

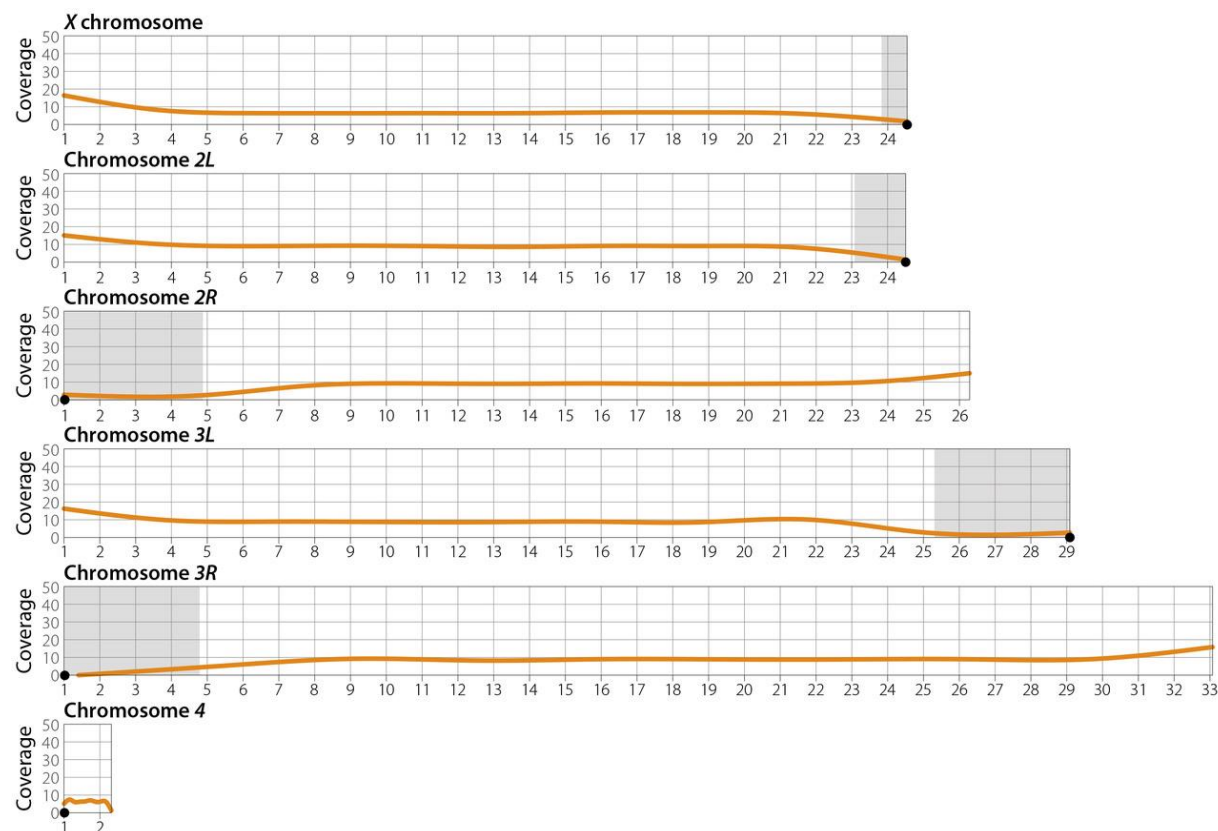
Figure S1

Figure S1 Depth of coverage was calculated using reads that uniquely map to non-repetitive regions of release 6 of the *D. melanogaster* genome. Black circles indicate centromeres, gray boxes are the approximate position of heterochromatin. Coverage was calculated in 5-kb non-overlapping windows. Coverage is relatively even across all five chromosome arms, suggesting the absence of euchromatic sequence on the B chromosomes.

Figure S2

Figure S2 Fluorescent *in situ* hybridization (FISH) on chromosome spreads from neuroblast squashes. Probes recognizing the AAGAG simple satellite repeat hybridized to the B chromosomes (A), but probes for the AACAC repeat (B), AACATAGAAT repeat (C), AAAAG repeat (D), or the intergenic spacer (IGS) between ribosomal DNA repeats (E and F) did not hybridize to the B chromosomes. See Table S2 for probe details. Scale bar = 5 μ m

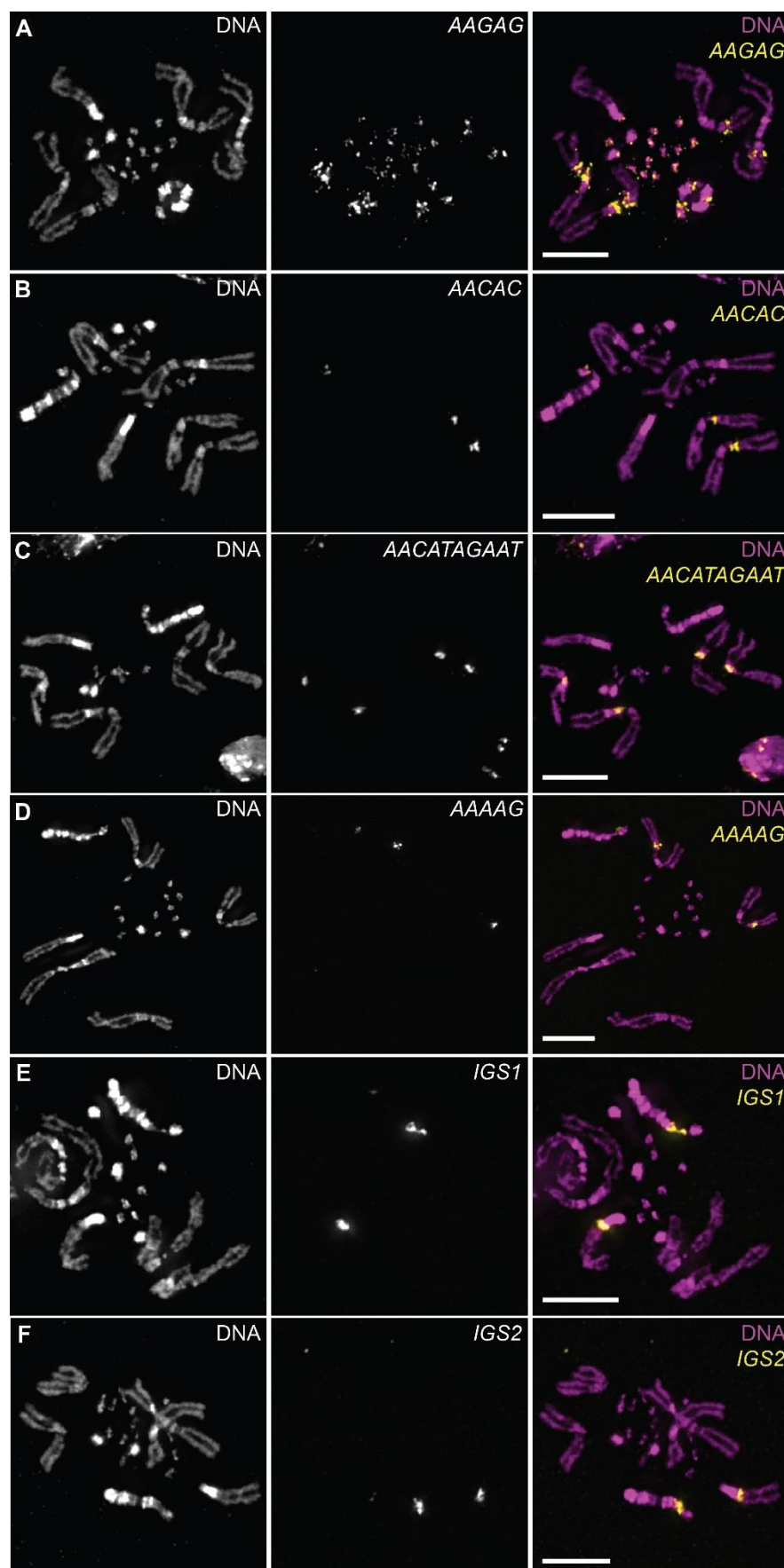


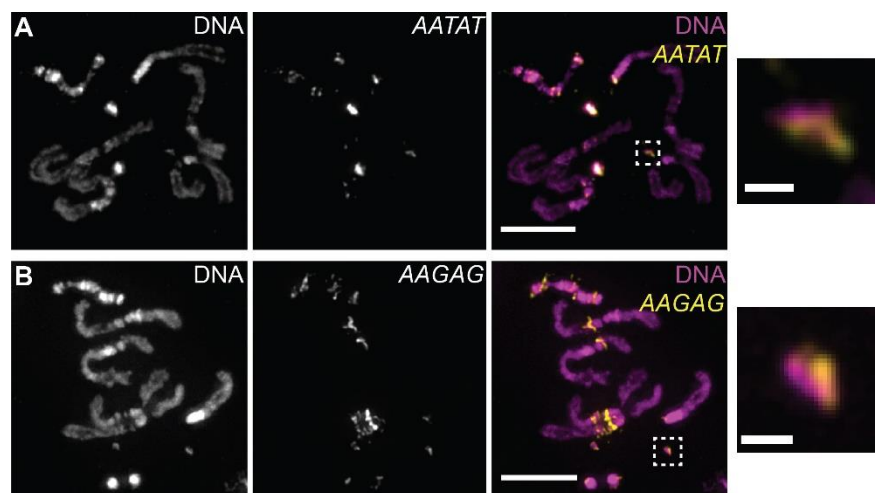
Figure S3

Figure S3 The B2 chromosome carries the same suite of satellite repeats as the B1 chromosome. FISH probes recognizing the *AATAT* (A) and the *AAGAG* (B) satellite repeat hybridize to the B2 chromosome in metaphase chromosome spreads. Scale bar in whole spreads = 5 μm; scale bar in magnifications = 0.5 μm.

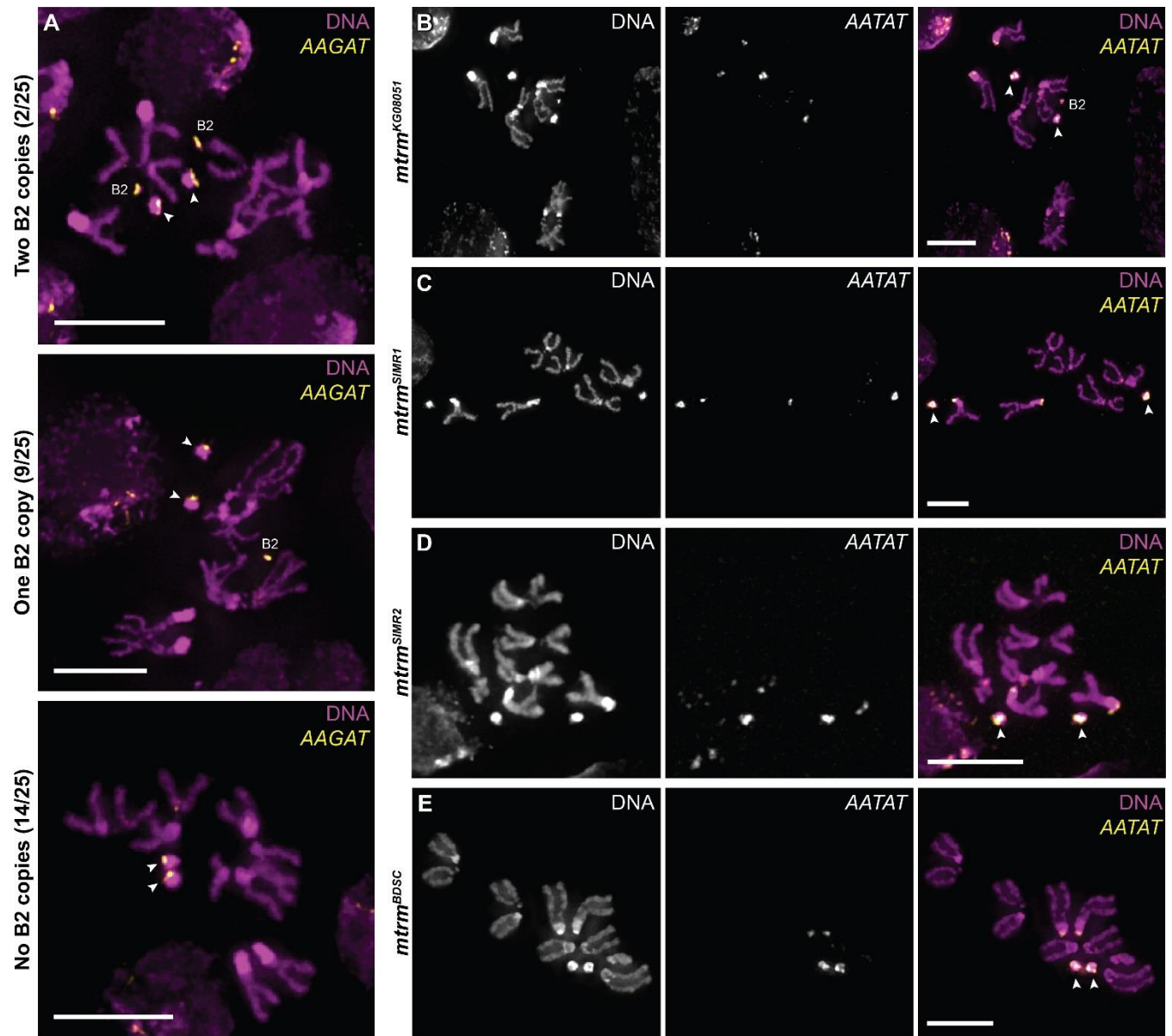
Figure S4

Figure S4 B chromosomes are only found in the laboratory *mtrm*^{KG08051} stock. (A) FISH with the AAGAT probe on metaphase chromosome spreads from ovary tissue of *mtrm*^{KG08051} females.

Examination of 25 individuals showed two carried two copies of the B2 chromosome, nine carried one copy, and the remaining 14 did not carry any. FISH with the AATAT probe on metaphase chromosome spreads from ovary tissue of *mtrm*^{KG08051} (B), *mtrm*^{SIMR1} (C), *mtrm*^{SIMR2} (D), and *mtrm*^{BDSC} (E) females.

Table S1 *Drosophila melanogaster* stocks used in this study.

Name	Genotype	Type of B chromosome (if present)	Average number of B chromosomes (if present)	Comments
<i>mtrm</i> ¹²⁶	<i>y w/y⁺ Y; mtrm</i> ¹²⁶ / <i>TM3, Sb Ser; sv</i> ^{spa-pol}	B1	10–12	Stock with original B (B1) chromosomes; documented in Bauerly <i>et al.</i> (2014)
<i>mtrm</i> ^{KG08051}		B2	1	Kept in our laboratory collection
<i>mtrm</i> ^{SIMR1}	<i>y</i> ¹ ; <i>P{SUPor-P}Exo70</i> ^{KG08051}	None found	N/A	Housed in the Stowers Institute collection
<i>mtrm</i> ^{SIMR2}	<i>mtrm</i> ^{KG08051} <i>ry</i> ⁵⁰⁶ / <i>TM3, Sb</i> ¹ <i>Ser</i> ¹	None found	N/A	Housed in the Stowers Institute collection
<i>mtrm</i> ^{BDSC}		None found	N/A	Obtained from Bloomington Drosophila Stock Center; stock number 14932
wild-type (WT)	<i>y w/y⁺ Y; sv</i> ^{spa-pol}	None	N/A	Hawley lab WT

Table S2 Fluorescent probes used for FISH in this study.

Probe name	Sequence 5'→3'	Fluorophore ^a
AATAT	AATATAATATAATATAATATAATATAATAT	Alexa Fluor 546
AAGAG	AAGAGAAGAGAAGAGAAGAGAAGAGAAGAG	Alexa Fluor 488
AAGAT	AAGATAAGATAAGATAAGATAAGATAAGAT	Alexa Fluor 488
AACAC	AACACAACACAACACAACACAACACAACAC	Alexa Fluor 488
AAAAG	AAAAGAAAAGAAAAGAAAAGAAAAGAAAAG	Alexa Fluor 488
AATAACATAG	AACATAGAATAACATAGAATAACATAGAAT	Alexa Fluor 488
IGS1	GTATGTGTTTCATATGATTTTGGCAATTATA	Alexa Fluor 555
IGS2	ATATTCCCATATTCTCTAAGTATTATAGAG	Alexa Fluor 555

^a Probes are conjugated to an Alexa Fluor dye at the 5' end and were produced by Integrated DNA Technologies (IDT) except for the two IGS probes, which were produced by Eurofins.

Table S3 Depth of coverage across chromosomes.

Chromosome or arm	Estimated size (bp)	Mapped reads ^a	% of all mapped reads	Depth of coverage (x)
X	23,542,271	2,128,775	16%	14
2L	23,513,712	1,770,270	14%	11
2R	25,286,936	2,067,093	16%	12
3L	28,110,227	2,017,347	15%	11
3R	32,079,331	2,325,354	18%	11
4	1,348,131	683,865	5%	76
Y	3,667,352	625,677	5%	26
All	133,880,608	13,031,938	100%	10

^a All read lengths were 150 bp.

Table S4 Alignment of unmapped reads to contigs.

Chromosome	Base pairs with more than 100 reads aligning to them	Chromosome size	Percentage of chromosome covered by more than 100 reads
chrUn_DS485957v1	877	1,013	87%
chrUn_DS483562v1	40,698	50,625	80%
chrUn_DS485102v1	918	1,240	74%
chrUn_DS483906v1	2,293	3,924	58%
chrUn_DS485695v2	285	564	51%
chrUn_DS485504v1	462	1,123	41%
chrUn_DS483770v1	2,481	6,193	40%
chrUn_DS484689v1	659	1,650	40%
chrX_DS485647v1_random	423	1,086	39%
chrUn_DS485995v1	344	1,004	34%
chrX_DS484701v1_random	526	1,625	32%
chrX_DS485351v1_random	353	1,161	30%
chrUn_DS485073v1	381	1,259	30%
chrUn_DS484972v1	389	1,310	30%
chrX_DS484765v1_random	416	1,516	27%
chrUn_DS485390v1	306	1,152	27%
chrX_DS485514v1_random	293	1,121	26%
chrUn_DS485433v1	283	1,140	25%
chrUn_DS485341v1	278	1,162	24%
chrUn_DS485255v1	273	1,190	23%
chrX_DS484555v1_random	415	1,916	22%
chrUn_DS485958v1	219	1,013	22%
chrUn_DS485340v1	242	1,163	21%
chrUn_DS484751v1	303	1,533	20%
chrX_DS484187v1_random	526	2,671	20%
chrUn_DS485249v1	233	1,192	20%
chrUn_DS484861v1	267	1,395	19%
chrUn_DS484567v1	360	1,900	19%
chrX_DS484626v1_random	335	1,786	19%
chrUn_DS485984v1	187	1,006	19%
chrX_DS485855v1_random	188	1,037	18%
chrX_DS483818v1_random	865	4,917	18%
chrUn_DS485386v1	200	1,153	17%
chrX_DS484995v1_random	216	1,296	17%
chrX_DS484529v1_random	314	1,947	16%
chrUn_DS484748v1	243	1,538	16%
chrX_DS484679v1_random	265	1,683	16%
chrUn_DS484938v1	203	1,334	15%

Chromosome	Base pairs with more than 100 reads aligning to them	Chromosome size	Percentage of chromosome covered by more than 100 reads
chrX_DS484610v1_random	261	1,813	14%
chrX_DS485141v1_random	165	1,228	13%
chrUn_DS484280v1	319	2,438	13%
chrUn_DS485773v1	135	1,054	13%
chrX_DS484809v1_random	183	1,453	13%
chrX_DS485599v1_random	131	1,100	12%
chrX_DS484556v1_random	226	1,916	12%
chrY_CP007111v1_random	4,039	34,521	12%
chrUn_DS484106v1	330	2,884	11%
chrUn_DS485086v1	142	1,250	11%
chrUn_DS484923v1	148	1,343	11%
chrX_DS484955v1_random	144	1,323	11%
chrX_DS484582v1_random	198	1,870	11%
chrX_DS484677v1_random	175	1,687	10%
chrX_DS485081v1_random	129	1,252	10%
chrX_DS485765v1_random	108	1,056	10%
chrUn_DS484007v1	327	3,262	10%
chrX_DS484507v1_random	192	1,978	10%
chrX_DS485735v1_random	99	1,063	9%
chrUn_DS484484v1	182	2,020	9%
chrUn_DS484180v1	240	2,697	9%
chrUn_DS485919v1	86	1,021	8%
chrX_DS484057v1_random	259	3,076	8%
chrX_DS484730v1_random	131	1,560	8%
chrX_DS484672v1_random	135	1,703	8%
chrX_DS484695v1_random	129	1,637	8%
chrUn_DS484068v1	233	3,013	8%
chrX_DS485454v1_random	82	1,135	7%
chrX_DS484388v1_random	159	2,209	7%
chrX_DS484618v1_random	128	1,800	7%
chrUn_DS483954v1	247	3,554	7%
chrUn_DS483918v1	260	3,818	7%
chrX_DS484545v1_random	131	1,928	7%
chrX_DS484429v1_random	136	2,113	6%
chrX_DS484433v1_random	130	2,109	6%
chrX_DS484664v1_random	105	1,713	6%
chrX_DS484361v1_random	139	2,282	6%
chrY_DS484094v1_random	177	2,922	6%
chrY_DS483677v1_random	747	12,513	6%
chrX_DS483955v1_random	210	3,553	6%

Chromosome	Base pairs with more than 100 reads aligning to them	Chromosome size	Percentage of chromosome covered by more than 100 reads
chrX_DS484647v1_random	102	1,743	6%
chrUn_DS483755v1	382	6,936	6%
chrX_DS484580v1_random	91	1,871	5%
chrUn_DS484183v1	130	2,688	5%
chrUn_DS484226v1	118	2,576	5%
chrX_DS484305v1_random	101	2,389	4%
chrX_DS483803v1_random	218	5,232	4%
chrX_DS484046v1_random	125	3,123	4%
chrX_DS484023v1_random	115	3,206	4%
chrX_DS484600v1_random	66	1,841	4%
chrUn_CP007100v1	329	10,091	3%
chrX_DS485898v1_random	33	1,028	3%
chrX_DS484430v1_random	62	2,113	3%
chrX_DS484344v1_random	60	2,322	3%
chrUn_DS484320v1	59	2,369	2%
chrUn_CP007120v1	1,855	76,973	2%
chrUn_DS484591v1	43	1,854	2%
chrX_DS483655v1_random	299	13,549	2%
chrUn_DS484054v1	65	3,080	2%
chrX_DS484178v1_random	56	2,698	2%
chrX_DS485801v1_random	21	1,048	2%
chrUn_DS485897v1	20	1,028	2%
chrX_DS484697v1_random	31	1,636	2%
chrX_DS484166v1_random	48	2,750	2%
chrUn_DS485297v1	20	1,178	2%
chrX_DS483950v1_random	56	3,582	2%
chrY_DS484956v1_random	20	1,322	2%
chrUn_DS484720v1	20	1,586	1%
chrX_DS484725v1_random	18	1,573	1%
chrUn_DS483650v1	155	13,906	1%
chrUn_DS484241v1	28	2,547	1%
chr4	11,185	1,348,131	1%
chrUn_DS484309v1	18	2,384	1%
chrUn_DS485261v1	8	1,189	1%
chrY_CP007110v1_random	207	33,316	1%
chrUn_DS483707v1	151	25,840	1%
chrUn_CP007083v1	148	25,537	1%
chrUn_DS483705v1	145	27,456	1%
chrUn_DS483681v1	65	12,368	1%
chrUn_DS483921v1	20	3,806	1%

Chromosome	Base pairs with more than 100 reads aligning to them	Chromosome size	Percentage of chromosome covered by more than 100 reads
chrUn_DS484093v1	11	2,922	0%
chrUn_CP007088v1	133	37,106	0%
chrUn_DS483774v1	20	6,041	0%
chrX_CP007103v1_random	89	33,320	0%
chrY_CP007119v1_random	20	11,498	0%
chrY_CP007112v1_random	67	39,041	0%
chrUn_DS483675v1	20	12,536	0%
chrUn_DS483736v1	20	14,006	0%
chrX_DS484393v1_random	3	2,201	0%
chrY	4,689	3,667,352	0%
chrUn_CP007091v1	21	20,763	0%
chrX	22,771	23,542,271	0%
chrUn_CP007095v1	20	25,560	0%
chrY_CP007116v1_random	20	25,805	0%
chrUn_CP007081v1	60	88,768	0%
Total base pairs	114,673		