LETTER

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Strict evolutionary conservation followed rapid gene loss on human and rhesus Y chromosomes

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The human X and Y chromosomes evolved from an ordinary pair of autosomes during the past 200–300 million years[1–3]. The human MSY (male-specific region of Y chromosome) retains only three percent of the ancestral autosomes' genes owing to genetic decay[4,5]. This evolutionary decay was driven by a series of five 'stratification' events. Each event suppressed X–Y crossing over within a chromosome segment or 'stratum', incorporated that segment into the MSY and subjected its genes to the erosive forces that attend the absence of crossing over[2,6]. The last of these events occurred 30 million years ago, 5 million years before the human and Old World monkey lineages diverged. Although speculation abounds regarding ongoing decay and looming extinction of the human Y chromosome[7–10], remarkably little is known about how many MSY genes were lost in the human lineage in the 25 million years that have followed its separation from the Old World monkey lineage. To investigate this question, we sequenced the MSY of the rhesus macaque, an Old World monkey, and compared it to the human MSY. We discovered that during the last 25 million years MSY gene loss in the human lineage was limited to the youngest stratum (stratum 5), which comprises three percent of the human MSY. In the older strata, which collectively comprise the bulk of the human MSY, gene loss evidently ceased more than 25 million years ago. Likewise, the rhesus MSY has not lost any older genes (from strata 1–4) during the past 25 million years, despite its major structural differences to the human MSY. The rhesus MSY is simpler, with few amplified gene families or palindromes that might enable intrachromosomal recombination and repair. We present an empirical reconstruction of human MSY evolution in which each stratum transitioned from rapid, exponential loss of ancestral genes to strict conservation through purifying selection.

The human Y chromosome no longer engages in crossing over with its once-identical partner, the X chromosome, except in its pseudoautosomal regions. During evolution, X–Y crossing over was suppressed in five different chromosomal regions at five different times, each probably resulting from an inversion in the Y chromosome[2,3]. Each of these regions of the Y chromosome then began its own individual course of degeneration, experiencing deletions and gene loss. Comparison of the present-day X and Y chromosomes enables identification of these five evolutionary 'strata' in the MSY (and X chromosome); their distinctive degrees of X–Y differentiation indicate their evolutionary ages[2,3]. The oldest stratum (stratum 1) dates back over 240 million years (Myr)[2] and is the most highly differentiated, and the youngest stratum (stratum 5) originated only 30 Myr ago and displays the highest X–Y nucleotide sequence similarity within the MSY[3]. The five strata and their respective decay processes, over tens to hundreds of millions of years of mammalian evolution, offer replicate experiments of nature from which to reconstruct the trajectories and kinetics of gene loss in the MSY.

Only the human and chimpanzee MSYs had been sequenced before the present study, and they are separated by just 6 Myr of evolution. We decided to examine the MSY of a much more distant relative, the rhesus macaque (*Macaca mulatta)*, to enable us to reconstruct gene loss and conservation in the MSY during the past 25 Myr. We sequenced the rhesus MSY using bacterial artificial chromosome (BAC) clones and the SHIMS (single-haplotype iterative mapping and sequencing) strategy that has previously been used in the human and chimpanzee MSYs[4,11–13] as well as in the chicken Z chromosome[5]. The resulting sequence is comprised of 11.0 megabases (Mb), is complete aside from three small gaps and has an error rate of about one nucleotide per Mb. We ordered and oriented the finished sequence contigs by fluorescence *in situ* hybridization and radiation hybrid mapping (Supplementary Figs 1–6, Supplementary Table 1, Supplementary Files 1, 2 and Supplementary Note 1).

We then compared the structure of the rhesus Y chromosome to that of the human and chimpanzee (Fig. 1). The rhesus Y chromosome has virtually no heterochromatin apart from the centromere, and the euchromatic segment of the MSY is notably smaller compared to that of the human and chimpanzee (Fig. 1). The single pseudoautosomal region (PAR) in rhesus corresponds to the short-arm PAR in human and to the single PAR in chimpanzee. The precise boundary between PAR and MSY is identical in the three species (Supplementary Fig. 7), confirming that stratification in all three lineages concluded before the divergence of apes from Old World monkeys.

The euchromatic portions of the rhesus, human and chimpanzee MSYs are comprised primarily of two distinct sequence classes: X-degenerate and ampliconic. The X-degenerate regions, relics of shared X–Y ancestry, are dotted with single-copy homologues of X-linked genes. The X-degenerate regions are relatively well conserved among the rhesus, human and chimpanzee MSYs, with large blocks of homology that are readily identifiable (Supplementary Figs 8 and 9). Indeed, the X-degenerate regions are the only portions of the rhesus and human MSYs whose sequences can be aligned over distances of greater than 50 kb. We found rhesus–human nucleotide divergence there to be 9.4% (Supplementary File 3). This is markedly higher than the 6.5% divergence that is observed when the rhesus and human female genomes are compared[14]. The difference probably reflects the restriction of the MSY to the male germ line, where base-pair substitutions are more frequent than in the female germ line[15]. From these data, we calculate the male-to-female mutation rate ratio (α_m) to be 2.78 (95% confidence interval 2.74–2.81), in agreement with previous but less precise estimates[14,16]. The X-degenerate sequences in rhesus, human and chimpanzee are not entirely colinear, as large-scale rearrangements have occurred in each lineage (Supplementary Figs 8–10).

For all three species, the MSY's ampliconic regions are composed of long, nearly identical repeat units that are arrayed in either direct or

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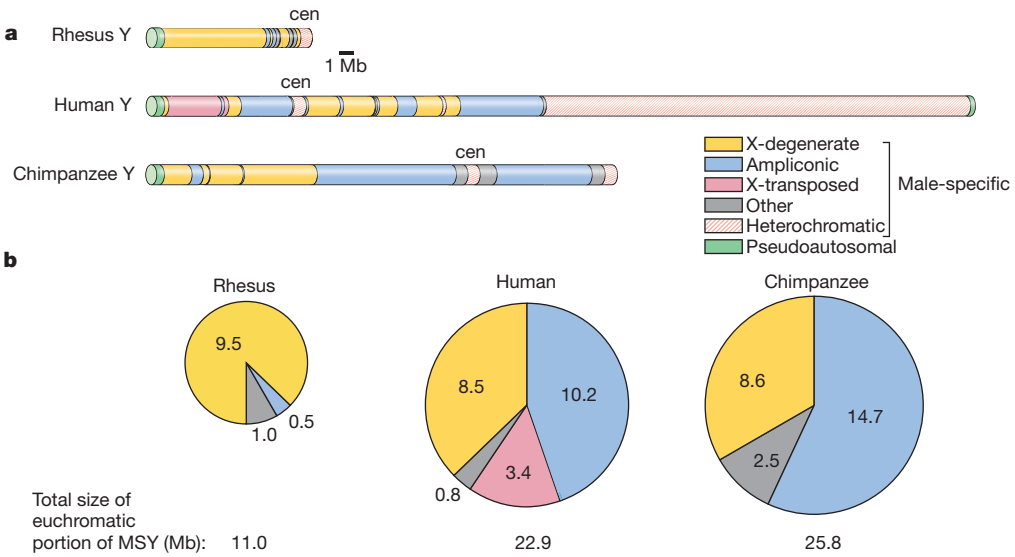

Figure 1 | Comparison of rhesus, human and chimpanzee Y chromosomes. **a**, Schematic representations of rhesus, human and chimpanzee Y chromosomes, to scale. Other, single-copy, male-specific sequences that are neither X-degenerate nor X-transposed. **b**, Sizes (in Mb) of euchromatic sequence classes in MSYs. cen, centromere.

inverted orientation and undergo frequent gene conversion—a process that is thought to slow or prevent the decay of genes that reside there[4,17]. Ampliconic genes display testis-specific expression patterns, consistent with their having critical roles in spermatogenesis[4,11,18]. Only 0.5 Mb of the rhesus MSY euchromatin is ampliconic, compared to 10.2 Mb and 14.7 Mb in human and chimpanzee, respectively (Fig. 1, and Supplementary Figs 11 and 12). In human and chimpanzee, the ampliconic regions of the MSY feature large palindromes, each composed of two inverted repeats (arms) separated by a short spacer. The human and chimpanzee MSYs have 8 and 19 palindromes that span 5.5 Mb and 7.5 Mb, respectively[4,13]. By contrast, the rhesus MSY has only three palindromes and these collectively span 437 kb (Supplementary Table 2 and Supplementary Fig. 13). Two of the rhesus MSY palindromes are orthologues of human MSY palindromes, demonstrating that these structures have been maintained for at least 25 Myr (Supplementary Fig. 13).

We identified protein-coding genes in the rhesus MSY using three complementary approaches. First, we electronically searched the rhesus MSY for homologues of all known human and chimpanzee MSY genes and pseudogenes. Second, we searched for homologues of all known human X-linked genes, to identify any X–Y shared genes that had been lost in both the human and chimpanzee MSY but retained in the rhesus MSY. Third, we searched for additional rhesus-specific MSY genes using electronic prediction tools and high-throughput sequencing of rhesus testis complementary DNA (245 Mb in total). We validated each putative gene by verifying transcriptional activity (Supplementary Fig. 14) and, where applicable, by comparing its predicted open reading frame to that of its human orthologue (Supplementary Table 3).

We then compared the catalogues of MSY genes in rhesus, human[4] and chimpanzee[13] to infer gene loss and conservation during the past 25 Myr. To root this analysis in a deep evolutionary context, we first reconstructed which of the modern rhesus MSY genes were present on the common autosomal ancestor of X and Y (Fig. 2, Supplementary Table 4 and Supplementary Note 2). Most 'ancestral' MSY genes would be expected to have a homologue both on the human X chromosome and on the chicken autosomes (chromosomes 1 and 4) that share common ancestry with mammalian X and Y chromosomes[3,5]. Indeed, 33 genes and pseudogenes in the rhesus, human or chimpanzee MSY have their most closely related human homologues on the X chromosome (Fig. 2), and 29 of these also have homologues within syntenic regions of chicken chromosome 1 or 4. Analyses of a more distant outgroup, *Xenopus tropicalis*, revealed that two of the four rhesus MSY genes lacking homologues on chicken chromosome 1 and 4 (*TSPY* and *AMELY*) are X–Y ancestral; they were lost in the chicken lineage after divergence from mammals (Supplementary Note 2). A few human MSY genes with X homologues are recent additions to the MSY rather than remnants of the ancestral autosome pair; *PCDH11Y* and *TGIF2LY* are located in the human-specific X-transposed region[4], and the X-linked homologue of *VCY* is found only in simian primates[19]. We found a total of 30 ancestral MSY genes and pseudogenes in rhesus, human or chimpanzee (Fig. 2).

Within strata 1–4, which collectively comprise the bulk of the human MSY, the rhesus and human MSYs possess precisely the same 18 ancestral genes (Fig. 2). This notable and unanticipated identity leads us to conclude that, 25 Myr ago, in the last common ancestor of rhesus and human, MSY strata 1–4 also carried these 18 ancestral genes (Table 1 and Supplementary Table 5), and that no loss of ancestral genes occurred subsequently in either lineage (Supplementary Note 3). We note that, within strata 3 and 4, the rhesus and human MSYs carry a total of six ancestral pseudogenes that seem to have lost their function more than 25 Myr ago (Supplementary Fig. 15).

The evolutionary stability of ancestral genes in strata 1–4 could be explained by purifying selection, which, in the absence of sexual recombination, would have preserved critical ancestral genes for tens or even hundreds of millions of years. We demonstrated previously that purifying selection preserved MSY genes during the past 100,000 years of human population expansion and migration[20]. Comparing human and rhesus, we find that most ancestral genes display a ratio of nonsynonymous substitution rate to synonymous substitution rate that is significantly less than one (Supplementary Note 4, Supplementary Table 3 and Supplementary Fig. 16), demonstrating purifying selection during the past 25 Myr.

The pattern of gene loss and conservation in stratum 5, formed only 5 Myr before the rhesus and human lineages split, is remarkably different from the pattern in the four older strata. Within the past 30 Myr, four ancestral genes have been inactivated or deleted from stratum 5 of the MSY in both rhesus and human (Fig. 2 and Supplementary Note 5). A fifth ancestral gene, *MXRA5Y*, remains active in rhesus (Supplementary Fig. 11) but was inactivated by an intragenic deletion in the human lineage (Supplementary Fig. 17). Apart from *MXRA5Y*, all differences in MSY gene content between rhesus and human involve genes that were added to the human MSY subsequent to the ape–Old World monkey split (Fig. 2 and Supplementary Table 5).

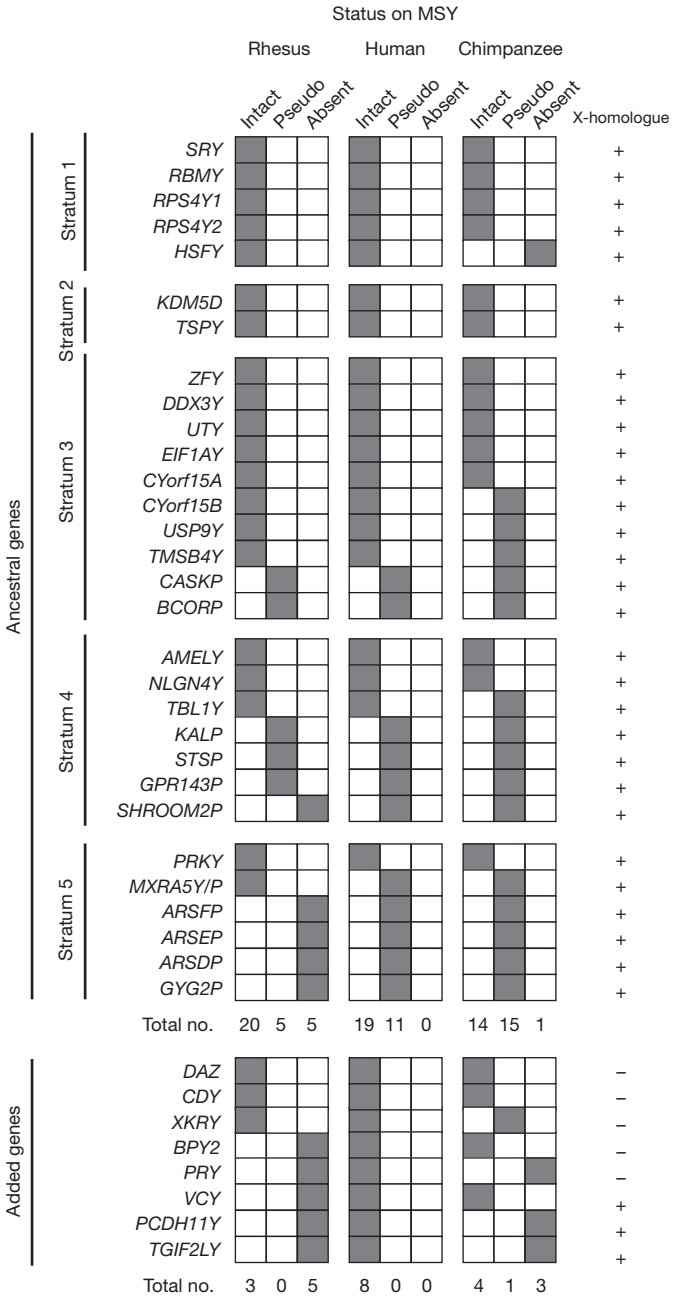

Figure 2 | Inventories of genes, both ancestral and added, in rhesus, human and chimpanzee MSYs. Ancestral genes grouped by stratum (1–5). In rhesus, human and chimpanzee, current status of each MSY gene is indicated by shading in one of three columns: present and intact, inactivated pseudogene, or absent or deleted. Total numbers of intact genes, pseudogenes (pseudo), and absent genes—both ancestral and added—are tallied for each species. For each MSY gene, whether the most closely related human homologue is located on the X chromosome is shown (right).

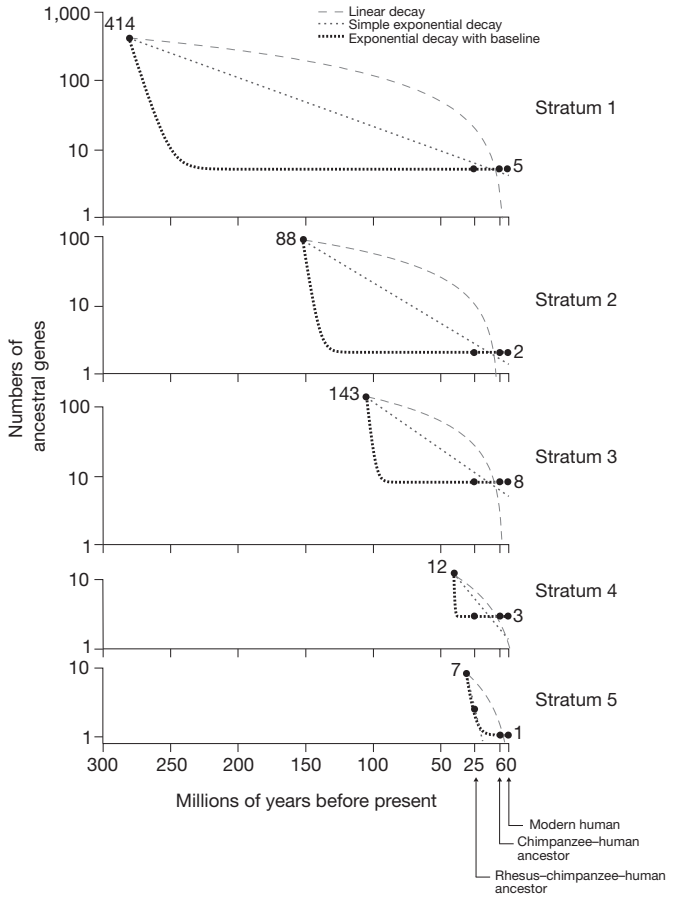

Figure 3 | Kinetics of ancestral gene loss during evolution of five human MSY strata. Gene numbers are plotted on a log scale on the *y* axis, and time (in Myr before present) is plotted on the *x* axis. Filled circles show inferred or observed gene numbers in (from left to right) X–Y ancestral chromosome (at time of stratum formation), rhesus–chimpanzee–human ancestral MSY (25 Myr ago), chimpanzee–human ancestral MSY (6 Myr ago), and modern human MSY. Dotted and dashed lines represent best-fit curves to data points using each of three decay models as indicated.

Returning to strata 1–4, we note that five ancestral genes have been inactivated or lost from the chimpanzee MSY during the past 6 Myr[12,13], in sharp contrast to the strict conservation of ancestral gene content in rhesus and human (Fig. 2). We previously proposed that in the chimpanzee lineage promiscuous mating behaviour[21], sperm competition and intense sexual selection that focused on the MSY drove rapid evolution and amplification of MSY sequences that are associated with spermatogenesis[12,13]. Furthermore, we speculated that in the chimpanzee lineage inactivated alleles of some ancestral genes became fixed in the population through 'genetic hitchhiking'; casualties of positive but indiscriminate selection operating in the absence of sexual recombination in the MSY[12,13,22]. Among primate species, chimpanzees have a high testis-weight to body-weight ratio, a useful indicator of the degree of sperm competition[23,24]. Although the rhesus

Table 1 | Stratification of X–Y ancestral gene loss in primate MSYs

	Age of stratum (millions of years) (from refs 2, 3)	Number of ancestral genes on human X chromosome*	Number of ancestral genes on MSY			
			Last common ancestor†	Rhesus	Human	Chimpanzee
Stratum 1	240–320	414	5	5	5	4
Stratum 2	130–170	88	2	2	2	2
Stratum 3	80–130	143	8	8	8	5
Stratum 4	38–44	12	3	3	3	2
Stratum 5	29–32	7	2–7	2	1	1

* Gene numbers from ref. 5, Supplementary Table 4 and Supplementary Note 2.
† Gene counts in MSY of a hypothetical rhesus–human–chimpanzee ancestor deduced from observed gene counts in extant species.

is similarly promiscuous and has an even higher testis-weight to body-weight ratio, the rhesus MSY shows little evidence of intense sexual selection. We suggest that in the rhesus lineage, such selection was focused on spermatogenesis factors that are encoded elsewhere in the genome. This would also account for the virtual absence in rhesus of the MSY sequence amplification that is prominent in human and even more pronounced in chimpanzee (Fig. 1).

Our knowledge of all five strata of the MSY, gained through our comprehensive comparisons of ancestral gene content in the rhesus, human and chimpanzee MSYs, enabled us to reconstruct the kinetics and trajectory of human MSY evolution. For each of the five MSY strata, we estimated ancestral gene numbers at three points in the human evolutionary lineage: in the last common ancestor of human and chimpanzee (6 Myr ago), in the last common ancestor of human and rhesus macaque (25 Myr ago) and at the time of the stratum's formation, when X–Y differentiation was initiated (from ~30 to >240 Myr ago; Table 1). For each stratum, we plotted these three estimated numbers against evolutionary time, together with the observed number of ancestral genes in modern human, and fit a curve (Fig. 3 and Supplementary Fig. 18). For each of the five strata, a simple two-parameter model, using an exponential decay equation that includes a baseline constant, provides an excellent fit to our data (Fig. 3 and Supplementary Table 6). According to this reconstruction, ancestral gene decay within each stratum proceeded rapidly at first—with an ancestral gene half-life of less than 5 Myr (Supplementary Table 6)—but then decelerated markedly, as the ancestral gene count reached a stable level far below its starting point. In our reconstruction, strata 1–4 had already reached a stable level before the human lineage diverged from rhesus; after divergence from rhesus, gene loss in the human lineage was limited to stratum 5, the youngest stratum, which stabilized before the human lineage diverged from chimpanzee.

Our empirical reconstruction of MSY evolution is at odds with a linear model[7,9,10] and with a simple random decay (exponential) model[25], both of which project a continual decline of MSY gene content and cannot account for the stability of gene content that we observe over the past 25 Myr (Fig. 3). Our data are better explained by more complex models for MSY gene loss that incorporate a combination of evolutionary forces[26]. Sequencing additional Y chromosomes from animals that represent more divergent mammalian lineages will enable refinement of our reconstruction of MSY gene kinetics in the human lineage.

METHODS SUMMARY

BAC selection and sequencing. The SHIMS (single-haplotype iterative mapping and sequencing) strategy[11] was used to assemble a path of sequenced clones selected from the CHORI-250 BAC library (http://bacpac.chori.org) and a custom BAC library (RMAEX) constructed by Amplicon Express (http://www.genomex.com).

Fluorescence *in situ* hybridization analysis. Assays were performed on rhesus fibroblast cell line PR00112 from Coriell Institute for Medical Research (http://ccr.coriell.org). Extended metaphase fluorescence *in situ* hybridization (FISH) and interphase FISH were performed as previously described[27].

Radiation hybrid mapping. Nine sequence-tagged site (STS) markers (Supplementary Table 7) were tested on a 10,000-rad panel consisting of 185 hybrid clones[28]. A genetic map was constructed and analysed statistically using RHMAPPER 1.22 (ref. 29).

Generation of complementary DNA for polymerase chain reaction with reverse transcription (RT–PCR) and 454 sequencing. cDNA was generated from total RNA that was isolated from male rhesus tissues using the RNeasy kit (Qiagen). For 454 sequencing, cDNA was normalized using the Trimmer kit (Evrogen).

Alignments and dot plots. Rhesus and human Y sequences were aligned using Stretcher (http://bioweb2.pasteur.fr/docs/EMBOSS/stretcher.html) with a gap open penalty of 20 and a gap extend penalty of 1. Dot-plot analyses were performed using custom Perl codes (http://jura.wi.mit.edu/page/papers/Hughes_et_al_2005/tables/dot_plot.pl).

Calculation of α_m. The male-to-female mutation rate ratio was calculated from the human–rhesus Y divergence rate and the human–rhesus autosomal divergence rate using a previously described method[15,30].

Modelling ancestral MSY gene loss. We fit a one-phase exponential decay model with a baseline constant (shown below) to our data (gene numbers shown in Table 1) using nonlinear regression analysis in GraphPad Prism 5.0. Parameters for each stratum are given in Supplementary Table 6.

One-phase exponential decay model:

$$N(t) = (N_0 - b)e^{-Kt} + b$$

Where $N(t)$ is the number of genes at time t, N_0 is the number of genes within given stratum in ancestral autosomal or pseudoautosomal portion of genome, K is the decay constant and b is the baseline (approximated by the number of active ancestral genes within that stratum on human Y chromosome).

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions J.F.H., H.Sk., W.C.W., S.R., R.A.G., R.K.W. and D.C.P. planned the project. J.F.H., H.Sk., L.G.B., T.J.C. and N.K. performed BAC mapping, radiation hybrid mapping and real-time polymerase chain reaction analyses. T.G., R.S.F., S.D., Y.D., C.J.B., C.K., Q.W., H.Sh., M.H., D.V., L.V.N., A.C., L.C., J.V., H.K. and D.M.M. were responsible for BAC sequencing. J.F.H. and H.Sk. performed comparative sequence analyses. T.P. performed FISH analyses. J.F.H. and D.C.P. wrote the paper.

Author Information cDNA sequences of rhesus Y genes have been deposited in GenBank (http://www.ncbi.nlm.nih.gov) under accession numbers FJ527009–FJ527028 and FJ648737–FJ648739. 454 testis cDNA sequences have been deposited in GenBank under accession number SRA039857. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to J.F.H. (jhughes@wi.mit.edu).

METHODS

BAC selection and sequencing. The SHIMS (single-haplotype iterative mapping and sequencing) strategy[11] was used to assemble a path of sequenced clones selected from the CHORI-250 BAC library (http://bacpac.chori.org) and a custom BAC library (RMAEX) constructed by Amplicon Express (http://www.genomex.com). The rate of error in the finished sequence was estimated by counting mismatches in overlapping clones.

FISH analysis. All assays were performed on rhesus fibroblast cell line PR00112 obtained from the Coriell Institute for Medical Research (http://ccr.coriell.org). Extended metaphase FISH and interphase FISH were performed as previously described[27].

Radiation hybrid mapping. Nine STS markers (Supplementary Table 7) were tested on a 10,000-rad, male whole-genome panel consisting of 185 hybrid clones[28]. The average retention frequency of the markers tested was 16%, ranging from 10–27%. A genetic map was constructed and analysed statistically using RHMAPPER 1.22 (ref. 29).

RT–PCR. Total RNA was isolated from male rhesus tissues (brain, prostate, liver, lung and spleen testis; Alpha Genesis) using the RNeasy kit (Qiagen) and cDNA was generated. RT–PCR primer sequences and product sizes are listed in Supplementary Table 8.

454 sequencing of testis cDNA. Rhesus testis cDNA was generated from total RNA isolated using the RNeasy kit (Qiagen). The cDNA was normalized using the Trimmer kit (Evrogen) and sequenced on a 454 FLX Titanium machine.

Alignments and dot plots. Rhesus and human Y sequences were aligned using Stretcher (http://bioweb2.pasteur.fr/docs/EMBOSS/stretcher.html) with a gap open penalty of 20 and a gap extend penalty of 1. Dot plot analyses were performed using custom Perl codes (http://jura.wi.mit.edu/page/papers/Hughes_et_al_2005/tables/dot_plot.pl).

Calculation of α_m. The male-to-female mutation rate ratio was calculated using the human–rhesus Y divergence rate (9.40%, 312,840 substitutions per 3,330,847 sites examined) and the human–rhesus autosomal divergence rate (1.385×10^8 substitutions per 2.248×10^{10} sites examined; hg18-rheMac2 alignments downloaded from http://www.genome.ucsc.edu). Miyata's formula was then used to calculate α_m (refs 15, 30):$Y/A = 2\alpha_m/(1 + \alpha_m)$. Confidence intervals for ratios of divergence rates were calculated as previously described[30].

Modelling ancestral MSY gene loss. We modelled the numbers of ancestral genes within individual MSY strata as a function of time in millions of years before the present by fitting a one-phase exponential decay model with a baseline constant (below) to our data (gene numbers shown in Table 1) using nonlinear regression analysis in GraphPad Prism 5.0. Parameters for each stratum are given in Supplementary Table 6.

One-phase exponential decay model:

$$N(t) = (N_0 - b)e^{-Kt} + b$$

Where $N(t)$ is the number of genes at time t, N_0 is the number of genes within a given stratum in the ancestral autosomal or pseudoautosomal portion of genome, K is the decay constant and b is the baseline (approximated by the number of active ancestral genes within that stratum on human Y chromosome).