estimated, from fingerprinting analysis. Results from STS-content mapping are shown with black dots. Primers are labeled by their BAC end or gene of origin. (b) Metaphase (left) FISH results are shown for BAC clone 326L15, confirming the placement of the single-copy/low-copy region on the short arm of the Y chromosome. Dual-labeled interphase FISH results (right) indicate two BAC clones, labeled in green (326L15) and red (259I15), showing single dots/signals typical of single-copy genes. (c) A schematic of the domestic cat Y chromosome shows the chromosomal assignment and approximate distribution of the different classes of single-copy X-degenerate and multicopy [12] genes.

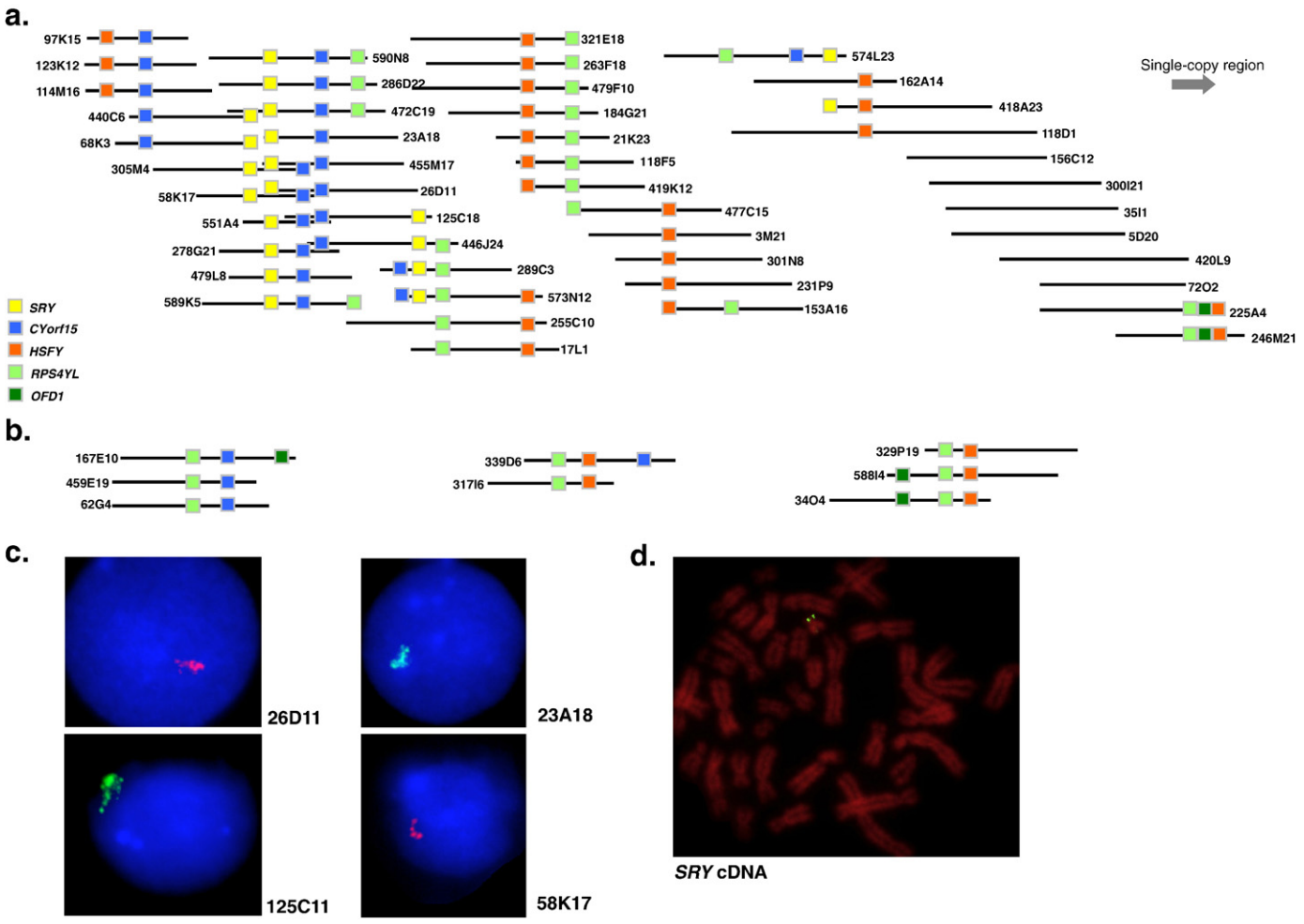


Fig. 3. Physical map for the low-copy X-degenerate region of the domestic cat Y chromosome. (a) BAC clones (represented by horizontal lines, labeled with their library address) are ordered, and overlaps estimated, from fingerprinting analysis. Results from STS-content mapping are shown with color-coded boxes, each color referring to a unique gene (see key to the left). Placement of the boxes within each clone was chosen to minimize the potential number of gene copies inferred from the data. (b) Three small contigs containing low-copy genes. (c) Interphase FISH results for four SRY-containing BAC clones showing multiple signals (dots), indicative of low-copy status, by comparison to results in Fig. 2b typical of single-copy BACs. (d) Successful FISH results using a 700-bp *SRY* cDNA probe on cat metaphase spreads are consistent with multicopy status (see text for discussion).

from these same BACs show signals consistent with a single-copy status (i.e., one or two signals/probe, Fig. 2b). We obtained no evidence that previously characterized multicopy genes (*TSPY, FLJ36031Y, TETY1, TETY2, CUL4BY*) were present in the X-degenerate BAC contigs shown in Figs. 2a and 3. This is consistent with previous FISH results that mapped these genes predominantly to the long arm of the Y chromosome [12] (Fig. 2c).

Three X-degenerate genes that are present in one (*SRY*) or two (*HSFY, CYorf15*) copies in human are present in greater than four copies in the domestic cat (Figs. 3a and 3b). These results make sense in light of published RH-mapping data, which indicated these three genes were potentially present in more than a single copy [12]. We estimate that *SRY* is present in as many as four copies based on the recovery of 20 *SRY*-positive BAC clones, approximately four times the number expected for a single-copy gene in the 10× male library (in which the Y chromosome is present at only fivefold redundancy). This number will likely be refined with future DNA sequencing efforts as BAC libraries are not always representative of gene copy number. Nonetheless, STS-content mapping data also suggest the presence of at least four nonoverlapping sets of BACs that are positive by PCR for the *SRY* coding sequence and its flanking regions: three shown in Fig. 3a and another *SRY*-containing singleton clone. DNA sequencing of *SRY*-positive PCR products from each BAC clone produced identical gene sequences that were identical to the GenBank domestic cat *SRY* reference sequence. Similar projections based on the number of positive BAC clones and clone overlaps (Figs. 3a and 3b) indicate that *CYorf15* and *HSFY* may be present in as many as six and eight copies, respectively.

FISH mapping of *SRY*-containing BAC clones onto metaphase and interphase spreads revealed multiple clustered signals on the short arm of the cat Y (Figs. 3c and 3d). As stated above, we cannot determine whether the multiple FISH signals are due to multiple gene copies or to other repetitive sequences located in the BAC clones. However, FISH mapping of a 700-bp SRY probe does produce signals on metaphase spreads (Fig. 3d). A FISH signal from such a short probe is a general indicator of multicopy status, whereas single-copy genes are typically unable to produce signals [27]. By comparison, we were unable to obtain a FISH signal using a nearly full-length (~7 kb) *USP9Y* cDNA probe on metaphase chromosomes (data not shown). Taken together, these and published FISH results suggest that very little of the cat Y chromosome may be truly single copy, except sequences immediately within and surrounding the eight putative single-copy genes (Fig. 2a).

Additional gene discovery using cDNA selection of BAC pools

Five pools of BAC clones were used in cDNA selection experiments to identify additional transcripts from these genomic contigs. Table 2

Table 2
Y chromosome gene fragments obtained by cDNA selection from BAC pools

BAC pool	Genes (No. of fragments) recovered
588I4, 34O4, 17L1	*CYorf15* (1), *HSFY* (27), *OFD1* (7), *RPS4YL* (2)
472C19, 419P21, 23A18	*CYorf15* (6), *EIF1AY* (4)
118L11, 472B16, 296G13, 250B1, 308A3	*DDX3Y* (5), *USP9Y* (15), *UTY* (3)
35I1, 246M21, 418A23	*HSFY* (8), *JARID1D* (12), *UBE1Y* (1), *RPS4YL* (2)
184G21, 129O24	*EIFS23Y* (7), *HSFY* (3)

summarizes the results of cDNA sequences obtained from five BAC pools. Using this approach, we were able to verify the existence of the majority of X-degenerate genes assigned to these BAC clones with STS-content mapping data alone. In addition, we obtained cDNA fragments similar to two known genes, *OFD1* and *RPS4Y1,* which had previously been mapped only to the human Y chromosome. *OFD1* is an X-linked gene in placental mammals, and in humans (and possibly other primates) there is a Y-linked pseudogene. *RPS4Y* is Y-linked in simian primates and marsupials, but has not been detected in other eutherians [8]. Only one of the three feline *RPS4Y*-like (*RPS4YL*) cDNA fragments aligned to a known exon of the human *RPS4Y* or *RPS4X* coding sequences (supplementary material). The remaining two aligned only to intronic regions, suggesting the gene structure and boundaries may have changed with respect to the primate genes. Transcription profiles for feline *OFD1* and *RPS4YL* genes show that both are broadly expressed in different feline tissues (Fig. 1b).

Phylogenetic analysis of X-Y gene pairs and the evolutionary strata hypothesis revisited

For each domestic cat X-Y gene pair nucleotide alignment, we obtained orthologous sequences from the human, mouse, rat, and dog genomes, plus sequences from other species in GenBank when available. We then estimated a maximum likelihood tree from the full cDNA alignments of each X-Y gene pair to assess whether X and Y genes each form monophyletic groups (Fig. 4). In nearly all cases we observed reciprocally monophyletic clusters of X and Y genes, except for *EIFS23Y, ZFY,* and *AMELY* (Fig. 4, supplementary material). This probably reflects either sequence homogenization due to gene conversion in *ZFX*/*Y* or *EIF2S3X*/*Y* [28,29] or recent movement of the pseudoautosomal boundary across the *AMELX/Y* genes [30].

For those gene pairs, or portions of genes, that show X-Y reciprocal monophyly we performed Bayesian relaxed-clock dating analyses to estimate the time of divergence of the X and Y gene lineages. Despite evidence for gene conversion based on whole-cDNA-based phylogenetic analyses, we were able to include *ZFX*/*ZFY* in this analysis because a portion of the transcript (corresponding to the 5′-most exons) produces X-Y reciprocal monophyly and does not appear to be affected by gene conversion. We were unable to generate a *SOX3* sequence from cat using RT-PCR from a variety of cat tissue cDNAs, and the gene was not intact in the cat 1.9× genome assembly.

Fig. 5 shows the estimated divergence times for each X-Y gene pair and their relationship to independent molecular divergence estimates for various mammalian lineages based on nuclear and mitochondrial gene segments. Three important observations are apparent from these results. First, point estimates for the two gene pairs assigned to Stratum 1, *SOX3/SRY* and *CUL4BX/Y,* place their divergence prior to the earliest splits between therian (marsupial and eutherian) mammals and, in the case of *CUL4BX/Y,* the origin of extant mammals. The 95% confidence intervals for both estimates overlap current molecular estimates for the origin of mammals and the subclass Theria (marsupials and eutherians), but nonetheless confirm that genes from Stratum 1 began diverging early in mammalian evolution. Second, there is no distinction between the X-Y divergence estimates for genes in Strata 2 and 3, as previously suggested based on K_S-derived divergences [4,26]. Indeed, the 95% confidence intervals of the two Stratum 2 gene pairs, *JARID1C/D* and *UBE1X/Y,* overlap completely with the different Stratum 3 genes. This implies that both classes of genes diverged around the same time, prior to the origin of eutherian mammals, but after the marsupial/eutherian split ~170–195 Mya [31–33]. Third, divergence times estimated from the *AMELX/Y* gene pairs (96 Mya, 95% CI 83–118) fit the description of a distinct evolutionary stratum that originated after the origin of placental mammals [3,5]. This result correlates well with evidence that *AMELX/Y* was the site of a former pseudoautosomal boundary that has moved at different rates in different eutherian lineages [30,34,35].

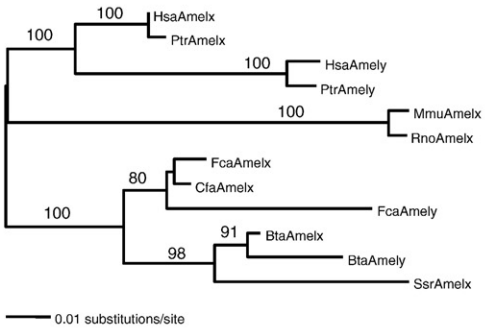
Discussion

Dynamics of X-degenerate gene birth and death

Because of the ongoing degradation of male-specific Y chromosome regions, X-degenerate gene repertoires should change over evolutionary time in a lineage-specific manner. Table 3 lists those single- or low-copy (nonampliconic) X-degenerate genes examined in this study that have been mapped in multiple mammals and are thought to have been present in the ancestral placental mammal Y chromosome [13]. The pattern of decay in different lineages is apparent, with the rate of gene loss in rodents and human being more pronounced than in the cat lineage, which has retained nearly all of the ancestral Y chromosome genes. Similarly, gene copy number has expanded in some lineages, particularly as genes acquire testis-restricted or more limited expression profiles. Examples include *UBE1Y* gene in horse (N. Paria, pers. comm.), *Zfy* in mouse, and *SRY* in rat and cat [14–19].

Because sperm defects are well described in the domestic cat and its wild relatives, we sought to determine whether any X-degenerate genes might be candidate spermatogenesis genes based on expression profiling. Our prediction was that genes that display a testis-limited expression profile are more likely to have an important role in spermatogenesis. Our results instead suggest that nearly all feline single-or low-copy X-degenerate genes are expressed in a broad array of tissues and likely perform cellular housekeeping roles, similar to their human counterparts [3,4,26]. This is in contrast to multicopy cat Y chromosome genes, and most human ampliconic Y genes, which share a testis-restricted transcription profile and remain strong candidates as fertility-related genes [4,12,26]. The exception to this dichotomy is *SRY:* we found that feline *SRY* is expressed in adult testis, as in humans [36], but it is also expressed in adult brain, similar to rodents [37,38]. From a structural standpoint, therefore, the cat Y chromosome can be divided into two functional partitions: (1) the short arm contains ubiquitously expressed single-and low-copy genes (except *SRY*), most shared by some or all placental mammals, compressed into a small physical space, and (2) the long arm contains several classes of novel testis-specific, multicopy genes that may be repeated tens to hundreds of times and are likely involved in normal spermatogenesis [12]. *SRY,* with its pericentromeric position (Fig. 3; [12]), therefore appears to be a transition between the single-and the multicopy regions.

SRY is the sex-determining gene in extant therian mammals and is found as a single-copy gene in the human and mouse Y chromosome sequences and has been assumed to be so in other eutherian mammals [13]. Our data show that *SRY* is present as multiple functional copies on the domestic cat Y chromosome and add to a growing body of evidence that *SRY* is present in multiple copies in many species, including several rodent lineages [14,15,17–19] and rabbit [16]. This implies that the single-copy status of *SRY* in human and mouse may be the exception rather than the rule in eutherians and will be informed by additional investigation into the *SRY* copy number in other eutherian mammals. From a functional standpoint it is not unexpected that *SRY,* with its critical role in sex determination, would commonly be found in multiple copies: testis-specific, ampliconic genes on the human Y chromosome have presumably amplified as a mechanism to buffer against mutations in any single copy, thus maintaining enough intact copies to perform the gene's normal function [3].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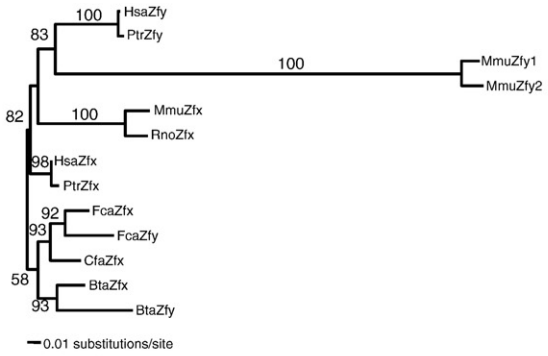
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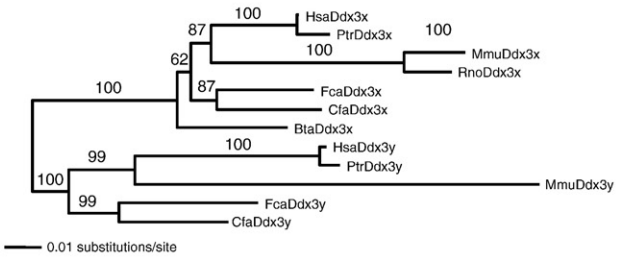
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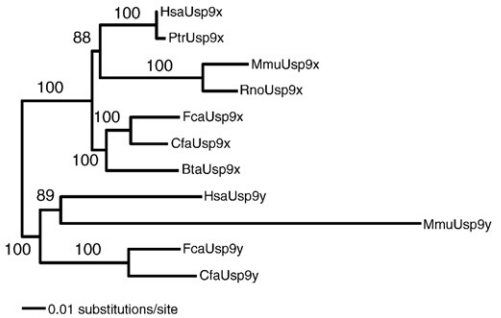
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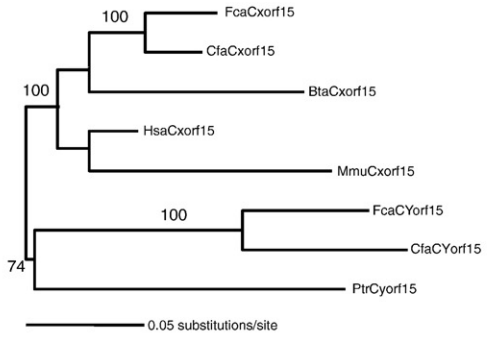
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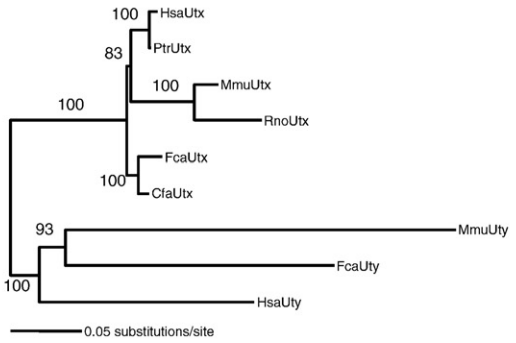
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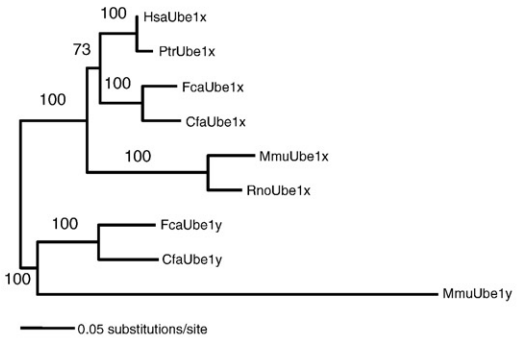
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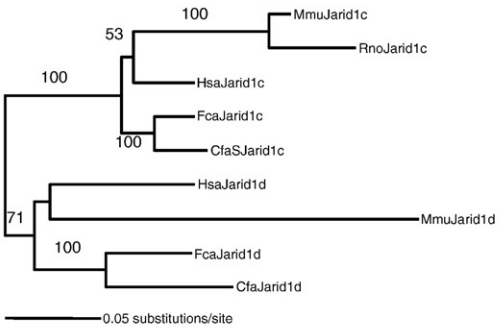
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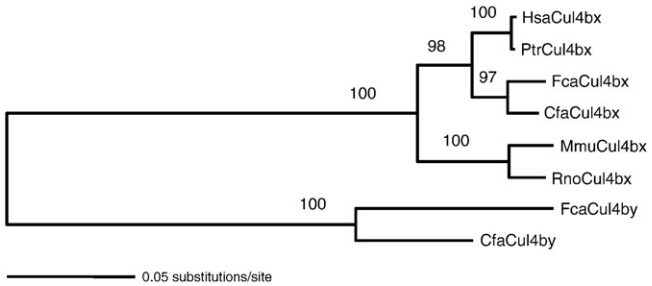
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RnoCul4bx
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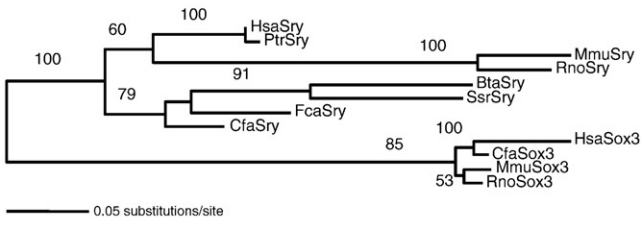
0.05 substitutions/site


HsaSry
PtrSry
MmuSry
RnoSry
BtaSry
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FcaSry
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0.05 substitutions/site

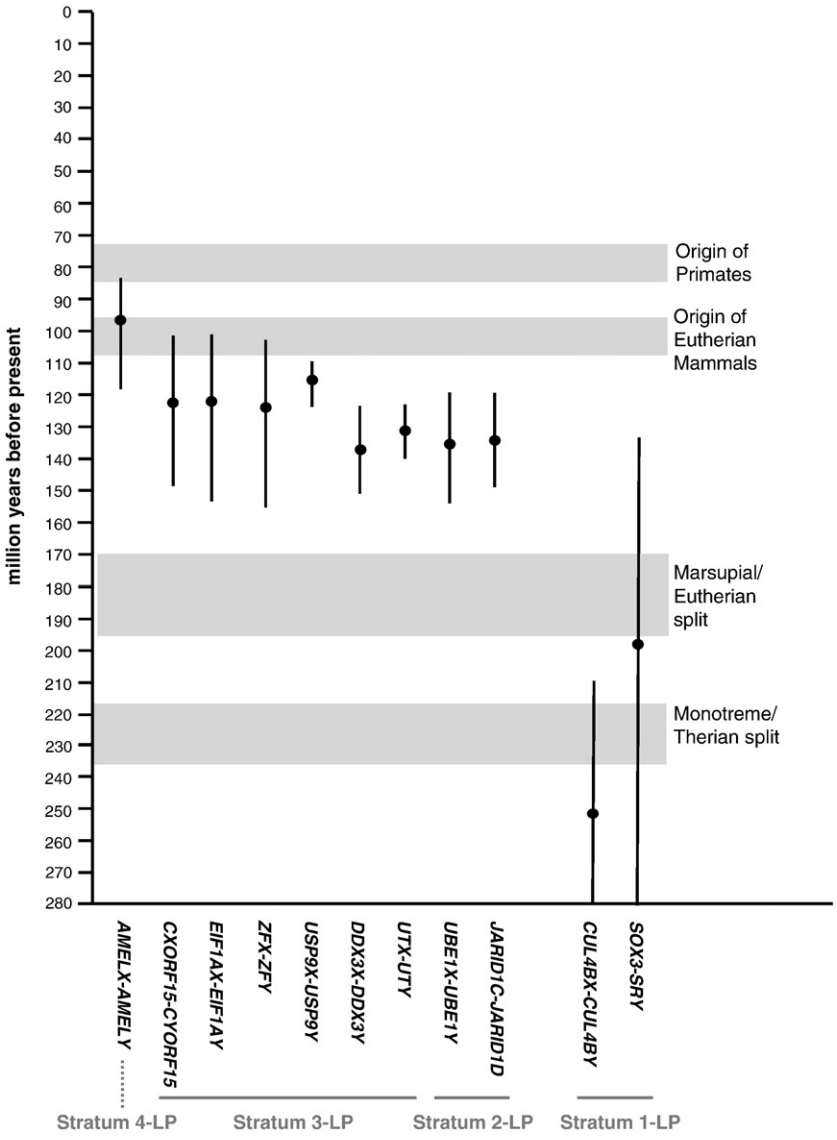

Fig. 5. Plot of molecular divergence times for X and Y gene pairs shown in Fig. 4 and supplementary material. Only those gene pairs that showed reciprocal monophyly of both X and Y gene orthologues were used for divergence time estimation. The *ZFX-ZFY* divergence is based on a 401-bp segment from the 5′ end of the transcript that produces X–Y monophyly. Black dots represent point estimates, and black vertical bars represent 95% credibility intervals from MULTIDIVTIME. Horizontal gray bars indicate presumed divergence times for different mammalian clades based on published estimates [7,31–33].

Revisiting the ages of mammalian X chromosome evolutionary strata

The identification of multiple evolutionary strata on the human X chromosome [5] has been confirmed by analysis of the complete human MSY sequence [4] and appears to be a central theme in the evolutionary process of sex chromosome degeneration across diverse organisms [39,40]. However, several factors may bias estimation of these boundaries, including (1) the number of sequences used in the calculations, (2) the number and type of calibration points, (3) the rate variation between X and Y gene copies, and (4) the assumption that K_S values are robust predictors of divergence time. Our data and previous results [2] indicate that X and Y gametologues evolve at different rates, with the Y chromosome copies evolving at a higher rate, as well as both X and Y genes evolving at different rates across eutherian lineages (Fig. 4). The recent advent of Bayesian methods that relax the assumption of a molecular clock [41], and the ability to incorporate multiple fossil calibration points, has improved estimates of mammalian divergence times [42]. We apply these methods here for the first time to the evolutionary strata hypothesis.

Our Bayesian estimates for X-Y gene divergence times support the distinctiveness of Stratum 1 genes and indicate a divergence of *SRY*/*SOX3* and *CUL4BX/Y* at 198.2 (95% CI=130–300) and 251.6 (95% CI=209–293) Mya, respectively (Fig. 5). Both estimates predate the age of origin of therian mammals, by comparison to divergence estimates obtained by independent studies of nuclear gene sequences [32,33]. Our results generally support Lahn and Page's hypothesis that the first stratum was formed early in mammalian evolution [4,5]. The short aligned region of *SRY/SOX3* will always preclude a precise estimation of their divergence time. Despite the large overlapping 95% confidence intervals, our point estimate for the *SRY/SOX3*

Fig. 4. Phylogenetic trees for aligned X and Y gene sequences from different eutherian mammals. Each tree represents the maximum likelihood (ML) tree, obtained using PAUP [52]. Hsa, *Homo sapiens* (human); Ptr, *Pan troglodytes* (chimpanzee); Fca, *Felis catus* (cat); Cfa, *Canis familiaris* (dog); Bta, *Bos taurus* (cow); Ssr, *Sus scrofa* (pig); Mmu, *Mus musculus* (mouse); Rno, *Rattus norvegicus* (rat). ML bootstrap support values ≥50%(100 replicates) are shown on each branch. The outgroups have been excluded for visual purposes. Additional gene trees are in the supplementary material.

Table 3
Presence/absence and copy number of eutherian X-degenerate genes analyzed in this study[a]

Gene	Human	Chimp	Mouse	Rat	Cat	Horse	Cow	Pig
AMELY	F-1	F-1	**A/Ps**	?	F-1	U	F-1	U
OFD1	**A/Ps**	**A/Ps**	**A/Ps**	**A/Ps**	F-M	A/Ps	A/Ps	A/Ps
CYorf15	F-2	F-2	**A/Ps**	**A/Ps**	F-M	F-1	?	?
EIF1AY	F-1	F-1	**A/Ps**	**A/Ps**	F-1	F-1	U	U
ZFY	F-1	F-1	F-2	F-1	F-1	F-1	U	U
EIF2S3Y	**A/Ps**	**A/Ps**	F-1	F-1	F-1	?	?	U
JARID1D	F-1	F-1	F-1	F-1	F-1	F-1	F-1	F-1
UBE1Y	**A/Ps**	**A/Ps**	F-1	F-1	F-2?	F-M	U	U
USP9Y	F-1	**A/Ps**	F-1	F-1	F-1	F-1	U	U
DDX3Y	F-1	F-1	F-1	F-1	F-1	F-1	U	U
UTY	F-1	F-1	F-1	F-1	F-1	F-1	U	U
RPS4Y	F-2	F-2	**A/Ps**	**A/Ps**	U-M	**A/Ps**	**A/Ps**	**A/Ps**
CUL4BY	**A/Ps**	**A/Ps**	**A/Ps**	**A/Ps**	F-M	F-M	?	?
SRY	F-1	F-1	F-1	F-M	F-M	F-1	F-?	F-?

F, functional (number of copies follows dash; M, multiple copies but exact number unknown). A/Ps, absent or detectable pseudogene. U, present on Y but functional status unknown.

[a] This list includes only those genes found in at least one other nonprimate mammalian species.

divergence (198.2 Mya) is in accord with recent comparative mapping studies of monotreme sex chromosomes, which hypothesize that, in the absence of a detectable *SRY* gene in monotremes and the autosomal location of *SOX3, SRY* likely arose after monotremes diverged from therians, but prior to the divergence of eutherians and marsupials [43,44].

Most striking, however, is the absence of statistically significant differences in genetic divergence estimates for gene pairs previously assigned to Stratum 2 and Stratum 3 (Fig. 5). This contrasts with earlier studies that claimed that Stratum 2 was formed prior to the origin of therians, but after the monotreme divergence, while Stratum 3 was formed sometime prior to the eutherian radiation [3,4,26]. Skaletsky et al. [4] noted, however, that the boundary between Stratum 2 and Stratum 3 was blurred, and this is substantiated by our findings. This is notable because both marsupials and eutherians have retained *UBE1Y* [9] and *JARID1D* ([45]—though based only on Southern blot data), despite the fact that our dating analyses indicate that the placental versions of these genes diverged long after the therian divergence and only 20–30 million years prior to the placental radiation. Our results imply that the ancient mammalian pseudoautosomal boundary moved (shrank) at different rates in marsupials and eutherians, resulting in convergent retention of Y orthologues of these two (or possibly one if *JARID1D* is nonfunctional in marsupials) genes in both therian lineages. One prediction of this hypothesis is that different eutherian orders that have undergone independent shortening of the pseudoautosomal region may have convergently retained the same, rather than random, sets of genes on the Y chromosome. The characterization of Y chromosomes from a greater diversity of eutherians, marsupials, and monotremes, will no doubt provide a more complete picture of the mechanisms of evolutionary strata formation and the timing of Y chromosome gene emergence.

Methods

Full-length cDNA sequencing of domestic cat X and Y gametologues

To generate full-length X chromosome transcripts, human and dog X chromosome cDNA sequences were queried against the cat 1.9× trace archives by BLAST. Sequence traces that matched with greater than 98% sequence similarity were used to create an initial cat sequence contig, aligned against the dog and human reference sequences. Overlapping sets of PCR primers were then designed with Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and used to obtain the full-length cDNA sequence, and verify the trace sequences, using Invitrogen's one-step RT-PCR kit. Y sequence primers were designed using alignment of feline Y cDNA fragments [12,25] and full-length human Y cDNA sequences and were mismatched with the cat X gametologue cDNA sequences. Primers were queried against the NCBI nucleotide collection with BLASTN to confirm that the closest match to the primer sequence was the target gene. All Y chromosome primers were tested in male and female domestic cat genomic DNAs to ensure Y specificity, before use in RT-PCR. RACE was then used to extend the RT-PCR-generated sequences through the 5′ and 3′ end of each gene. Reactions were performed using the GeneRacer system (Invitrogen) at the manufacturer's specifications and 1 μg of total testis RNA. Total RNA was extracted using the RNAqueous 4PCR kit (Ambion). First-round and nested gene-specific PCR primers were designed using Primer3 [46]. We used 2 μl of a 1:100 dilution of first-round product for nested PCR, incorporating a second, internal gene-specific primer. Cycling conditions for the GeneRacer reactions followed the manufacturer's suggested profiles and were modified where needed to improve specificity. Products were visualized on 1–1.5% agarose gels with ethidium bromide in 0.5× TBE buffer. PCR products containing multiple bands were gel-purified using spin columns and cloned using Invitrogen's TOPO TA Cloning Kit for Sequencing. Plasmid DNA was extracted using either Invitrogen's SNAP Miniprep Kit or Qiagen's REAL Prep 96-well Kit and digested with *Eco*RI. Plasmids containing appropriate-sized inserts were sequenced using T3 and T7 primers on an ABI 3730 sequencer. cDNA sequences were trimmed of vector and poor-quality sequences. Repetitive sequences were identified and masked by RepeatMasker (http://www.RepeatMasker.org) using the cat repeat library. The remaining sequences were assembled into contigs using Sequencher (GeneCodes). Human and dog cDNA orthologues were downloaded from GenBank and served as reference sequences in building contigs for each gene. Discontiguous MegaBLAST searches against the NCBI nucleotide databases (genome, nr, and est) and protein BLAST searches (nr, RefSeq, and SwissProt) were used to confirm sequence orthology and to confirm intron–exon boundaries.

RT-PCR-based gene expression profiling

We obtained gene expression profiles for each gene using RT-PCR on a suite of total (tRNA) or messenger (mRNA) RNAs obtained from the following panel of adult feline tissues: testis, heart, brain, thymus, muscle, lung, and kidney. RNA was isolated from each tissue using either the RNAqueous PCR kit (Ambion) or the Fast-Track mRNA isolation kit (Invitrogen). RT-PCR was performed using Invitrogen's Superscript III One-Step RT-PCR System with Platinum *Taq*, 40 pmol of each primer, and 50 ng of tRNA or mRNA. Products were visualized on 1.5% gels with ethidium bromide in 0.5× TBE buffer. When possible, primers were designed to span known introns to distinguish between genomic DNA contamination occasionally observed in RNA preparations. In cases in which genes lack introns, RT- (reverse transcriptase negative) amplifications were used as controls.

BAC library screening

Overgo primers were designed using the full-length cat Y cDNA sequences with the Overgo Maker program (http://www.genome.wustl.edu/tools/?overgo.html) and tested for gene specificity by BLAST. Overgo primers were radioactively labeled using a modification of the BACPAC hybridization protocol (http://www.chori.org/bacpac/) and used to probe high-density filters from the RPCI-86 male feline BAC library to identify clones containing the genes of interest. A 10-μl labeling reaction containing 1 μM forward primer, 1 μM reverse primer, 0.05 mM dGTP, 0.05 mM dTTP, 2 U Klenow fragment DNA polymerase, 1× DNA polymerase buffer (Roche), and 150 Ci/mmol each of [^{32}P]dATP and [^{32}P]dCTP was incubated at 37 °C for 30 min. Unincorporated nucleotides were removed using Sephadex G-10 gravity-flow columns.

The labeled overgo probes were pooled and added to a 2× hybridization solution (20× SSPE, 10% SDS, 5% milk, and 100× Denhardt's) containing 50% formamide. Filters were washed at 65 °C for 15 min in prewash solution (0.1× SSPE, 1.0% SDS) and then with a 1× prehybridization solution (20× SSPE, 10% SDS, 5% milk, 100× Denhardt's) for a further 15 min at 65 °C. Probes were hybridized onto the prepped filters at 42 °C for 16 h. After overnight hybridization, filters were washed three times for 15 min with 2× SSPE. The filters were exposed to film over intensifying screens for 2 days at −80 °C and the autoradiograms developed and scored.

BAC clone DNA isolation

DNA from clones identified as containing genes of interest was isolated using a modified large-construct extraction method. Single colonies were picked and grown in 1 ml of 2YT+12.5 mg/ml chloramphenicol for 7 h at 37 °C with 250-rpm shaking. Five hundred microliters of this starter culture was used to inoculate 50 ml of 2YT+12.5 mg/ml chloramphenicol, which was incubated for 16 h at 37 °C with 150-rpm shaking. The remainder of the starter culture was used to make glycerol stocks and stored at −80 °C. DNA was extracted from the pelleted cultures using a standard alkaline-lysis protocol (Qiagen). Extractions were resuspended in TE and treated with an RNase A/T1 enzyme (Fermentas) at 37 °C for 1 h. Samples were then cleaned using a standard phenol–chloroform extraction and ethanol precipitated, and the resulting DNA pellets were rehydrated in water at room temperature for a minimum of 48 h.

BAC-end sequencing and fingerprinting

BAC-end sequencing was performed using a modified version of a protocol published in [47]. Samples from glycerol stocks of the BACs of interest were used to inoculate 1 ml 2× LB+12.5 mg/ml chloramphenicol in a 96-well deep-well plate and incubated at 37 °C for 21 h at 320 rpm. Three microliters of preculture was used to inoculate 1.2 ml 2× LB+12.5 mg/ml chloramphenicol in a 96-well deep-well plate, and these cultures were incubated at 37 °C for 17 h with shaking at 320 rpm. Cells were pelleted by centrifugation and DNA was extracted using Qiagen's REAL Prep 96-well kit. The DNA of selected BACs was extracted and the BACs were individually fingerprinted as described in [48]. Fingerprint overlap was established and BAC clone order derived with FPC [49].

STS-content mapping

Identified BAC clones were screened with STS markers from known cat Y chromosome genes [12,25]. The number of primer pairs used varied by the length of the gene involved, but at least two marker pairs were used per gene, usually derived from the 5′ and 3′ ends of the full-length cDNA sequences. In most cases these primers spanned introns to rule out amplification of processed pseudogenes. PCRs were performed in 10 μl total volume with 0.25 units of RedTaq polymerase (Sigma-Aldrich), 4 pmol of each primer, and 10 ng of extracted BAC DNA. Reactions were run with an initial hot start of 1 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 15 s at 58 °C, and 90 s at 72 °C, and completed with a final extension for 5 min at 72 °C. Products were visualized on 1% agarose gels with ethidium bromide in 0.5× TBE buffer and scored for presence/absence.

Direct cDNA selection

cDNA selection was performed following the protocol of Del Mastro and Lovett [50], described in Murphy et al. [12]. Briefly, after a prehybridization step with domestic cat Cot-1 DNA to block repetitive elements, adult domestic cat testis cDNA was hybridized to biotin-labeled pools of DNA from separate BAC contigs. PCR-amplified primary selected cDNA was purified and then subjected to a second round of selection. The final secondary selected PCR-amplified cDNAs were cloned en masse into the TOPO-TA cloning vector (Invitrogen). Plasmid DNA was isolated from approximately 48 clones per BAC pool as described and sequenced using universal vector sequencing primers.

Fluorescence in situ hybridization

BAC or cDNA clones were labeled with biotin and/or digoxigenin (Bio-Nick and Dig-Nick kits; Roche Molecular Biochemicals) and hybridized to male cat metaphase or interphase chromosomes. Images were captured and analyzed with a Zeiss Axioplan2 fluorescence microscope equipped with Cytovision/Genus version 2.7 (Applied Imaging).

Phylogenetic analysis

Nucleotide and amino acid sequences of X and Y gene orthologues were retrieved from GenBank for human, chimpanzee, mouse, rat, dog, cattle, and relevant outgroups (opossum, platypus, chicken, zebrafish). Dog Y chromosome sequences were largely assembled from testis EST sequences that were verified as male-specific by PCR testing with male and female genomic DNAs. Sequences were aligned with CLUSTAL-X [51] and then adjusted by eye. Regions of ambiguous alignment were excluded from phylogenetic analyses. Sequences were analyzed with neighbor-joining and maximum likelihood (ML) in PAUP* [52]. Heuristic searches were performed for each X-Y gene data set by using starting neighbor-joining trees, followed by tree-bisection reconnection branch swapping. Models of sequence evolution for each gene were estimated using Modeltest [53] and then optimized after subsequent likelihood searches in PAUP*. One hundred heuristic bootstrap replicates were performed for each data set under the same conditions as the ML analysis.

X–Y divergence time estimation

Divergence dates and 95% confidence intervals were estimated for each reciprocally monophyletic X–Y gene pair using a Bayesian approach for estimating posterior probabilities of divergence times [41]. Trees were rooted with either marsupial or, in cases in which the eutherian X–Y gene is also X-linked in marsupials, other vertebrate autosomal homologues of each X–Y gene pair, though these out-group taxa were removed in the final analysis. Branch lengths for the input topologies were estimated with the *estbranches* program [41]. In each case, the maximum likelihood topology was used, with nodes constrained where necessary (i.e., in cases of insufficient sequence variation to recover known superordinal clades accurately) to reflect current consensus on mammalian interordinal relationships [42]. Divergence times were estimated using the program *multidivtime.* Markov chain Monte Carlo analyses were run for 1 million generations, including a burn-in of 100,000 generations, to allow Markov chains to approach stationarity. States were sampled every 100 generations. We used the following 95% confidence intervals of eutherian divergence dates from previous molecular studies of large nuclear gene concatenations [7] as constraints on in-group nodes (upper and lower, respectively): 94–81 Mya for Primates–Rodentia (Euarchontoglires), 60–50 Mya for Carnivora, 101–88 Mya for Euarchontoglires–Carnivora, and 21–13 Mya for mouse–rat. A minimum age for the therian divergence was set at 173 Mya [31]. The prior for the in-group root was varied depending upon which gene pairs were analyzed. For Stratum 1 gene pairs, which presumably originated before mammals diverged [5], the prior was set at 227 Mya (based on the average age of the origin of extant mammals estimated from nuclear gene data sets [32,33]). For Stratum 2 and Stratum 3 gene pairs, which presumably originated around or after the therian divergence [5], the prior was set at 182 Mya based on the average age of the therian divergence from nuclear gene data sets [31–33].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygeno.2008.06.012.

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