

Eighty million years of rapid evolution of the primate Y chromosome

Received: 1 July 2022

Accepted: 15 December 2022

Published online: 02 June 2023

 Check for updates

Yang Zhou¹, Xiaoyu Zhan¹, Jiazheng Jin¹, Long Zhou^{2,3}, Juraj Bergman^{4,5}, Xuemei Li^{1,6}, Marjolaine Marie C. Rousselle⁵, Meritxell Riera Belles⁵, Lan Zhao⁷, Miaoquan Fang¹, Jiawei Chen¹, Qi Fang¹, Lukas Kuderna⁸, Tomas Marques-Bonet^{8,9,10,11}, Haruka Kitayama¹², Takashi Hayakawa^{13,14}, Yong-Gang Yao^{15,16,17,18,19}, Huanming Yang^{1,6,20,21,22}, David N. Cooper²³, Xiaoguang Qi⁷, Dong-Dong Wu^{17,18,19,24}, Mikkel Heide Schierup⁵ & Guojie Zhang^{2,3,24,25} ✉

The Y chromosome usually plays a critical role in determining male sex and comprises sequence classes that have experienced unique evolutionary trajectories. Here we generated 19 new primate sex chromosome assemblies, analysed them with 10 existing assemblies and report rapid evolution of the Y chromosome across primates. The pseudoautosomal boundary has shifted at least six times during primate evolution, leading to the formation of a Simiiformes-specific evolutionary stratum and to the independent start of young strata in Catarrhini and Platyrrhini. Different primate lineages experienced different rates of gene loss and structural and chromatin change on their Y chromosomes. Selection on several Y-linked genes has contributed to the evolution of male developmental traits across the primates. Additionally, lineage-specific expansions of ampliconic regions have further increased the diversification of the structure and gene composition of the Y chromosome. Overall, our comprehensive analysis has broadened our knowledge of the evolution of the primate Y chromosome.

Sex chromosomes usually carry the master genes that determine sex and play prominent roles in many evolutionary processes, including genomic conflict, adaptation and speciation^{1–3}. This is reflected in a very different genomic evolution of X and Y chromosomes as compared with autosomes^{4,5}. The sex chromosomes of therian mammals share a common origin from a pair of autosomes around 180 million years ago (MYA)^{6,7} and have undergone subsequent episodes of recombination suppression, leading to massive structural and sequence divergence between the X and Y (refs. ^{8,9}). The Y chromosome has been subject to unique selective forces. It contains the pseudoautosomal region (PAR), which is shared with the X chromosome and a non-recombining male-specific region, which has experienced extensive gene loss compared with other genomic regions while also accumulating repetitive sequences^{3,8}. All eutherian sex chromosomes share three ‘evolutionary strata’ corresponding to

cessation of recombination at different evolutionary timepoints^{9,10}. Recent sequence analysis of the human and other mammalian Y chromosomes has suggested that some primate species, including humans, contain two additional strata that appeared during primate evolution^{7,10,11}. These studies also revealed a preponderance of large palindromes on the Y chromosome that homogenize through the continuous action of intrachromosomal gene conversion^{8,12–14}, as well as a high turnover of genes in multiple, very similar copies, termed ampliconic genes (AGs). However, owing to the highly repetitive nature of the Y chromosome and the inherent difficulty in assembling it¹⁵, high-quality Y chromosomes have until now been published only for ten primate species, with considerable phylogenetic bias towards the great apes^{8,13,16–19}.

In this Article, to explore primate Y chromosome evolution more broadly, we analysed Y chromosomes across a more diverse set of

primate lineages. We generated the X-linked and Y-linked sequence assemblies for 19 previously uncharacterized primate species using long-read sequencing, thereby increasing the number of available Y chromosomes from 10 to 29 primate species. Our new dataset includes species from all the major primate lineages (Fig. 1 and Supplementary Data 1), including 2 prosimian species from the Lorisidae and Daubentonidae, 5 New World monkeys (NWMs) from the Atelidae, Callitrichidae and Cebidae, 9 apes from the Hylobatidae and Hominidae, and 13 Old World monkeys (OWMs), that is, Cercopithecidae, covering both subfamilies Cercopithecinae and Colobinae. The divergence times of these species range from 1.9 million years (Myr) between species from the same genus (for example, between *Papio hamadryas* and *Papio anubis*), to 81.8 Myr between the prosimians and simians²⁰. With this unique dataset, we were able to identify the conserved and lineage-specific patterns of change in different regions of the Y chromosome during primate diversification.

Results

Structural variation of Y chromosomes across the primates

The sex chromosome dataset is based on the male individuals of a larger set of de novo assembled genomes (Supplementary Data 1)²⁰. Briefly, long-read genome sequencing technologies, including PacBio and Nanopore, together with short-read sequencing, were used to create and polish primary genome assemblies²⁰. In five species (*Colobus guereza*, *Hylobates pileatus*, *Nasalis larvatus*, *Nycticebus pygmaeus* and *Saguinus midas*), Hi-C technology was further used to scaffold the assembled contigs.

On the basis of the depth difference between male and female sequencing reads, we first identified the X- and Y-linked sequences from the long-read assembly (Fig. 1a and Methods). As the Y chromosome is usually enriched in large segmental duplications and repeats, assemblers are prone to collapse these sequences in the resulting assembly¹⁵. We addressed this by performing additional de-collapsing of the identified Y-linked sequences from coverage information to obtain more complete and accurate Y chromosome assemblies (Supplementary Notes and Supplementary Data 2). Finally, we also performed manual examination for sex-linked sequences in each species using Hi-C data, 10X-linked reads and long-read mappings (Methods). The X chromosome assemblies are all close to being complete (on average 97% coverage of the human X chromosome), but due to their rapid evolution, Y chromosome completeness is more difficult to evaluate. We used published male karyotype images for 25 species to estimate the lengths of their Y chromosomes (Methods). Of the 15 newly sequenced species where such karyotype images were available, the Y assemblies for 10 species were within 53.7–100% of these length estimates, suggesting that a major fraction of the euchromatin region of Y chromosomes has been assembled (Supplementary Fig. 1 and Supplementary Data 2).

In contrast to the X chromosome, the Y chromosome length varies widely across mammalian species^{16,21,22}. Here, by estimating the size of the Y chromosome based on the karyotype image data collected from a number of studies, we extend this observation to a broader range of primates. The sizes of the Y chromosomes from karyotype images varies by more than sixfold from 16.24 Mb (*Papio hamadryas*) to 109.66 Mb (*Nycticebus pygmaeus*) (mean 39.48 Mb, standard deviation 22.91, Supplementary Data 2). The human Y chromosome consists of four major male-specific sequence classes, viz. the X-degenerate, X-transposed, ampliconic and heterochromatic regions⁸. Of these, the X-degenerate region mainly contains the single-copy genes derived from the proto sex chromosomes and which has undergone X/Y divergence due to recombination suppression. The X-transposed region is highly identical to its X counterpart and is thought to have originated via an X-to-Y transposition after the human–chimpanzee split⁸. The ampliconic regions feature copies of highly identical (up to 99.9% identity) sequences, which are often arranged in a palindromic structure. The fourth class, the heterochromatic region, contains sequences that are compacted

during cell division and highly enriched in satellite sequences²³. Previous studies with few primate species showed that the length and composition of Y chromosomes varied across species^{16,17}. Here we provided a broader view with data from more lineages and found that the size variation among the primate Y chromosome is mainly due to variation in the sizes of the heterochromatic and ampliconic regions (standard deviation 23.23 and 4.91 in the heterochromatic and ampliconic regions, respectively, compared with standard deviation 4.63 in the X-degenerate region; Fig. 1b,c and Supplementary Data 3). As has been previously reported in studies that focused on the great apes and other mammals^{16,21,22}, the length of the X-degenerate region is broadly similar across lineages. Of note, two NWMs, *Saguinus midas* and *Cebus albifrons*, with complete Y chromosome assemblies of similar size as estimated from karyotype, have short heterochromatic regions but very large ampliconic regions accounting for 25.00% and 45.24% of their Y chromosomes, respectively. Indeed, NWMs tend to have larger ampliconic regions compared with OWMs and apes (Supplementary Fig. 2). We confirm that the large X-transposed region is unique to human⁸. However, a smaller number of different X-transposed genes (Supplementary Fig. 3) are found in seven diverse species across the primate phylogeny. The wide phylogenetic distribution and the uniqueness of these genes suggest that X transposition has occurred independently several times during primate evolutions ('Evolutionary strata of primate sex chromosomes').

PAB shifts during the primate sex chromosome evolution

It is well established that recombination suppression between the sex chromosomes gradually evolves over time, leading to a reduction in PAR size and the emergence of new evolutionary strata^{7,10,24}. The localization of the PAR boundary (PAB) is therefore crucial for elucidating strata evolution in primates. On the basis of the difference in sequencing coverage between male and female individuals (Supplementary Figs. 4–7), we defined the PAB in each species and found large variation in PAR length, particularly between the Strepsirrhini and Simiiformes. The Strepsirrhini exhibit the longest PAR of 8.28 Mb (Fig. 2 and Supplementary Data 3), while the common marmoset (*Callithrix jacchus*) exhibits the shortest PAR at 0.98 Mb, covering only 0.67% of its X chromosome²⁵. The PAB is generally conserved among the Catarrhini (located in the third intron of the *XG* gene), and the boundary position varies only slightly across the Platyrrhini.

We further inferred, by parsimony, the PAB in the ancestral nodes of the primate phylogeny and the minimum number of independent subsequent reductions (Fig. 2). Compared with the outgroup species, treeshrew (*Tupaia belangeri*)^{26,27}, recombination suppression events have shortened the PAR in the most recent common ancestor (MRCA) of primates leading to two PAR genes in the treeshrew being located in the sex-differentiated region (SDR) of primates. The PAR was further reduced in the Simiiformes, with the relocation of the PAB between *PRKX* (PAR gene) and *NLGN4X* (SDR gene), thus generating a new evolutionary stratum spanning at least nine genes that is shared across the Simiiformes. The PAR has continually reduced in size during the diversification of the Simiiformes, resulting in the independent emergence of new evolutionary strata in several lineages. The reduction of the PAR is most striking in the common marmoset where a large segment of the ancestral PAR sequence on the Y chromosome became translocated into the SDR of this species²⁵. Sexually antagonistic selection is one proposed evolutionary force that favours recombination suppression, that is, the reduction of PAR size²⁸. Previous small-scale studies suggested that the different level of sex antagonism may be associated with the variation of PAR in primates²⁹. However, we found no correlation between PAR length and primate life history traits, such as male-to-female body mass ratio (Supplementary Fig. 8). Indeed, we find no evidence supporting the conclusion that differences in sexual dimorphism between these two clades contributed to the varied PAR length in primates.

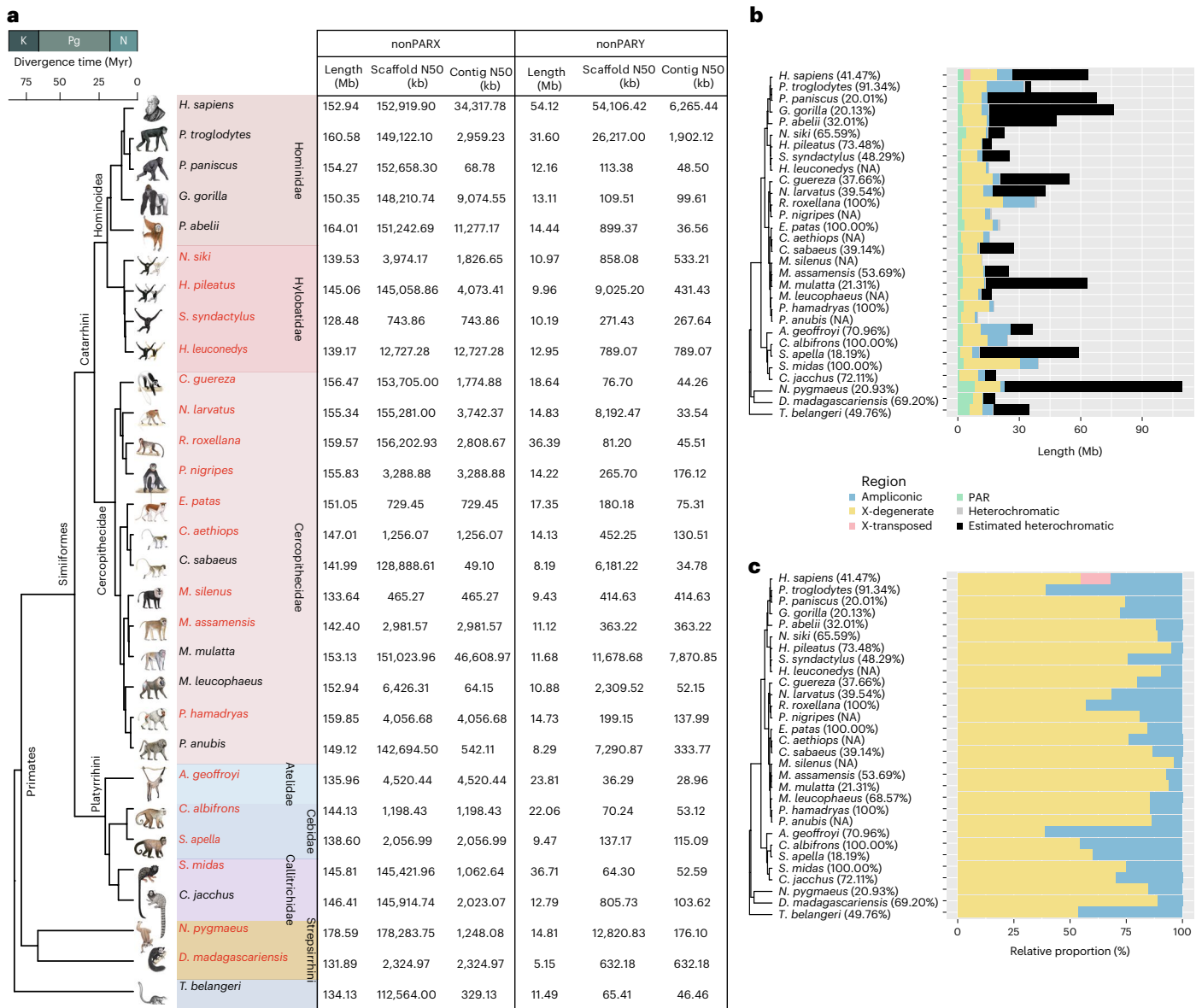


Fig. 1 | Overview of the X and Y assemblies used in this study. a Metrics of the nonPARX and nonPARY assembly. **b, c**, Length (**b**) and relative proportions (**c**) of X-degenerate, X-transposed, ampliconic and heterochromatic regions in the Y chromosomal sequences from each species. The estimated completeness of the Y chromosome is given in parentheses after each species. The extent

of the heterochromatic region is estimated from the unassembled length of the Y chromosome. Relative proportions are calculated as the ratio of each type of region to the total euchromatic regions. Primate pictures are copyrighted by Stephen D. Nash/IUCN/SSC Primate Specialist Group, and are used with their permission in this study.

Across all 30 species we investigated, we found that the PAB shifted at least six times in the ancestral nodes or individual lineages (Fig. 2). The position of the PAB within the third intron of the *XG* gene is conserved in extant OWMs and apes, as including humans, great apes and the rhesus macaque^{16,30} (Supplementary Figs. 5 and 6). By contrast, the PAB has shifted more frequently across the NWMs. The boundary was inferred to be located in a region between *CD99* and *XG* in the Platyrrhini, and the PAR length in the Callitrichidae lineage is further reduced (Fig. 2 and Supplementary Figs. 4 and 7).

We also investigated the evolution of the human-specific PAR2, which harbours three protein-coding genes (*SPRY3*, *VAMP7* and *IL9R*). A previous study proposed that the *IL9R* had been translocated to the X chromosome in the primate common ancestor³¹, but we found the gene to be autosomal in both the treeshrew and the

Strepsirrhini (Fig. 2), suggesting that the translocation occurred in the Simiiformes MRCA. *VAMP7* and *IL9R* are X-specific in all non-human primate species, except in the green monkey *Chlorocebus sabaeus*. In the green monkey, this region spans about 0.14 Mb and exhibits similar male and female normalized sequencing depths (Supplementary Fig. 9a), suggesting that it is located within a PAR. The *Chlorocebus sabaeus* PAR2 is much shorter than the ~0.33 Mb PAR2 of humans and does not include the *SPRY3* gene. Furthermore, in *Chlorocebus aethiops* and *Pan troglodytes*, which are closely related to *Chlorocebus sabaeus* and human, respectively, the orthologous region of PAR2 is X-specific, showing differentiated male and female sequencing depths (Supplementary Fig. 9b,c). These various pieces of findings suggest an independent evolution of PAR2 in *Chlorocebus sabaeus*.

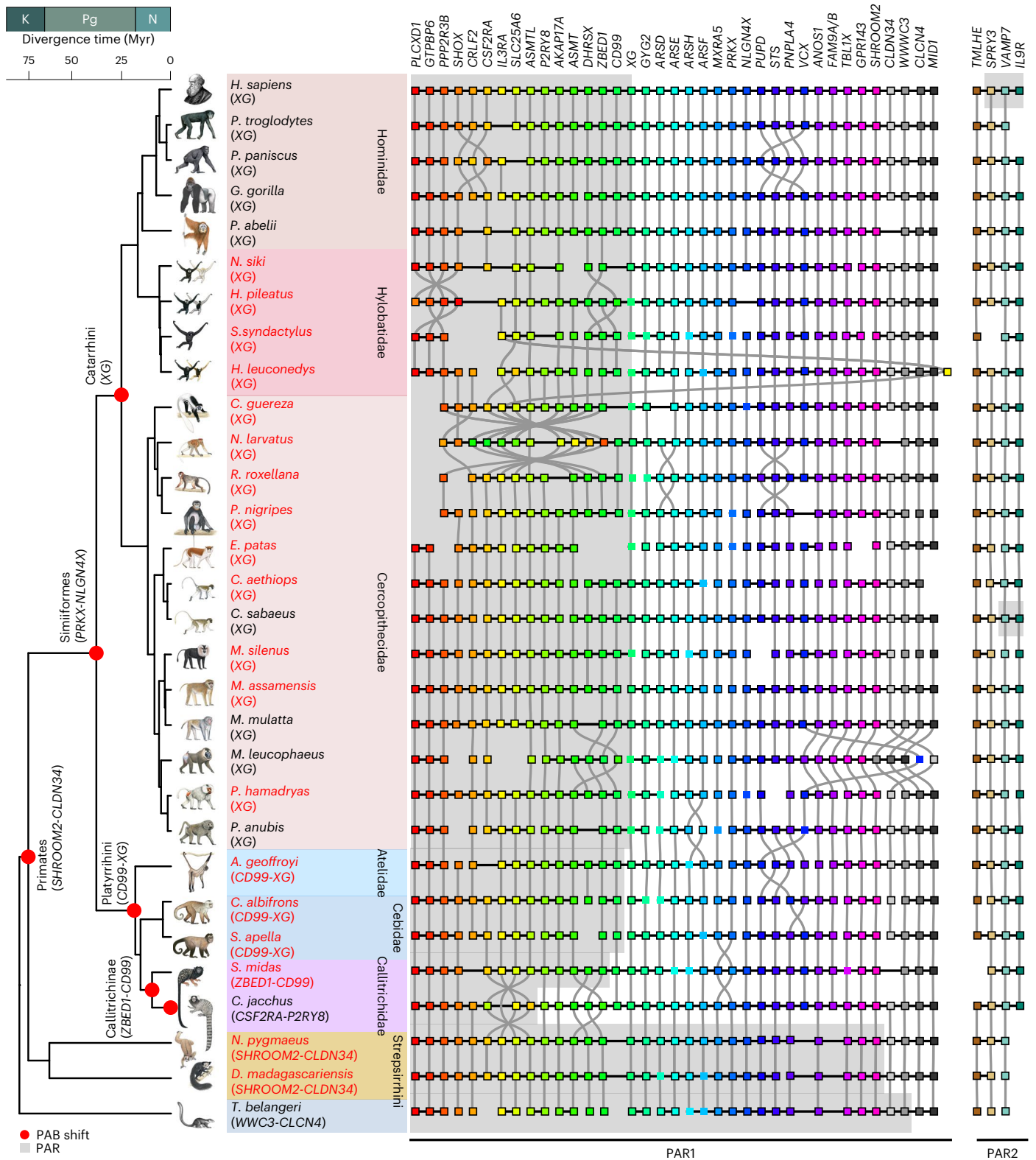


Fig. 2 | The evolution of the primate PAR. PABs of PAR1 are given in parentheses below each species name, in the format of 'PAR gene - nonPARX gene', except in the Catarrhini where the PABs are within XG. Genes that are fragmentally assembled in the genome are shown as boxes without a black border. The evolutionary nodes with inferred PAB shifts are labelled with red dots. PAR2 is found only in *Homo sapiens* and *Chlorocebus sabaeus*. PAR genes are denoted by grey shading. Genes on different scaffolds were arranged according to their synteny with the human X chromosome. Genes are linked by black lines

in each species if they were assembled in the same scaffold. Orthologous gene relationships across species are linked by grey lines, while different orthologues are encoded with different colours. Only genes known to be present in >25 species are shown. Multi-copy genes are presented as one box. Geological epoch: K, Cretaceous, Pg, Paleogene, N, Neogene. Species name colour: black, published assembly, red, assembly produced in this study. Primate pictures are copyrighted by Stephen D. Nash/IUCN/SSC Primate Specialist Group, and are used with their permission in this study.

Evolutionary strata of primate sex chromosomes

Combining the gametologue phylogeny and the pairwise synonymous substitution rate (dS) of the gametologue pairs in each species, we confirmed that all primates share the three ancestral strata (S1–S3), which evolved in the common ancestor of the Eutheria as previous reported^{7,10,11} (Fig. 3a and Supplementary Data 4). In contrast to the prosimians, which maintain the ancestral state of the Eutheria, the PAB shift in the Simiiformes MRCA led to the emergence of S4, which is unique to the clade, whereas species in the Platyrrhini and Catarrhini have each independently evolved another new stratum (S5) (Fig. 3a,b). The common marmoset is the only species with an additional stratum, S6, due to the recent expansion of the SDR²⁵. Both the non-synonymous and synonymous substitution rates between X and Y gametologues increase with stratum age (Fig. 3c and Supplementary Fig. 10), but the dN/dS ratio is at its highest for the youngest strata, indicating less constrained evolution for these genes, as was expected owing to their relative recent origin (Supplementary Fig. 10). The GC content of the corresponding sequence follows a decreasing trajectory in Y-linked gametologues with stratum age, particularly at the third codon positions, probably due to older strata having evolved for a longer time in the absence of recombination-associated GC-biased gene conversion (Supplementary Fig. 11).

Among the 29 primates, we inferred 20 ancestral gametologue pairs in S1–S3 including three (*MBTPS2X/Y*, *TAB3X/Y* and *BCORX/Y*) newly discovered in this study^{7,11,16,19}. However, it should be noted that these new gametologue pairs are present in only one or two species. Briefly, we discovered *BCORX/Y* in *Sapajus apella* ($dS = 0.4521$) and *Daubentonia madagascariensis* ($dS = 0.2968$), and *MBTPS2X/Y* and *TAB3X/Y* in *D. madagascariensis* ($dS = 0.3300$ and $dS = 0.3114$). The pairwise dS values of these X/Y gametologues are similar to those S3 X/Y gametologues (for example, *EIF1AX/Y*) that are intact in most primates (Supplementary Data 4). The gametologue phylogeny also confirms their ancestral origin as being derived from the proto sex chromosome (Supplementary Fig. 12). At least 15 of these ancient gametologue pairs have been retained in over half of the extant primates under study (Fig. 3a). These surviving Y-linked genes have been suggested to play important roles in relation to male viability, and are dosage sensitive with a higher likelihood of haploinsufficiency¹¹. One of the most conserved X/Y gametologue pairs was the assumed first therian X/Y gametologue pair, *SOX3/SRY*, both of which are highly conserved in therians^{7,11}. As a member of the SOX family, SOX3 interacts with other transcription factors via the high-mobility-group (HMG) domain and participates in gonad and neural development^{32,33}. Its Y counterpart *SRY* is the therian sex-determining gene^{34,35}. We found that all species retain their *SRY* as expected; however, the X gametologue, *SOX3*, has become pseudogenized in *Saguinus midas* (Fig. 3a). We confirmed with short reads that four indels in *Saguinus midas* *SOX3* resulted in a change in the open reading frame, leading to the fragmentation of the HMG domain and the loss of the SOXp domain (Supplementary Fig. 13). It would be worthwhile to study the functional consequence of the loss of *SOX3* in this species and whether another gene may have been recruited to complement its pseudogenization of *SOX3*.

Our data suggest that primate S4 evolved specifically in the Simiiformes but was absent in the Strepsirrhini. We detected three X/Y gametologue pairs in this stratum that have been retained in at least one of the extant species examined, including *VCX/Y*, which is present in human, chimpanzee and two *Hylobatid* species, *Nomascus siki* and *Symphalangus syndactylus*. Previous studies have suggested that *VCY* was acquired in the human–chimpanzee common ancestor due to gene conversion with its X counterpart *VCX*^{21,36,37}. We confirmed this conclusion, but our findings suggest an independent origin for *VCY* in the *Hylobatidae*. In contrast to the gametologue pair in human and chimpanzee, the *VCX/Y* pair in the *Hylobatidae* has a relatively high pairwise dS value similar to those in other S4 gametologues

(Supplementary Data 4), suggesting its more ancient origin and absence of gene conversion.

Previous studies have suggested that a relatively young stratum, S5, evolved in the common ancestor of the Catarrhini^{7,16}. Our data provide evidence for the convergent evolution of a similar stratum in the Platyrrhini lineage. While these two strata span the same genomic region and contain up to four X/Y gametologue pairs, they have originated independently after the lineages split 42.8 MYA²⁰ (Fig. 3a), according to the gametologue phylogenetic topology (Fig. 3b and Supplementary Fig. 14). In the *PRKX/Y* tree, X/Y gametologues cluster in groups of Catarrhini and Platyrrhini rather than by chromosomal origin (Fig. 3b). Human *PRKX* plays a role in kidney development, angiogenesis and other developmental processes³⁸. Only partial *PRKX* genes are evident in the genome of five primate species (Fig. 3a), indicating potential independent loss of this gene in these lineages. Additional development-related transcriptomic data need to be investigated to learn more about the underlying consequences. In addition, we found that all the other S5 X/Y gametologue pairs, *MXRA5X/Y*, *ARSD*, *ARSE*, *ARSF*, *ARSH* and *GYG2*, cluster by groups of Hominoidea, Cercopithecidae and Platyrrhini (Fig. 3b and Supplementary Fig. 14). Although gene conversion is known to obscure gene phylogeny construction^{39,40}, our finding that all of the S5 genes (*MXRA5X/Y* to *GYG2*) show similar phylogenetic signals suggests that these X/Y divergences occurred independently across the three clades, in contrast to the situation with *PRKX/Y* divergence. In addition, we discovered nine new insertions of X/Y gametologue pairs into S1–S3 strata that were present in one or a few species. These gametologues have low levels of pairwise dS (Fig. 3a and Supplementary Data 4) and their X and Y counterparts cluster together (Supplementary Fig. 15), suggesting their recent duplication from X to Y. Out of the six X-transposed Y genes for which transcriptome data were available, four of them were found to exhibit a testis-specific expression pattern (Supplementary Fig. 16).

Lastly, we used gametologue diversity to quantify the relative change in the per base pair genomic mutation rate for each species over the past 180 Myr since the formation of the first stratum. Specifically, we compared the observed dS values of each stratum with our expectation dS_{exp} that is based upon mutation rates that have been estimated by parent–offspring sequencing studies (Supplementary Data 5). The ratio dS/dS_{exp} quantifies the change that the genome-wide mutation rate experienced at different timepoints defined by stratum formation, relative to the extant mutation rate. Notably, dS/dS_{exp} decreased across S1–S3 (Fig. 3d), in line with previous evidence of a mutation rate slowdown in primates relative to the Simiiformes MRCA⁴¹, especially for the apes where the discrepancy between dS and dS_{exp} is at its highest⁴². On average, a ~3.3-fold higher mutation rate than that estimated by parent–offspring sequencing studies would be required to achieve the dS level observed for S1. In similar vein, a 0.9–2.9-fold difference in mutation rates would be needed to account for the observed dS of S2–S5 since their formation.

Genomic re-arrangement in Simiiformes-specific strata

Structural variations between the X and Y chromosomes have been proposed to contribute to the recombination suppression between them. On the basis of the observation that pairwise dS values of the X/Y gametologue clusters vary greatly among strata along the X chromosomes¹⁰, previous studies hypothesized that the Y chromosome has experienced stepwise recombination suppression region by region^{10,28}. The oldest stratum S1 is suggested to have evolved at the MRCA of all therians ~181 MYA; eutherian S2/3 formed later, beginning ~117 MYA⁷. With X/Y chromosomes available from more species, we are able to extend the investigation more broadly to additional taxa, and to assess the genomic re-arrangements of the X and Y chromosomes in different primate species. The primate X chromosomes have a high degree of conserved synteny in relation to the outgroup and have rarely experienced large re-arrangements over the past 81.76 Myr;

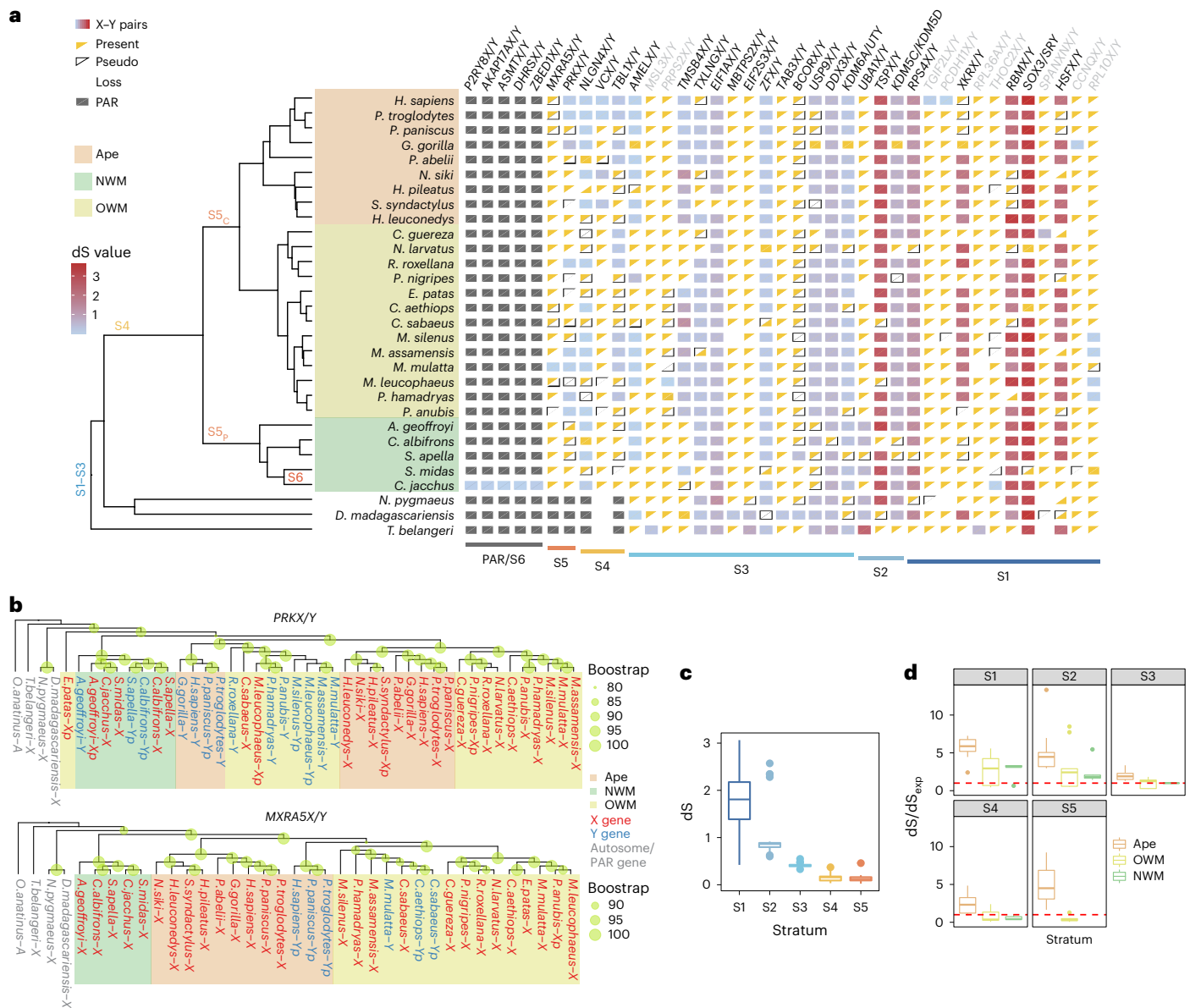


Fig. 3 | Evolutionary strata of primates. a, X/Y gametologues and the evolutionary strata in which they are located. The gametologue pair box is colour coded according to the X/Y pairwise dS value if both X-linked and Y-linked gametologues were intact. If only the X or Y gametologue was available, the upper triangle represents the presence of the X-linked gene while the lower triangle represents the presence of the Y-linked gene. Yellow: gene with intact open reading frame, blank: lost gene, white with black outline: pseudogene, grey: PAR gene. Gametologues have intact copies in both the X and Y but without dS values because they were missing in the assemblies and have been recovered from raw sequencing reads and RNA-seq data. The approximate placement of strata on branches is based on the topology of individual gene trees and dS values, after consideration of their placement in previous studies^{7,10,11}. S5_c and S5_p indicate the stratum 5 convergently evolved in Catarrhini and Platyrrhini, respectively. Gametologue pair names colour: black, derived from the proto sex chromosome, grey: Y newly transposed from X. **b**, Phylogenetic trees of two examples of S5 X/Y gametologues, *PRKX/Y* and *MXRA5X/Y*. Gene names are colour coded according to chromosomes (red, X; blue, Y; grey, PAR). Leaf

node background is colour coded according to lineage (yellow, OWM; red, apes; green, NWM). Pseudogenes are also included and are marked with a 'p' suffix. **c**, Distributions of dS values calculated using the KaKs_Calculator for concatenated stratum sequences of each species. $n = 30, 30, 30, 19$ and 8 in the order from S1 to S5 (treeshrew and prosimians are included in S1–S3). Box plots show median, quartiles (boxes) and range (whiskers). **d**, Mutation rate evolution across different strata. The distribution of the dS/dS_{exp} ratio for each stratum and species across different timepoints defined by strata formation events. The dS values were calculated from the X/Y alignment of concatenated CDSs in a given stratum. The dS_{exp} values represent the expected rates of synonymous substitution based on mutation rates estimated by parent–offspring sequencing studies (Supplementary Data 5). The red dashed line represents the dS/dS_{exp} ratio of 1, expected in the case of no mutation rate change. In S1–S3, $n = 9, 13$ and 5 for ape, OWM and NWM, respectively. In S4, $n = 7, 9$ and 3 for ape, OWM and NWM, respectively. In S5, $n = 3$ and 5 for ape and OWM, respectively. Box plots show median, quartiles (boxes) and range (whiskers).

one notable exception was an -14 Mb inversion on the long arm of the pygmy slow loris (*Nycticebus pygmaeus*) X chromosome, which was further confirmed with Hi-C map data (Supplementary Fig. 17). The highly conserved X chromosome that resembles the primate ancestral

stage therefore provides a background for the investigation of structural alterations to the Y chromosomes following the formation of the Simiiformes-specific strata, S4 and S5. Owing to their comparatively recent formation, most of the alignable regions between the

X and Y chromosomes are found in these two strata (Supplementary Fig. 18). Utilizing the Hi-C scaffolded or chromosome-scale Y assemblies from eight primate species covering the NWMs, OWMs and apes, along with the human X as the outgroup, we detected 21 conserved syntenic blocks under 50 kb resolution across species within these two regions and the PAR, and found a high frequency of inversions in these regions that had originated during their diversification (Fig. 4a). For instance, S4 was likely to have originated via an inversion⁹ at Simiiformes MRCA (Fig. 4a). Further, S5 of Catarrhini first experienced four inversions that shuffled the sequence order of both S4 and S5, and then acquired several lineage-specific changes in each extant species (Fig. 4a). We found that OWMs, especially *Rhinopithecus roxellana*, largely maintained the Catarrhini ancestral Y structure after the evolutionary emergence of S5. By contrast, the Y chromosomes of the Hominoidea experienced more structural changes resulting in more diverse Y structures in S4 and S5 among the extant species. Similar results were found from the reconstruction under 30 kb resolution (Supplementary Fig. 19).

The extensive genomic re-arrangement of the Y chromosomes motivated us to investigate whether the chromatin configuration of the homologous regions between the two sex chromosomes was altered after recombination suppression³. Here, on the Hi-C interaction map⁴³, we estimated the chromatin configuration differences between the X/Y homologous regions of the new SDR, that is, S4 and S5. Of the five species for which we could detect sufficiently long homologous sequences between the X and Y, four showed significant changes in chromatin configuration after transformation from the PAR to SDR, when compared with the control distribution represented by the difference in chromatin configurations between autosomal alleles (FDR-corrected P value < 0.01; Fig. 4b,c, Supplementary Fig. 20 and Supplementary Data 6). Such chromatin contact change on the sex chromosomes was also observed in the recently emerged stratum in the Z/W system of the emu⁴⁴. Nevertheless, this pattern does not appear to be present in the strata that have evolved very recently in the eel X/Y chromosomes⁴⁵, which still maintain high similarity between X and Y homologous sequences and might be too young to have established such a difference in chromatin configuration. By contrast, when measuring the chromatin configuration difference between Simiiformes and Strepsirrhini (or treeshrew) along the X chromosome, we did not find a significant difference between the new SDR and the ancestral SDR (Wilcoxon rank-sum test, P value > 0.05; Supplementary Fig. 21 and Supplementary Data 7 and 8). Additionally, it would appear that both the new SDR and the ancient SDR on the X chromosome maintain the same level of topologically associating domain (TAD) boundary conservation across the Simiiformes (Fig. 4d, Supplementary Fig. 22 and Supplementary Data 9). Specifically, about 50% of TAD boundaries are conserved between Simiiformes and treeshrew in both the new SDR (S4 and S5) and the ancestral SDR (S1–S3). Between Simiiformes and pygmy slow loris, about 60% of TAD boundaries are conserved in both the new and the ancestral SDR. These results suggest that the configuration difference between the X and Y in new strata may be largely attributable to alterations on the Y chromosome, probably after rather than before the

recombination suppression. It also supports the association between epigenetic modification changes and heterochromatinization during Y chromosome evolution³.

Evolutionary dynamics of genes on the Y chromosome

In contrast to the X chromosome, which has maintained most of the ancestral gene properties of the proto sex chromosome, the Y chromosome has experienced dramatic gene loss facilitated by recombination inhibition, and therefore possesses markedly different gene properties between extant primate species. It has been shown that degeneration proceeds quite rapidly on newly formed Y chromosomes, but the rate decreases in evolutionarily older Y chromosomes^{11,16}. This degeneration model was confirmed by the different preservation pattern of genes between the therian ancient strata and the Simiiformes-specific strata. While most Y-linked genes in S1–S3 were lost before the Simiiformes radiation, the remaining 20 genes were preserved in most primates over the last 81 Myr, with relatively few (on average 38.89%) being lost in individual branches of the primate phylogeny (Fig. 5a and Supplementary Figs. 23 and 24). By contrast, S4 and S5 experienced a much more rapid gene loss in Simiiformes species with on average 84.79% and 94.93% gene loss over the past 81 and 42 Myr, respectively (Fig. 5a, Supplementary Fig. 23 and Supplementary Data 10 and 11), probably due to a stronger Hill–Robertson interference effect when more genes are present in younger strata⁴⁶. Of the at least 16 ancestral genes in S4 and S5, a maximum of 4 have been maintained as gametologue pairs within the same species. With more primate species, we were able to refine the degeneration model of these two young strata on the basis of the number of the surviving Y chromosomal genes in each evolutionary node. The observed patterns within S4 and S5 are generally consistent with the hypothesized degeneration model^{11,16} (Fig. 5b and Supplementary Data 11). Notably, more Y-linked genes of the Platyrrhini have undergone degeneration in both S4 and S5, resulting in fewer Y-linked genes being present in this region (on average 0.80 and 0.20 in Platyrrhini versus 1.31 and 0.41 in Catarrhini for S4 and S5, respectively) (Fig. 5a). We were also able to trace at which ancestral nodes the degeneration of each Y-linked gene occurred (Supplementary Fig. 25). Thus, for example, in human the extant S4 Y-linked gene set had already formed at the Simiiformes MRCA; by contrast, the S5 Y-linked genes experienced a two-step inactivation that occurred at the MRCA of Catarrhini and apes.

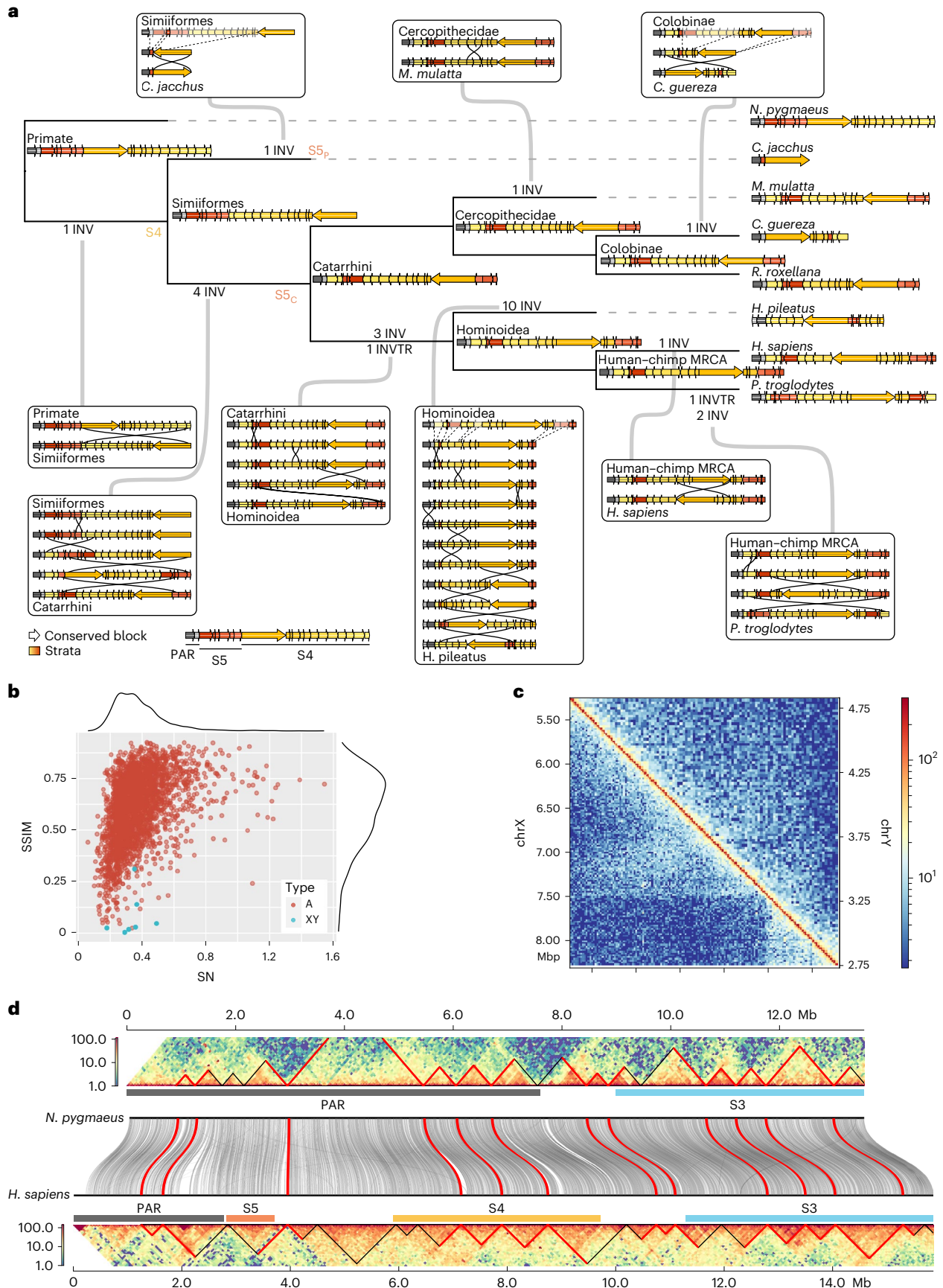
Although our observation indicates that most genes in S4 and S5 were lost ancestrally at Simiiformes MRCA, one exception is provided by *NLGN4Y* of S4 which was still present in over a half of extant Simiiformes, suggesting that the gene may have played an important role in this lineage. A recent study postulated that one of the amino acid differences, Pro93Ser, between the human *NLGN4X* and *NLGN4Y* proteins might be key to understanding the mechanism underlying the male bias observed in *NLGN4X*-associated autism spectrum disorder⁴⁷. We found that this amino acid substitution in *NLGN4Y* occurred in the ancestral node of Simiiformes and has been maintained in all descendant lineages except *Callithrix jacchus* and *Sapajus apella* (Supplementary

Fig. 4 | Structural and chromatin configuration changes in S4 and S5 during primate sex chromosome evolution. a, Reconstruction of the ancestral Y chromosome sequence order with conserved syntenic blocks under 50 kb resolution. A total of 21 conserved blocks were used in reconstruction. Each arrow block represents a conserved segment, colour coded in PAR, S5 and S4. The postulated genomic re-arrangement events marked at each branch were inferred by GRIMM and manual curation. INV, inversion; INVTR, inverted translocation.

b, Homologous blocks between X and Y (blue) harbour lower chromatin configuration similarity than the regions between homologous chromosomes (red), indicating that the contact map changed after X/Y divergence. **A**, diploid autosomal background; XY, X/Y homologous block. $n = 7$ and 2,335 for XY and A dataset, respectively. **c**, Comparison of Hi-C contact maps as an example of

an X/Y homologous block in *Macaca mulatta* (chrX:5,249,201–8,310,709(+), chrY:2,669,614–4,768,941(–)). Upper triangle depicts the Y Hi-C matrix, whereas the lower triangle depicts the X Hi-C matrix. **d**, TAD boundaries are conserved on the X chromosome after the evolutionary emergence of S4 and S5. Top: Hi-C map and TADs in *Nycticebus pygmaeus*. Middle: alignment between *Nycticebus pygmaeus* and *Homo sapiens*. Bottom: Hi-C map and TADs in *Homo sapiens*. Conserved TAD boundaries are highlighted with red lines, while species-specific TAD boundaries are delineated with black lines. In this example, six of the ten TAD boundaries in the recently degenerated S4 and S5 of *Homo sapiens* are conserved with those of the PAR of *Nycticebus pygmaeus*. This level is similar to that in the ancestral stratum S3 where six of the nine TAD boundaries are conserved between the two species.

Fig. 26). We also identified three other X/Y variants in this gene that were conserved across the Catarrhini (Supplementary Fig. 26). The high level of conservation of two of the X/Y variants in NLGN4Y implies that these amino acid residues have been important for male development in simians, with a hint of a potentially deleterious side effect by virtue of a contribution to the male bias in autism spectrum disorder.



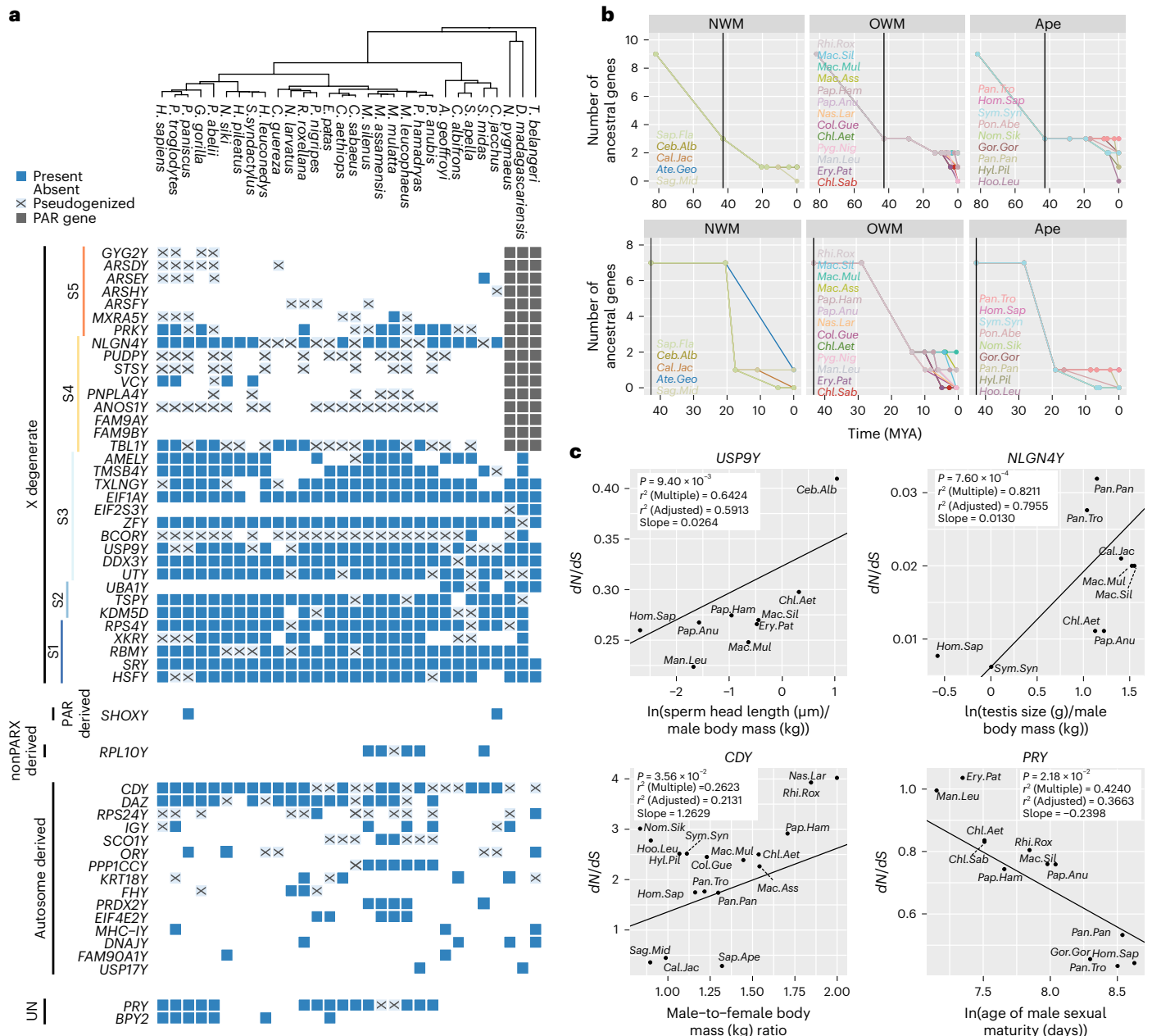


Fig. 5 | Dynamic evolution of primate Y chromosomes. **a**, Heat map depicts the state of Y-linked genes (present, pseudogenized or absent) in each species in this study. For genes outside S4 and S5, only Y-linked genes available in more than one species are shown. X-degenerate, PAR derived and nonPARX derived genes are ordered according to the genomic coordinates of their X-linked gametologues. Autosome-derived genes are ordered on the basis of the number of species carrying these genes. UN, unknown. **b**, Degeneration model for Y-linked genes in S4 and S5 during primate evolution. In both strata, the Y gene degeneration rate is fast initially, but then decelerates eventually reaching a stable level in recent

time. The degeneration rate of NWM is more rapid than that of OWM and apes in both S4 and S5. The Platyrrhini–Catarrhini split is marked by a black line. **c**, dN/dS values of the Y-linked genes *USP9Y*, *NLGN4Y*, *CDY* and *PRY* exhibit a correlation with the sperm head length (μm) to male body mass (kg) ratio, testis size (g) to male body mass (kg) ratio, male-to-female body mass ratio and age of male sexual maturity (days), respectively, with PGLS analysis. Log transformation was applied to all traits except body mass dimorphism ratio. Two-sided *F*-test is used in PGLS analysis, and we do not apply multiple testing correction to adjust the *P* value.

We also detected a significant correlation between the dN/dS values of several Y-linked genes and male developmental traits across the primates, for example, age of male sexual maturity, sperm morphologies, testis size and level of sexual dimorphism in relation to body mass (Fig. 5c, Supplementary Fig. 27 and Supplementary Data 12 and 13), after accounting for the phylogenetic covariance. These traits are widely believed to evolve in response to sexual selection which has been reported to favour genes on the Y chromosome that enhance male fertilization success^{48–50}. We have found that *USP9Y* and *NLGN4Y*

exhibit a positive correlation between dN/dS values and sperm head length and testis size (both scaled by body mass and log-transformed), respectively. Moreover, the evolution of *CDY* may correlate with sexual dimorphism in relation to body mass and the age of male sexual maturity. Interestingly, *CDY* is one of the few Y-linked genes that have not degenerated from the ancestral sex chromosome but were instead derived from an autosome prior to the adaptive radiation of the primates. In contrast to the autosomal gene *CDYL* from which *CDY* originated, the Y-linked gene has experienced a change from a ubiquitous

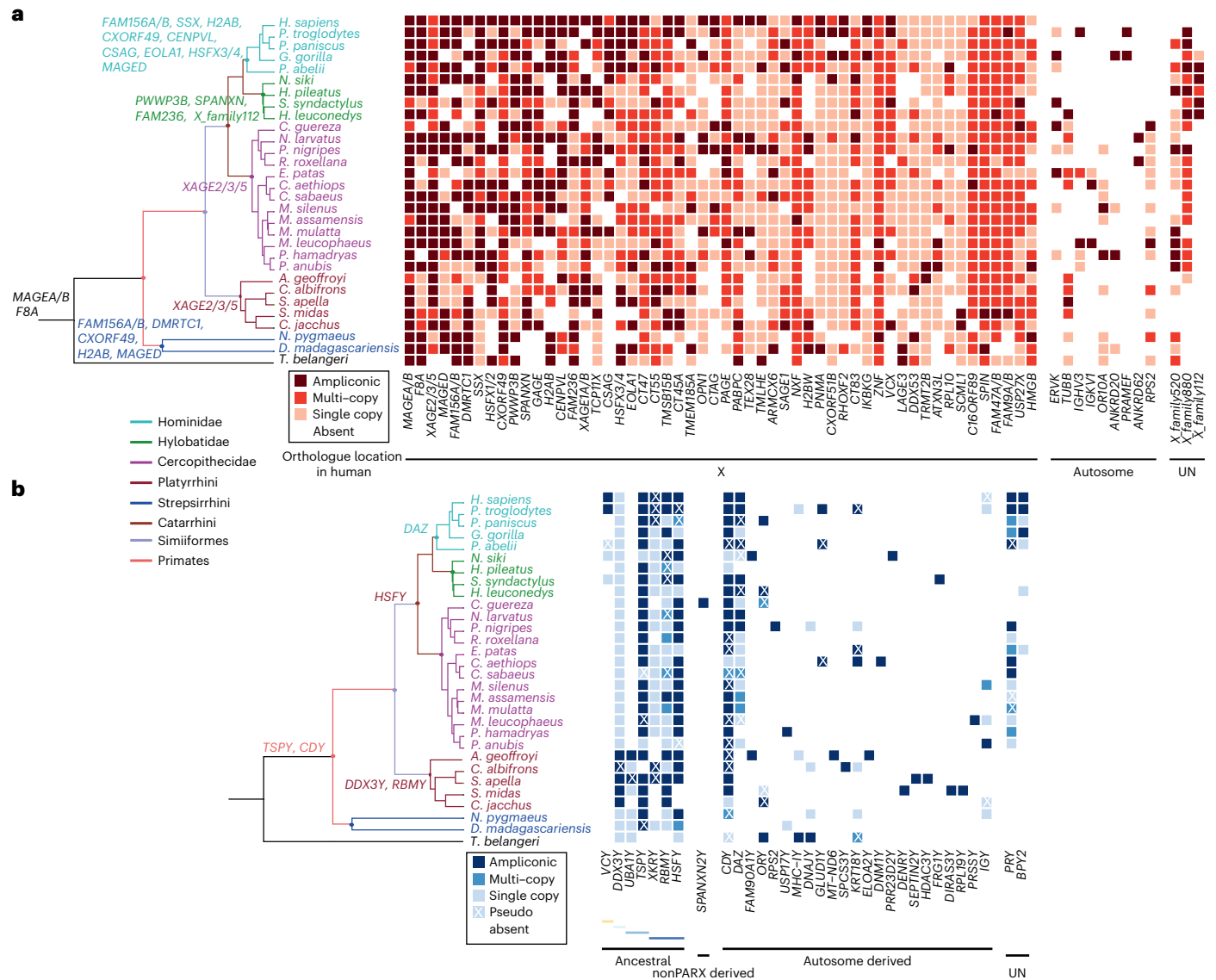


Fig. 6 | Comparison of X-linked (a) and Y-linked (b) families involving AGs across the primates and treeshrew. a. Comparison of the X-linked gene families involving AGs across the primates. Only families with AGs in more than one species are shown. Families were ordered by the number of species containing AGs. **b.** Comparison of the Y-linked gene families involved AGs. Pseudogenes are marked by crosses. Families were organized in the order of ancestral,

nonPARX derived, autosome derived and unknown (UN). Ancestral Y-linked genes were ordered by stratum age. The other three types of gene were ordered by the number of species containing AGs. Differently coloured branches in the phylogenetic tree represent different taxa. Genes in different colours represent different ancestral origins.

pattern of expression to testis-specific expression, an example perhaps of neofunctionalization⁵¹⁻⁵³. We were also able to detect dozens of new Y chromosomal genes that appeared during the process of primate diversification (Fig. 5a). These were mainly derived from the autosomes with only a few cases being duplicated from the X chromosome. Most of these genes are present in only one or a few species (Fig. 5a), suggesting that their acquisition was lineage specific (Supplementary Fig. 28).

AG evolution across primates

AGs, defined as genes with highly similar copies (sequence identity >99%), are common in most sex chromosome systems, including human, chimpanzee and mouse^{22,51,54} as well as *Drosophila*⁵⁵. These genes are predominantly expressed in the testis, are often important for male fertility^{54,56}, and have been proposed to be associated with sexual selection through traits such as mating system^{17,57} and sex-linked meiotic drive^{22,58}. We searched for AGs on both sex chromosomes in

all primate species by identifying genes located in genomic regions that occur as duplicated copies with high identity, or which have more than twofold sequencing depth compared with single-copy regions and hence may have been collapsed in the assemblies. Employing this method, we were able to detect AGs on both the X and Y chromosomes of all primate species (Supplementary Data 14 and 15), with the exception of the Y chromosome of the aye-aye (*D. madagascariensis*) which possesses a very small ampliconic region (Fig. 1b). We also identified genes characterized by multiple duplicated copies but with sequence identity lower than 99%, and found that these multiple copy genes generally belong to gene families that also harbour AGs (Fisher’s exact test, $P = 1.09 \times 10^{-11}$; Supplementary Data 16), suggesting that at least a portion of these multiple copy genes may once have been AGs but accumulated substitutions after the cessation of gene conversion.

In total, we identified 178 X-linked gene families across all primates that harboured AGs in at least one species. Becoming ampliconic

appears to be a highly species-specific phenomenon, with only 66 X-linked gene families containing AGs in more than one species (Fig. 6a). The MAGEA/B family is one of the most highly conserved gene families with AGs in 24 species spanning all major primate groups (Fig. 6a). This family also has AGs in mice⁵⁴ and members of the family are predominantly expressed in testis⁵⁹, suggesting that this AG family has been evolving at least since the Euarctomyrmecines, having been maintained ampliconic for more than 96 Myr⁶⁰.

As in the situation encountered with the X-linked AG families, from a total of 32 Y-linked families containing AGs in primates, 21 are AG in only one species. Nevertheless, we found that several Y-linked AGs, such as *TSPY*, *HSFY* and *CDY*, are AGs in over half of the primates studied (Fig. 6b). For example, *TSPY* is an important factor for spermatogenic efficiency⁶¹, and the amplification of *TSPY* might therefore have been favoured by sexual selection during the evolution of the primates. Of note, some AGs are only present in specific taxa. For instance, *DDX3Y* and *UBA1Y* were found to be AG specifically in NWM. In particular, *DDX3Y* was present as AGs in most investigated species of NWM. This gene is thought to be a major azoospermia factor in humans, and its knockout results in the reduction or even absence of germ cells⁶². The amplification of *DDX3Y* could be attributed to the multimale–multifemale and polyandry mating systems in NWM⁶³, which increase the degree of sperm competition. A previous study reported three waves of duplication and transposition of *DAZ*, another azoospermia factor, into the human Y chromosome⁶⁴, starting with the ancestors of the Catarrhini. Consistent with this, we found that *DAZ* constituted an AG family in most Catarrhini, with the exception of the Cercopithecoinae, in which multiple copies have accumulated mutations, probably due to a lack of gene conversion events between copies.

Previous studies have also found massive co-amplification of AGs on the X and Y chromosomes in mouse and bovine, suggesting a possible role in genomic conflicts over sex chromosome transmission^{22,58}. Interestingly, we detected two examples of co-amplified genes on the X and Y chromosomes, *HSFX/Y* and *VCX/Y*, in several primate lineages. The co-amplification of *VCX/Y* was found to be confined to human and chimpanzee, consistent with a previous study^{17,21}. By contrast, co-amplified *HSFX/Y* were found in 11 species from NWM, OWM and apes (Supplementary Data 17). This gametologue pair has also been reported to be co-amplified in bovine, with high copy number variation on the Y chromosome⁵⁸. It is possible that the co-amplification of *HSFX/Y* might have been the consequence of sexually antagonistic selection or meiotic drive.

Discussion

Our study fills a number of important phylogenetic gaps pertaining to the structure and gene properties of the sex chromosomes in primates and broadens our knowledge of sex chromosome evolution across the primate phylogeny. This unprecedented data resource has allowed us to identify the key changes that has occurred on the sex chromosomes in each evolutionary node and to disentangle the evolutionary patterns that occurred in the common ancestor of all primates as well as particular events in specific lineages. Compared with the X, our analyses revealed rapid dynamic change on the Y chromosome that accompanied the diversification of primates, for example, the two young strata (S4 and S5) that evolved in the Simiiformes ancestor and Catarrhini (apes and OWMs) ancestor respectively, while the NWMs also evolved an S5 stratum quite independently. Interestingly, the two new strata would appear to have experienced different rates of degeneration across primate groups, probably due to different background selection or rate of genetic drift. In line with previous findings in humans and a few other primates, we found that primates have not only expanded their male-specific regions by extending the X-degenerate region, but have also continually accumulated new gene copies either by translocation from the rest of the genome or by the

amplification of Y-linked genes, mediated potentially by palindromic sequences. These AG families are dominated by testis-specific genes that have remained highly identical as a consequence of the action of interallelic Y–Y gene conversion, suggesting a selective advantage conferred by their increased gene copy number. Interestingly, we observed that AG families were highly dynamic across primate lineages, possibly due to variation in sexual selection and background selection pressures between species. An accurate and complete indexing of all ampliconic and palindromic sequences would require a complete telomere to telomere assembly of the Y chromosome, which still remains a technical challenge even for the human genome¹⁵. Nevertheless, our study has illuminated many key evolutionary features of the Y chromosomes of primates. With improvements in sequencing technology and the sequencing of additional primate species, comparative analyses on a phylogenetic scale will enable us to acquire a much better understanding of the molecular basis of the diversity of sex-linked biological traits across the primates, with potential medical significance for studies on human reproduction and health that pertain to Y-linked genes^{14,57,65–67}.

Methods

Data type and species used in each analysis are listed in Supplementary Data 18.

Ethics, sample preparation and sequencing

Blood samples of female *Macaca silenus*, *Papio hamadryas*, *Erythrocebus patas* and *Colobus guereza* and a muscle sample of female *Hylobates pileatus* were collected from the Japan Monkey Centre, Japan. The use of genetic materials of Japan Monkey Centre was approved by the Research Ethics Committee from the Japan Monkey Centre (#2017-018) and performed in accordance with the Ethical Guidelines for Research at the Japan Monkey Centre (1 April 2016). DNA was extracted from the tissue samples with QIAGEN DNeasy Blood & Tissue Kit (50), following the manufacturer's instructions. Library preparation was performed with Illumina TruSeq DNA PCR-Free Kit (Illumina) and sequencing was run on the Illumina NovaSeq 6000 system (151 bp × 2 PE).

Identification of X-linked and Y-linked sequences

To identify the X-linked and Y-linked sequences in each primate species, we used a similar method to that employed in ref. 25. Male and female resequencing short reads were mapped to each genome using BWA MEM (v0.7.17) (ref. 68). For species lacking female short reads, we used short reads from female individuals from the same genus (Supplementary Data 1 and Supplementary Notes). Coverage was extracted with samtools (v.1.9) (ref. 69), normalized by the peak coverage, and was then calculated in 5 kb windows with bedtools (v2.29.2) (ref. 70). Scaffolds (>10 kb) of over 60% of windows with normalized F/M coverage ratio between 1.5 and 2.5 were identified as X-linked, and between 0.0 and 0.3 as Y-linked. Coverage of candidate X- and Y-linked scaffolds was also visualized with ggplot2 (v3.3.5) (ref. 71) and manually examined to delineate the PAR and nonPARX/Y within each scaffold. For species with PacBio or Nanopore long reads, we also mapped long reads to the genome, and further de-collapsed the nonPARY region with SDA (git commit 4ca0c0709dc86181afaeeee862543dc19a411eb3) (ref. 72), using the half value of the peak long reads coverage of the genome (Supplementary Notes). The sequences assembled by SDA were merged with other sequences using the method described in ref. 25 and polished with male short reads data by means of the freebayes-bcftools polishing pipeline (freebayes v1.3.1-17-gaa2ace8, bcftools v1.11) described in ref. 73. We further BLASTped the protein sequence translated from the nonPARY genes to the NR database (access date: 20190727) with diamond (v2.0.0) (ref. 74). The BLAST hit was then further processed with BASTA (v1.3.2.3) (ref. 75) for categorization. If over 80% of the genes on a nonPARY candidate scaffold were categorized to be from a non-primate source, the scaffold was removed.

Y gene set annotation

We combined the homologous method and the de novo method to annotate genes on nonPARY sequences. Homologous gene annotation was first performed with GeneWise (v2.4.1) (ref. ⁷⁶) using protein sets from the X and Y chromosomes of human, rhesus macaque and mouse collected from Ensembl 98. De novo gene annotation was performed with Augustus (v3.2.3) (ref. ⁷⁷), with pre-trained human parameters on N-masked sequences. We further excluded possible retrogenes or pseudogenes from the annotation using the following criteria: (1) containing more than two frameshifts or premature termination signals, (2) annotated as a single-exon gene while the query was a multi-exon gene, (3) the coding sequence (CDS) was covered with $\geq 60\%$ of repeats. These two datasets were further merged to construct a non-redundant gene set, and the dataset was then used to BLASTP the Swiss-Prot database (release date May 2020). Genes BLASTed to the database but with an alignment rate ≤ 0.7 were excluded, and gene names were obtained by their best hits on the Swiss-Prot database. Frameshift signals within genes that passed the above criteria were confirmed with male short reads from the same individual (Supplementary Notes and Supplementary Data 19 and 20). The absence of Y-linked genes was confirmed with male long reads and short reads (Supplementary Notes and Supplementary Data 21–23).

Y completeness evaluation

Karyotype images of several studied primates were collected from previous studies (Supplementary Data 2) and the expected size ratios between the X and Y chromosomes were calculated using the method described in ref. ⁷³. We used the size of identified X-linked and PAR scaffolds to calculate the size of the assembled X chromosome. The assembled X chromosome size and the expected X-to-Y ratio were then used to calculate the estimated Y chromosome size in each species. The completeness of the assembled Y-linked scaffolds was calculated as the proportion of the assembled ungapped Y-linked scaffold length relative to the expected Y chromosome length.

Y structural analysis

We split the Y-linked sequences into 5 kb windows and performed all-versus-all BLASTN sequence comparisons⁷⁸. If a BLASTN hit had an identity $>99\%$ and an alignment rate $>50\%$, the windows were considered as candidate ampliconic regions. We also used the male sequencing depth to identify windows that might still have collapsed in the assembly and hence could not be detected by means of the BLASTN method. We compared the mean sequencing depth of each window to the mean sequencing depth of the windows covering the single-copy orthologues in most species (*SRY*, *KDMSD*, *TXLNGY*, *XKRY*, *UTY*, *USP9Y*, *TMSB4Y*, *AMELY* and *NLGN4Y*). Where the sequencing depth fold change to the control depth was ≥ 2 , the window was considered to be a candidate ampliconic region. The two resulting ampliconic datasets were merged with bedtools to obtain a final ampliconic region. The X-transposed windows were defined by 5 kb windows that overlapped with X-transposed genes characterized in the strata analysis. We also BLASTNed the nonPARY 5 kb windows against the nonPARX sequences. Windows with a BLASTN hit of identity $>90\%$ and an alignment rate $>80\%$ were retained. These 5 kb windows were then merged with 'bedtools merge -d 15000'. Only merged regions of >1 Mb were retained as being X-transposed region. We further curated the human X-transposed region by taking the smallest continuous region that could cover the merged segments. Heterochromatic regions were defined as windows for which $>80\%$ of the sequences were satellite repeats, based on the definition in ref. ⁸. When combining the three datasets, we assigned the windows with the following priority: X-transposed, ampliconic, heterochromatic. The remaining regions were defined as X-degenerate. Estimated heterochromatic regions were defined as regions that were not assembled but were instead estimated from karyotype. To confirm that the large X-transposed

region is unique to human, we obtained each X-transposed gene and its flanking 50 kb region sequence. The X counterpart and the flanking 50 kb region were also obtained. The Y region was split into 100 bp windows and BLASTNed to the X region. Solar (v0.9.6) was used to chain the alignment with parameter '-a est2genome2' and the longest chained block was retained. Average identity in every neighbouring three windows (that is, 300 bp) was calculated and visualized by pyGenomeTracks (v3.7) (ref. ⁷⁹).

PAR identification and PAB inference

Some PARs could be identified by the sequencing depth method if the sequence was assembled with nonPARX or Y, but using only this method could have generated some false negatives. On the basis of the assumption that the PAR and its flanking region are relatively well conserved among primates, we used human as the reference and produced whole genome alignments to identify PARs in other species. We treated a scaffold as a candidate PAR scaffold if over 70% of the sequence was aligned to the human X and most of the scaffold was aligned to the human X 0–12 Mb region. We further confirmed the candidate as a true PAR if it had similar normalized male and female resequencing depth. To annotate genes on PAR1, PAR2 and their flanking region, genes from the first 12 Mb (from *PLCXDI* to *MIDI1*) and the last nine genes (from *TMLHE* to *IL9R*) on human chrX were used as queries. Homologous annotation was performed with GeneWise (v2.4.1) and Exonerate (v2.4.0) (ref. ⁸⁰), and de novo gene annotation was performed with Augustus (v3.2.3). Human PAR was obtained from <https://www.ncbi.nlm.nih.gov/grc/human>. To check if PAB is conserved among Simiiformes¹⁶, we first performed whole-genome pairwise lastZ (v1.04.00) (ref. ⁸¹) alignment between each species with human, with parameter set '-step=19 -hsptresh=2200 -inner=2000 -ydrop=3400 -gappedthresh=10000'. The human PAB location was then liftOvered into each species. We further examined the normalized male and female sequencing coverage under 200 bp window resolution. If the normalized F/M coverage ratio was changed abruptly at the liftOvered location, we considered the PAB conserved between human and the primate species. Marmoset PAR was obtained from ref. ²⁵. The ancestral PAB was inferred in a parsimonious way, under the assumption that once X/Y divergence had occurred and transformed a gene from PAR to SDR, it could not then revert to PAR. We also considered the evolutionary history of S4 and S5 in the inference.

Strata analysis

To establish X/Y gametologues, we searched for the closest homologues between X and Y genes using encoded amino acid sequences and running a BLASTP search, with e-value cut-off 1×10^{-5} . The gametologue pairs were further examined in terms of their hits to the Swiss-Prot database. Only pairs of both X-linked and Y-linked genes that BLASTPed to the same gametologue were retained for further analysis. We then aligned the CDSs of candidate X/Y gametologue pairs and their orthologues with PRANK (v170427) (ref. ⁸²), built phylogenetic trees with RAXML (v8.2.4) (ref. ⁸³) and manually examined to remove false positive gametologue pairs caused by paralogues. Additionally, we incorporated gametologues found in other studies²⁵. Gametologues recovered from raw reads in the Y gene-set annotation procedure were included but were not used for evolutionary analysis due to their high sequencing error rate. To calculate the pairwise *dS* value, we first aligned the protein sequence of each gametologue pair with PRANK and converted the protein alignment into the CDS alignment. Pairwise *dS* values were calculated with KaKs_Calculator (v2.0) (ref. ⁸⁴). Gametologue pairs were grouped into different strata according to the pairwise *dS* values and their assignment in human^{10,11}. To further confirm the evolutionary history of S4 and S5, we aligned the entire gametologue gene region, including both CDS and intron, with MAFFT (v7.471) (ref. ⁸⁵), and constructed the phylogenetic tree with RAXML.

***dS* analysis**

To increase the accuracy of estimation of species-specific population genetic parameters, we concatenated CDSs of gametologues within each stratum. To quantify the change of mutation rate between different strata and species, we calculated the *dS* of the concatenated sequences using the KaKs_Calculator. The expected values of *dS* (dS_{exp}) were based on empirically estimated extant mutation rates as shown in Supplementary Data 5.

The expected dS_{exp} values were calculated as

$$dS_{\text{exp}} = \frac{4(1 + 2\alpha)}{3(1 + \alpha)} \mu t,$$

where α is the male mutation bias (in this case we used $\alpha = 3$ for all strata and species, as determined for mammals⁸⁶), μ is the annual autosomal mutation rate and t is the time of stratum formation^{59,11}; S1 = 181 MYA, S2 = 117 MYA, S3 = 116 MYA, S4 = 42.8 MYA and S5 = 33 MYA. If the empirical μ was not available for a given species, we assigned it the μ of its closest available relative. Given that dS_{exp} is based on μ values estimated for current populations, the ratio dS/dS_{exp} is informative in relation to the extent of change in the mutation rate experienced throughout the 181 Myr period of stratum evolution, relative to the current empirical mutation rate.

Ancestral Y sequence order reconstruction in S4 and S5

X chromosome conservation was confirmed with lastZ alignment among multiple primates (Supplementary Notes). For eight species with high-level assemblies on both X and Y (*Nycticebus pygmaeus*, *Callithrix jacchus*, *Macaca mulatta*, *Rhinopithecus roxellana*, *Colobus guereza*, *Hylobates pileatus*, *Pan troglodytes* and *Homo sapiens*), pairwise alignments between X and Y sequences were performed using lastZ with the same parameter set as above. Dot plots were produced using custom Python scripts for reciprocal best MAF results. To reconstruct the ancestral sequence order, the Y assemblies in these eight species were aligned to human X with lastZ using the same parameters mentioned above. PAR sequences were added to the Y if PAR was linked with nonPARX in the assembly. Conserved segments under 50 kb or 30 kb were then extracted with DESCrambler (git commit c52d775), and the reconstruction was performed using the MLGO web server (<http://www.geneorder.org/server.php>)⁸⁷, which allows for segment gains/losses during evolution, with tree structure: '((NYCPYG,(CALJAC,(MACMUL,(RHIOX,COLGUE)),(HYLPIL,(PANTRO,HOMSAP))))),HOMSAPX)'. The initial reconstruction at *Simiiformes* MRCA contained SVs involving both S4 and S5, which contradicted the argument that S5 evolved after the Platyrrhini–Catarrhini split. Thus, we manually curated the reconstruction at *Simiiformes* MRCA. PAR segments of MACMUL were also curated in order to be included. Conserved blocks in both parental and child nodes were retained when we inferred the evolutionary events between each of the two closest nodes with GRIMM (v2.01) (ref. ⁸⁸) by manual curation.

Chromatin configuration evolution analysis

Hi-C reads were sampled into -30X for *Nycticebus pygmaeus*, *Callithrix jacchus*, *Macaca mulatta*, *Rhinopithecus roxellana*, *Colobus guereza*, *Hylobates pileatus*, *Homo sapiens* and *Saguinus midas*, and mapped to the corresponding genome with juicer (v1.6). The resulting 'merged_nodups.txt' were used to generate Hi-C contact maps under different resolutions (10 kb, 20 kb, 50 kb, 100 kb, 200 kb, 500 kb, 1 Mb, 2 Mb and 5 Mb) with cooler (v0.8.11) (ref. ⁸⁹), converted to h5 format and normalized using the KR method with hicexplorer (v3.7.2) (ref. ⁹⁰). Regional Hi-C maps were visualized by hicPlotMatrix in the hicexplorer package.

To obtain homologous blocks between X and Y within a species, Y-linked sequences were aligned to X-linked sequences with LastZ with the same parameter set as above. Reciprocal best results were obtained according to the guidance provided at <http://genomewiki.ucsc.edu/>

index.php/HowTo:Syntenic_Net_or_Reciprocal_Best. X/Y homologous blocks within S4 and S5 were obtained on the basis of the reciprocal NET result by 'netFilter' from kent package (v351). We required homologous blocks of ≥ 500 kb in both X and Y, and a length ratio of X and Y syntenic blocks between 0.2 and 5. Filtered homologous block pairs were manually examined with MAF alignment to exclude false positives. To evaluate the similarity of the Hi-C contact maps between homologous blocks, chess (v0.3.7) (ref. ⁴³) was used to calculate the structural similarity scores (SSIM) and signal-to-noise ratio (SN). When examining the changes between X and Y, we used the 25 kb diploid human autosomal Hi-C map generated by ref. ⁹¹. Chess was run in 1 Mb windows as a control, given that the median length of the X/Y homologue blocks is about 1.5 Mb. After excluding blocks with low SN (estimated as 10% percentile of the background), *P* values were calculated on the basis of the background SSIM distribution and adjusted with FDR. When examining the changes from PAR to nonPARX, we used the regions on the X chromosome that had diverged ancestrally as the control. Hi-C maps were at 20 kb resolution and the syntenic block was required to be ≥ 500 kb. Chess was run between each primate and *Nycticebus pygmaeus* or treeshrew. After excluding blocks with low SN, a Wilcoxon rank-sum test was used to test for significant SSIM differences between ancestral PAR (*Simiiformes* S4, S5 and PAR) and ancestral SDR that evolved before the primate MRCA. TAD boundaries were identified with hicexplorer in 50 kb resolution Hi-C map under 0.01 FDR-corrected *P* value cut-off, and visualized with hicPlotTADs. We determined the conservation of a boundary following a similar method to that in ref. ⁹². Specifically, TAD boundaries were lifted over to the coordinates in *Nycticebus pygmaeus* (or treeshrew) with CrossMap (v0.5.4) (ref. ⁹³). If the lifted boundary was within a 100 kb (two bins) region to a *Nycticebus pygmaeus* (or treeshrew) TAD boundary, the TAD boundary was defined as being conserved between the two species.

Y gene evolutionary analysis

All-versus-all BLASTP was performed with Y gene protein sequences from primates and mouse using BLAST+ (v2.2.31) under an 1×10^{-5} e-value cut-off and the gene family clustering was performed with mcl (v14-137) (ref. ⁹⁴) (Supplementary Notes). We manually examined the mcl output and curated the clusters based on the gene name and the pairwise BLASTP score. Y-linked genes recovered from raw reads and Y-linked gene states (presence/pseudo/absence) from refs. ^{7,11,21,95} were used to curate a presence/absence matrix. When drawing inferences as to whether a Y-linked gene was present in each primate ancestor, we first converted the gene number into a presence/absence matrix and inferred with Count (v10.04) (ref. ⁹⁶). Pseudogenes were treated as 'present' before inference, and converted back into 'pseudo' after Count inference. The X-degenerate Y-linked genes were inferred with Dollo parsimony, whereas the other Y-linked genes were inferred with Wagner parsimony. We also considered the evolutionary history of S5 when inferring the ancestral states of S5 genes. Gene loss was visualized with the 'ete3' Python package (v3.1.2) (ref. ⁹⁷).

Primate species' life history traits, including the age of male sexual maturity, male-versus-female body mass dimorphism, testis mass and morphology of sperm (sperm total length and head length) were collected from previous studies and four databases: AnAge⁹⁸, the Animal Diversity Web (<https://animaldiversity.org/>), All the World's Primates⁶³ and Pan-THEIRA⁹⁹ (Supplementary Data 12). Orthologous Y-linked genes were obtained from the MCL clustering result. If multiple copies were available for one species, we randomly selected one. CDS of orthologous genes were aligned with PRANK. The alignment was further cleaned by Gblock (v0.91b)¹⁰⁰ with default parameters and estimate *dN* and *dS* values for each branch with codeml (paml package¹⁰¹) under the free-ratios model. Root-to-tip *dN*, *dS* and *dN/dS* values were calculated. Only species with root-to-tip *dN/dS* ratios between 0.001 and 5 were retained for downstream analysis. To test the potential relationships between gene evolution (root-to-tip

dN/dS) and life history traits, the R package ‘caper’ (v1.0.1) was used to perform phylogenetic generalized least squares (PGLS) analysis between root-to-tip dN/dS and each trait. At least eight data points were required. Sperm total length, sperm head length and testis size were first scaled with male body mass, then log-transformed. The age of male sexual maturity was log-transformed. Outlier values that fell outside of the interquartile range were excluded and PGLS was re-run to confirm the regression significance. We required P values <0.05 , and plotted the transformed traits and dN/dS with the regression lines excluding the outliers.

AG analysis

If $>50\%$ of a gene overlapped with the ampliconic region, we considered it to be a candidate AG. Sequences of candidate AGs, including CDS and introns, were BLASTed to the ampliconic region again. Only genes with $>99\%$ identity and $>80\%$ alignment rate were retained. The BLASTed regions were merged with bedtools to remove redundancy. Candidate AGs that hit against only themselves were removed. To obtain the gene orthologous relationships between species, we clustered the X-linked or Y-linked genes using the same mcl workflow described in ‘Y gene evolutionary analysis’. Families that contained AGs in at least one species were retained for evolutionary analysis. For each family, if multiple genes were found in the same species but were not defined as ampliconic, these genes were classified as multiple copy. We calculated the number of gene families that contained both AGs and multi-copy genes, contained only AGs, contained only multi-copy genes, and contained neither AGs nor multi-copy genes. The resulting contingency table (Supplementary Data 16) was used for a Fisher’s exact test.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Primate long- and short-read sequencing data were obtained from the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) Database (<https://www.ncbi.nlm.nih.gov/sra/>) under accession code [PRJNA785018](#), [PRJNA658635](#) and [PRJEB49549](#), and the GSA database with project no. PRJCA003786. Sequencing data and curated assemblies used in this study have been deposited in the NCBI Assembly Database (<https://www.ncbi.nlm.nih.gov/assembly/>) under accession code [PRJNA790674](#) and the CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb) with accession number CNP0002500. Human diploid Hi-C mapping data were obtained from <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63525>. Other data are available in the main text or the supplementary data. Analytical data have been deposited into figshare with the link <https://doi.org/10.6084/m9.figshare.20115467.v1>.

Code availability

Custom scripts are available at: https://github.com/zy041225/primate_sex_chromosome.

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Acknowledgements

We thank all supervisors, collaborators and anyone else involved with the collection and processing of the primary datasets. We thank China National GeneBank for providing the computational resources. This study was supported by grants from Strategic Priority Research Program of the Chinese Academy of Sciences (XDB31020000 to G.Z.), International Partnership Program of Chinese Academy of Sciences (no. 152453KYSB20170002 to G.Z.), Villum Investigator Grant (no. 25900 to G.Z.), National Natural Science Foundation of China (31822048 to D.-D.W.), Yunnan Fundamental Research Project (2019FI010 to D.-D.W.) and The Animal Branch of the Germplasm Bank of Wild Species of Chinese Academy of Science (the Large Research Infrastructure Funding to D.-D.W.).

Author contributions

G.Z. conceived the project. D.-D.W., H.K., T.H., Y.-G.Y., La.Z., X.Q., L.K. and T.M.-B. coordinated and were involved in sample collection, extraction and sequencing. Y.Z., X.Z., J.J., Lo.Z., J.B., X.L., M.M.C.R., M.R.B., M.F., J.C. and Q.F. performed the analyses. G.Z., M.H.S. and H.Y. supervised the project. G.Z., D.N.C., M.H.S., Y.Z., M.R.B., J.B., M.M.C.R., Y.-G.Y. and T.M.-B. wrote the manuscript with input from all the authors.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at <https://doi.org/10.1038/s41559-022-01974-x>.

Correspondence and requests for materials should be addressed to Guojie Zhang.

Peer review information *Nature Ecology & Evolution* thanks Qi Zhou and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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¹BGI-Shenzhen, Shenzhen, China. ²Centre for Evolutionary & Organismal Biology, and Women's Hospital, Zhejiang University School of Medicine, Hangzhou, China. ³Liangzhu Laboratory, Zhejiang University Medical Center, Hangzhou, China. ⁴Section for Ecoinformatics & Biodiversity, Department of Biology, Aarhus University, Aarhus C., Denmark. ⁵Bioinformatics Research Centre, Aarhus University, Aarhus C., Denmark. ⁶College of Life Sciences, University of Chinese Academy of Sciences, Beijing, China. ⁷College of Life Sciences, Northwest University, Xi'an, China. ⁸Institute of Evolutionary Biology (UPF-CSIC), PRBB, Barcelona, Spain. ⁹Catalan Institution of Research and Advanced Studies (ICREA), Barcelona, Spain. ¹⁰CNAG-CRG, Centre for Genomic Regulation (CRG), Barcelona Institute of Science and Technology (BIST), Barcelona, Spain. ¹¹Institut Català de Paleontologia Miquel Crusafont, Universitat Autònoma de Barcelona, Barcelona, Spain. ¹²Graduate School of Environmental Science, Hokkaido University, Sapporo, Japan. ¹³Faculty of Environmental Earth Science, Hokkaido University, Sapporo, Japan. ¹⁴Japan Monkey Centre, Inuyama, Japan. ¹⁵Kunming College of Life Science, University of the Chinese Academy of Sciences, Kunming, China. ¹⁶Key Laboratory of Animal Models and Human Disease Mechanisms of Chinese Academy of Sciences & Yunnan Province, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China. ¹⁷Center for Excellence in Animal Evolution and Genetics, Chinese Academy of Sciences, Kunming, China. ¹⁸National Resource Center for Non-Human Primates, Kunming Primate Research Center, and National Research Facility for Phenotypic & Genetic Analysis of Model Animals (Primate Facility), Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China. ¹⁹KIZ-CUHK Joint Laboratory of Bioresources and Molecular Research in Common Diseases, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China. ²⁰James D. Watson Institute of Genome Sciences, Hangzhou, China. ²¹Guangdong Provincial Academician Workstation of BGI Synthetic Genomics, BGI-Shenzhen, Shenzhen, China. ²²The Cancer Hospital of the University of Chinese Academy of Sciences (Zhejiang Cancer Hospital), Institute of Basic Medicine and Cancer (IBMC), Chinese Academy of Sciences, Hangzhou, China. ²³Institute of Medical Genetics, School of Medicine, Cardiff University, Cardiff, UK. ²⁴State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China. ²⁵Section for Ecology and Evolution, Department of Biology, University of Copenhagen, Copenhagen, Denmark. ✉ e-mail: guojiezhang@zju.edu.cn

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| <input type="checkbox"/> | <input checked="" type="checkbox"/> A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
<i>Give P values as exact values whenever suitable.</i> |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| <input type="checkbox"/> | <input checked="" type="checkbox"/> Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated |

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

- | | |
|-----------------|--|
| Data collection | Data collection did not involve any software or code. |
| Data analysis | Common bioinformatic and statistical analysis software packages were used, including: caper (v1.0.1), Gblock (v0.91), PRANK (v150803), GeneWise (v2.4.1), Augustus (v3.2.3), bedtools (v2.29.2), blast+ (v2.2.31), solar (v0.9.6), KaKs_Calculator (v2.0), RAxML (v8.2.4), MAFFT (v7.471), iqtree (v2.1.3), bwa (v0.7.17), SDA (git commit 4ca0c0709dc86181afaeeeee862543dc19a411eb3), ggplot2 (v3.3.5), samtools (v1.9), freebayes (v1.3.1-17-gaa2ace8), bcftools (v1.11), diamond (v2.0.0), BASTA (v1.3.2.3), exonerate (v2.4.0), pbmm2 (v1.2.0-1-g31b4be0), minimap2 (v2.17), juicer (v1.6), 3d-dna (v180922), straw (v0.0.8), matplotlib (v3.4.2), bowtie2 (v2.4.1), wtdbg2 (v2.3), lastz (v1.04.00), DESCRAMBLER (git commit c52d775), MLGO (http://www.geneorder.org/), GRIMM (v2.01), cooler (v0.8.11), hicexplorer (v3.7.2), liftOver (v351), netFilter (v351), chess (v0.3.7), CrossMap (v0.5.4), mcl (v14-137), Count (v10.04), ete3 (v3.1.2), hmmer (v3.1b2), paml (v4.8) |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

Primate long- and short-read sequencing data were obtained from National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) Database (<https://www.ncbi.nlm.nih.gov/sra/>) under accession code PRJNA785018, PRJNA658635 and PRJEB49549, and GSA database with project no. PRJCA003786. Sequencing data and curated assemblies in used this study have been deposited into National Center for Biotechnology Information (NCBI) Assembly Database (<https://www.ncbi.nlm.nih.gov/assembly/>) under accession code PRJNA790674 and CNGB Sequence Archive (CNSA) of China National GeneBank DataBase (CNGBdb) with accession number CNP0002500. Human diploid Hi-C map was obtained from: <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE63525>. Other data are available in the main text or the supplementary materials. Analysis data have been deposited into figshare with the link: <https://doi.org/10.6084/m9.figshare.20115467.v1>

Human research participants

Policy information about [studies involving human research participants and Sex and Gender in Research](#).

Reporting on sex and gender	Study only uses human genome (hg38) which contains both X and Y chromosomes.
Population characteristics	Study does not involve human population data
Recruitment	Study does not recruit human participants
Ethics oversight	Study only uses human genome (hg38) which contains both X and Y chromosomes.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

Life sciences Behavioural & social sciences Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	The determination of sample size for genome sequencing is not applied in this study. Bioinformatic analyses were performed with all available data.
Data exclusions	Bioinformatic analyses were performed with all available data.
Replication	Replication is not applied for genomes sequencing.
Randomization	Randomization for genome sequencing is not applied in this study.
Blinding	Blinding was not necessary for genome sequencing

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involvement
<input checked="" type="checkbox"/>	<input type="checkbox"/> Antibodies
<input checked="" type="checkbox"/>	<input type="checkbox"/> Eukaryotic cell lines
<input checked="" type="checkbox"/>	<input type="checkbox"/> Palaeontology and archaeology
<input type="checkbox"/>	<input checked="" type="checkbox"/> Animals and other organisms
<input checked="" type="checkbox"/>	<input type="checkbox"/> Clinical data
<input checked="" type="checkbox"/>	<input type="checkbox"/> Dual use research of concern

Methods

n/a	Involvement
<input checked="" type="checkbox"/>	<input type="checkbox"/> ChIP-seq
<input checked="" type="checkbox"/>	<input type="checkbox"/> Flow cytometry
<input checked="" type="checkbox"/>	<input type="checkbox"/> MRI-based neuroimaging

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals

Blood samples of female *Macaca silenus*, *Papio hamadryas*, *Erythrocebus patas*, *Colobus guereza* and muscle sample of female *Hylobates pileatus* were collected from Japan Monkey Centre, Japan.

Wild animals

Study did not involve wild animals.

Reporting on sex

Both sex genomic data are used in this study.

Field-collected samples

Study did not involve field-collected samples.

Ethics oversight

The use of genetic materials of Japan Monkey Centre was approved by the Research Ethics Committee of the Japan Monkey Centre (#2017-018) and performed in accordance with the Ethical Guidelines for Research at the Japan Monkey Centre (1 April 2016).

Note that full information on the approval of the study protocol must also be provided in the manuscript.