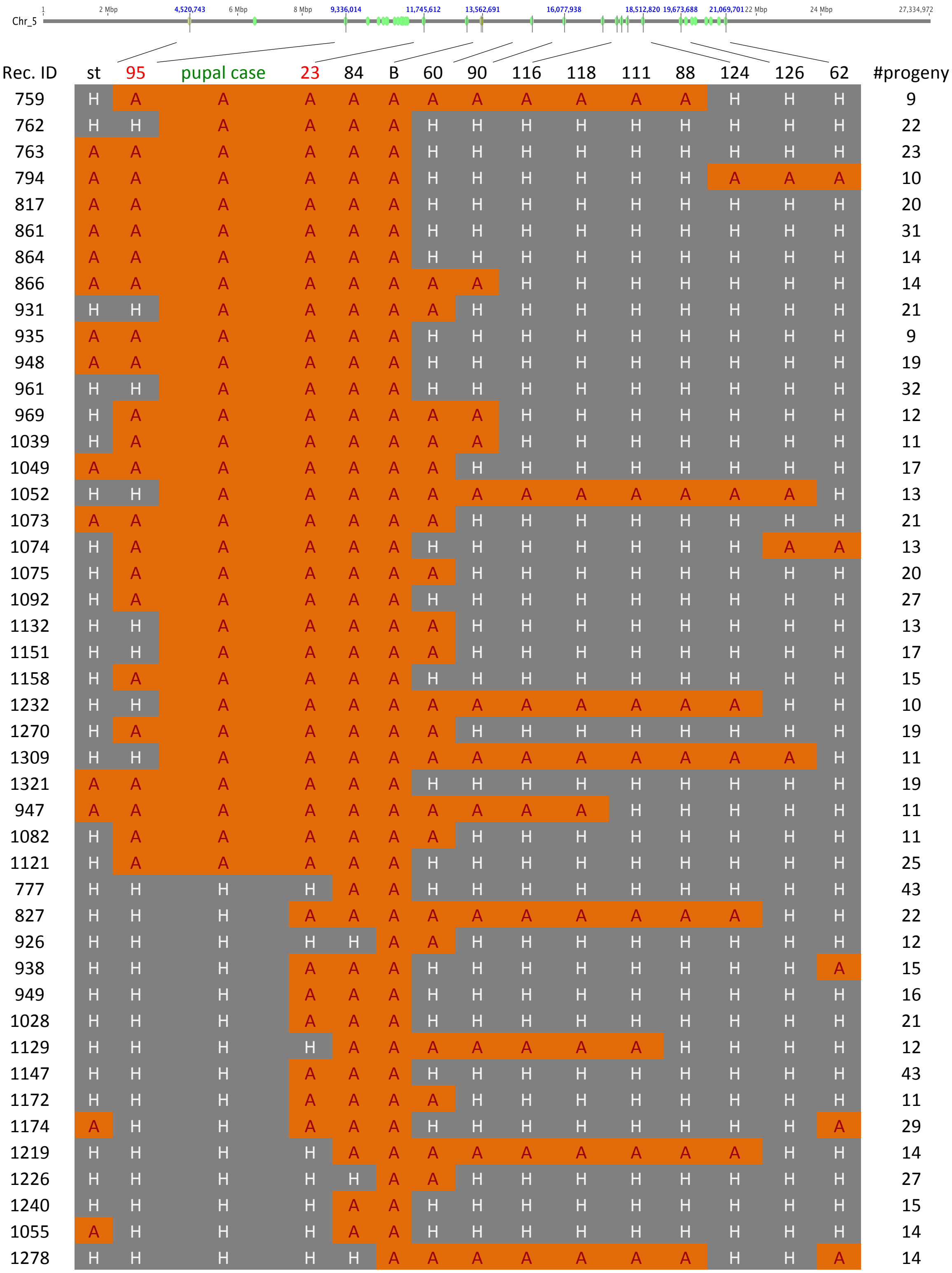
Photo credit: David Duneau

Figure S1: *D. americana* (left) and *D. virilis* (right) pupae at stage 3 (see Materials and Methods).



Rec. ID	st	95	pupal case	23	84	B	60	90	116	118	111	88	124	126	62	#progeny
759	H	A	A	A	A	A	A	A	A	A	A	A	H	H	H	9
762	H	H	A	A	A	A	H	H	H	H	H	H	H	H	H	22
763	A	A	A	A	A	A	H	H	H	H	H	H	H	H	H	23
794	A	A	A	A	A	A	H	H	H	H	H	H	A	A	A	10
817	A	A	A	A	A	A	H	H	H	H	H	H	H	H	H	20
861	A	A	A	A	A	A	H	H	H	H	H	H	H	H	H	31
864	A	A	A	A	A	A	H	H	H	H	H	H	H	H	H	14
866	A	A	A	A	A	A	A	A	H	H	H	H	H	H	H	14
931	H	H	A	A	A	A	A	H	H	H	H	H	H	H	H	21
935	A	A	A	A	A	A	H	H	H	H	H	H	H	H	H	9
948	A	A	A	A	A	A	H	H	H	H	H	H	H	H	H	19
961	H	H	A	A	A	A	H	H	H	H	H	H	H	H	H	32
969	H	A	A	A	A	A	A	A	H	H	H	H	H	H	H	12
1039	H	A	A	A	A	A	A	A	H	H	H	H	H	H	H	11
1049	A	A	A	A	A	A	A	H	H	H	H	H	H	H	H	17
1052	H	H	A	A	A	A	A	A	A	A	A	A	A	A	H	13
1073	A	A	A	A	A	A	A	H	H	H	H	H	H	H	H	21
1074	H	A	A	A	A	A	H	H	H	H	H	H	H	A	A	13
1075	H	A	A	A	A	A	A	H	H	H	H	H	H	H	H	20
1092	H	A	A	A	A	A	H	H	H	H	H	H	H	H	H	27
1132	H	H	A	A	A	A	A	H	H	H	H	H	H	H	H	13
1151	H	H	A	A	A	A	A	H	H	H	H	H	H	H	H	17
1158	H	A	A	A	A	A	H	H	H	H	H	H	H	H	H	15
1232	H	H	A	A	A	A	A	A	A	A	A	A	A	H	H	10
1270	H	A	A	A	A	A	A	H	H	H	H	H	H	H	H	19
1309	H	H	A	A	A	A	A	A	A	A	A	A	A	A	H	11
1321	A	A	A	A	A	A	H	H	H	H	H	H	H	H	H	19
947	A	A	A	A	A	A	A	A	A	A	H	H	H	H	H	11
1082	H	A	A	A	A	A	A	H	H	H	H	H	H	H	H	11
1121	H	A	A	A	A	A	H	H	H	H	H	H	H	H	H	25
777	H	H	H	H	A	A	H	H	H	H	H	H	H	H	H	43
827	H	H	H	A	A	A	A	A	A	A	A	A	A	H	H	22
926	H	H	H	H	H	A	A	H	H	H	H	H	H	H	H	12
938	H	H	H	A	A	A	H	H	H	H	H	H	H	H	A	15
949	H	H	H	A	A	A	H	H	H	H	H	H	H	H	H	16
1028	H	H	H	A	A	A	H	H	H	H	H	H	H	H	H	21
1129	H	H	H	H	A	A	A	A	A	A	A	H	H	H	H	12
1147	H	H	H	A	A	A	H	H	H	H	H	H	H	H	H	43
1172	H	H	H	A	A	A	A	H	H	H	H	H	H	H	H	11
1174	A	H	H	A	A	A	H	H	H	H	H	H	H	H	A	29
1219	H	H	H	H	A	A	A	A	A	A	A	A	A	H	H	14
1226	H	H	H	H	H	A	A	H	H	H	H	H	H	H	H	27
1240	H	H	H	H	A	A	H	H	H	H	H	H	H	H	H	15
1055	A	H	H	H	A	A	H	H	H	H	H	H	H	H	H	14
1278	H	H	H	H	H	A	A	A	A	A	A	A	H	H	A	14

Figure S2: Genotype table of recombinant backcross males from the first round of genotyping (see Materials and Methods). Genotypes at each marker are indicted by letter and color (orange/A = *D. americana* homozygote, gray/H = heterozygote). Pupal case genotype is inferred from progeny pupal case colors and is indicated at the putative location (green) between markers SSR95 and SSR23 (red).

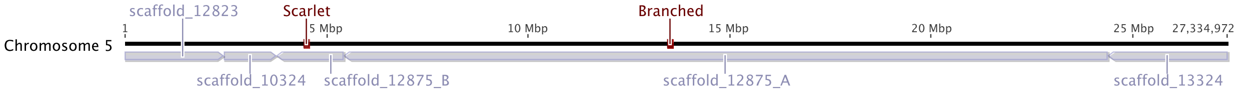


Figure S3: Correspondence of *D. virilis* genome scaffolds (release droVir2, UCSC) to chromosome 5 (Muller Element C; Schaffer *et al*. 2008).

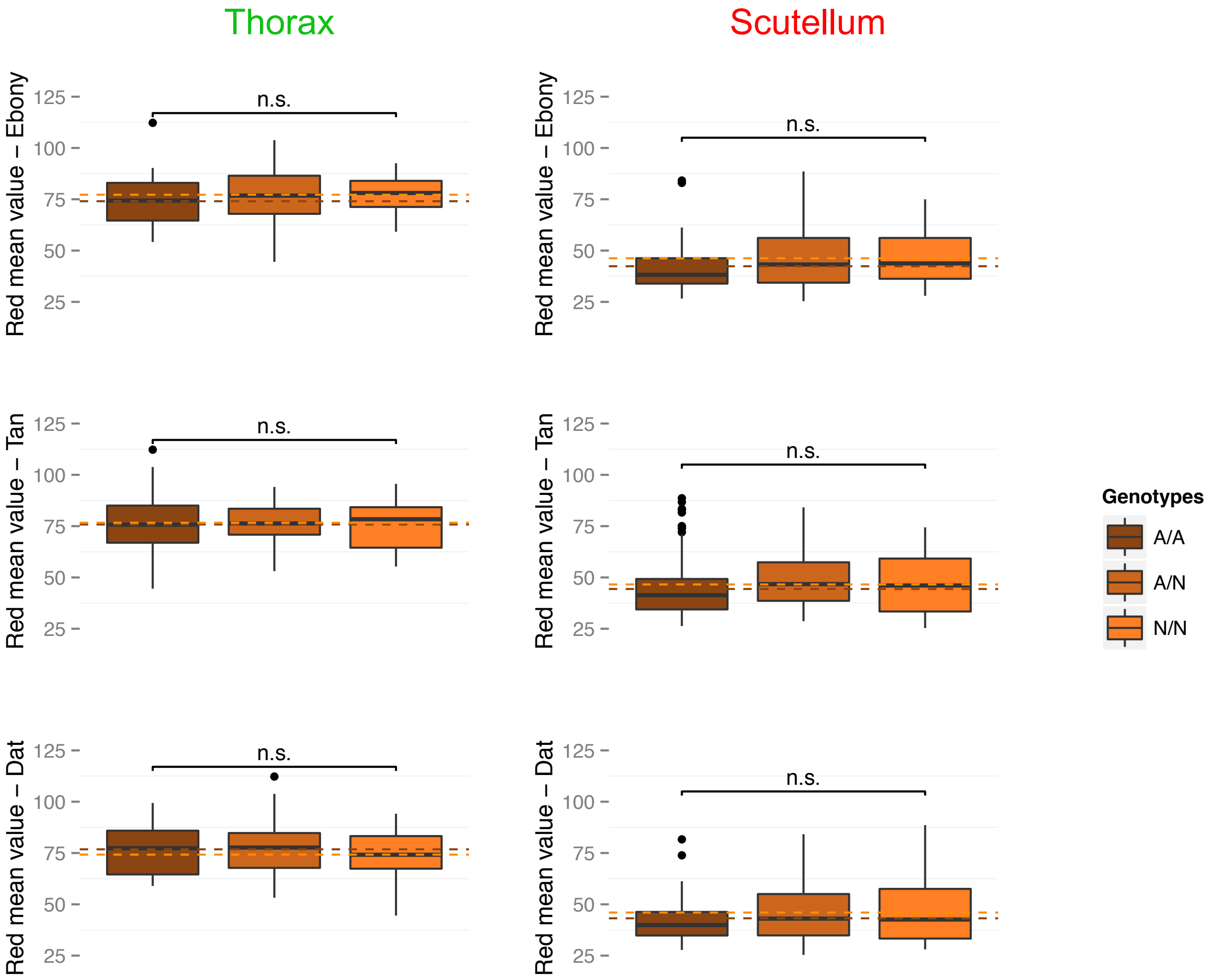


Figure S4: *Boxplot distribution of mean red values in the thorax and scutellum across genotypes for the three pigmentation genes surveyed*: Red mean values are partitioned into the three genotypic classes recovered in the F6 population (n=188). Dashed horizontal lines indicate the mean read value for A/A (brown) and N/N (orange) genotypes.

Table S1: List of genetic markers used in this study, their coordinates, and primer sequences.

Name(type*)	Chr_5 coords.	scaffold_12875 coords.	Forward primer	Reverse primer
***St* (VM)**	4519096-4520743	922609-924256	N/A	N/A
SSR58 (MS)	6545087-6545300	19509634-19509847	TGCCTAGCATTTGGCACTTA	AAAAGAGCGTGGCAAAGAAA
SSR95 (MS)	9335798-9336014	16718920-16719136	TGTGCCTGCTGACAAAACAT	ACACTGCCTGCTTGCATTTA
SSR138 (MS)	10017486-10017694	16037240-16037448	TCGTACCAATGTTGTCAATGC	ACAGGAAGTGGGTGGAAGTG
SSR139 (MS)	10361473-10361687	15693247-15693461	CAAAGATGGAGGAAGCCTCA	CCAGCGCACACTCATAGATT
SSR157 (MS)	10504504-10504720	15550214-15550430	TTTCGTTTGCATTTCCTGTG	GCGTTCGAACCTGAACAAAT
SSR155 (MS)	10604771-10604976	15449958-15450163	TGGGACAAATGTGCCAAATA	ATATGCTCGGTCGGAGATTC
SSR154 (MS)	10621121-10621371	15433563-15433813	CGGGCAGAATCAACTCGTAG	CCGCAGACACTTGAACCAAC
SSR150 (MS)	10864161-10864552	15190382-15190773	GCTATGTTTGCTGGCAGTGA	ATTGCCCAAGCGCATAAATA
SSR149 (MS)	10955634-10955876	15099058-15099300	CAGTACATCGGGCAATTGTG	TGTGGGTGAAGTCAATGTTGT
SSR148 (MS)	11054476-11054716	15000218-15000458	ACGTGCCACATTGCTCATAC	ACAACTTGTCGCGCACTAAA
SSR170 (MS)	11062637-11062844	14992090-14992297	TTTTTCGTTTTCTTCCGTTTTT	ATCTAAGCAAGCGGGTTTCA
SSR169 (MS)	11067738-11067901	14987033-14987196	CATCAAGGAGCTGGCCTATC	ACTTCTCCGCCGTACAACTG
SSR168 (MS)	11080856-11080998	14973936-14974078	CTCCTGGCTGAAAAGCAAAC	CGGCAAACACAATGACAATC
SSR165 (MS)	11083472-11083698	14971236-14971462	TTTGACCAGGGCATGACATA	AAGCACAAGCAAGCACACAC
SSR167 (MS)	11086448-11086593	14968341-14968486	TTCGAATTTCCGCTTGATTT	TATAGCCGGCTCGTTGAAGT
pF10 (SNP)	11089640-11089848	14965086-14965294	AGCTTTAGCTTCCCTTGTGC	TTGAAATTTCGCCGTCCTAC
pE10 (SNP)	11090368-11090570	14964364-14964566	CGCGATCGCAGCTATTCTT	TCCAGATGTGCGGTAAGTGT
pD10 (SNP)	11091748-11091976	14962958-14963186	TCGACGACGACATATTGAGC	GTCTGGCTTTCATCTAAGAGCA
pC10 (SNP)	11093207-11093456	14961478-14961727	AGGGATCGGCCAACTTATC	AAACCCCTCAAGGACAACTG
pB10 (SNP)	11095456-11095683	14959251-14959478	TACGCAACGCCTATCTGAAA	TCAAGCTGTCGTCGTAATCC
pA10 (SNP)	11096841-11097053	14957881-14958093	GATCGTCGCGACTTTCACTA	CGATGGGCAATAATATGGAG
SSR164 (MS)	11099701-11099906	14955028-14955233	TTGTGTTTGTGCTGATGCTG	GCAAAGCAAAAGCTGCGTA
SSR147 (MS)	11129608-11129795	14925139-14925326	TTAGGCGCTGTCACTCACAC	GCGTACAGCTGATAAGCACAG
SSR163 (MS)	11179710-11179876	14875058-14875224	TCAATGCACGAAAATTGGTC	GTTGCGGTAAAATGGATTCG
SSR162 (MS)	11205769-11205921	14849013-14849165	AGGTAAATGTTGGCCAATGC	TTCGAAAGGCATTTTGTTGA
SSR146 (MS)	11245420-11245584	14809350-14809514	GTGACAATTGTTCGCGAGTG	CGCCGTCTGAAAAGAGAAAC
SSR23 (MS)	11745404-11745612	14309322-14309530	AAACTGGCAGATGGGCATAG	CCACGATTTCAGAAGCACAA
SSR84 (MS)	13073885-13074040	12980894-12981049	CAGCATGGAGCATCTGTGTC	TGGAAGGGATGTCATGGACT
B (VM)	13512463-13562691	12492265-12542347	N/A	N/A
SSR60 (MS)	15084664-15084836	10970098-10970270	CAAAAGTGTTGCCTTGATGG	GGGTTCTAGCCCCCAAATAA
SSR90 (MS)	16077689-16077938	9976996-9977245	ACTTTGCCAAGCTGTGAAGG	GCGTCTCGTATGCTCTGCTA
SSR116 (MS)	17269263-17269460	8785474-8785671	CCCCATTGAAAGTTCATCCA	GTCAGGAGGCCACATTGTTT
SSR118 (MS)	17695072-17695271	8359663-8359862	GCCCAAAATTCTTAGCCAAA	TGGCTTGGGTACTGGTTTCT
SSR111 (MS)	17849127-17849347	8205587-8205807	TTTGATTGTTTCCCTCACTCG	TGTCATTGTCCTTGGCAAAA
SSR88 (MS)	18040111-18040269	8014665-8014823	CCAAAAGGCAGGACCATAAA	TTGCGTAGACACCACAAGGT
SSR124 (MS)	18512607-18512820	7542114-7542327	CGCTTAAACGATCCAACGAT	GTTCATAACCGGTGCTCGAT
SSR126 (MS)	19673479-19673688	6381246-6381455	AATTGCCAAAGAAACCACCA	AGCGGCTCGTCGTCTGTA
SSR128 (MS)	19839381-19839552	6215382-6215553	ACGACTTGTCGCTGATAGGC	CTGTTCTGGAACATGCAAGC
SSR129 (MS)	20026180-20026357	6028577-6028754	ACCATGAGCAGGCATCAGA	CCGGCAATCGTTCTTTAACT
SSR130 (MS)	20129136-20129321	5925613-5925798	ACACAGGTCCCAACACAACA	CAGAAAATGATGTGCGTCGT
SSR133 (MS)	20473430-20473645	5581289-5581504	CTCGCAACTTGGCAGACATA	TGCCCCAATGTAACTGCTATC
SSR134 (MS)	20601881-20602035	5452899-5453053	GCACCGCCATACCCATAAT	ATTTGGCCACCCATTAGACC
SSR136 (MS)	20858440-20858624	5196310-5196494	ATGCCCAACAATGCTAAACA	TCCCCGATGATCATTGTCTT
SSR62 (MS)	21069407-21069701	4985233-4985527	TGTTAGTTGGCAGCGCAAT	GATTATGCGTGTTGCAGTCG

**VM: visible marker, MS: microsatellite marker, SNP: single nucleotide polymorphism.*

Table S2: Statistical analysis of RT-qPCR results. Two sample *t*-tests (one-tailed) for each relevant comparison are shown. Significant *p*-values (<0.05) are indicated in red.

Samples compared (isoform)	t-value	*p*-value	Associated figure
Apre v. Vpre (A)	17.54	<0.00001	5b
Apup v. Vpup (A)	35.19	<0.00001	5b
Apos v. Vpos (A)	5.69	0.00046	5b
Apre v. Vpre (B)	4.63	0.00169	5b
Apup v. Vpup (B)	0.31	0.76582	5b
Apos v. Vpos (B)	2.99	0.01739	5b
Apre v. F1pre (A)	1.49	0.13674	5c
Apup v. F1pup (A)	0.27	0.40072	5c
Apos v. F1pos (A)	2.21	0.07916	5c
Vpre v. F1pre (A)	7.29	0.00915	5c
Vpup v. F1pup (A)	25.46	0.00081	5c
Vpos v. F1pos (A)	2.88	0.05133	5c
Apos v. F3Br (A)	2.05	0.05457	5c
Vpos v. F3Bl (A)	2.45	0.06719	5c
Apre v. Npre (A)	1.67	0.11882	5d
Apup v. Npup (A)	1.91	0.09801	5d
Apos v. Npos (A)	1.89	0.10008	5d
Apre v. Lpre (A)	7.92	0.00778	5d
Apup v. Lpup (A)	29.88	0.00056	5d
Apos v. Lpos (A)	32.38	0.00048	5d
Lpre v. Npre (A)	13.38	0.00277	5d
Lpup v. Npup (A)	36.6	0.00037	5d
Lpos v. Npos (A)	8.02	0.00761	5d
Lpre v. Vpre (A)	0.23	0.42108	5d
Lpup v. Vpup (A)	2.72	0.05652	5d
Lpos v. Vpos (A)	2.84	0.05235	5d

Table S3: Statistical analysis of adult pigmentation between *D. americana* and *D. novamexicana*. One-way ANOVA was performed for genotype at each of the three genes and mean red value at three adult landmarks. Significant *p*-values (<0.05) are indicated in red.

				Tukey HSD adjusted *p-value*		
Gene	**Landmark**	**F value**	**Pr (>F)**	**N-A**	**H-A**	**N-H**
ebony	Abdomen	4.969	0.00244	0.00221	0.0389	0.31829
	Scutellum	0.799	0.496	0.59866	0.46525	0.99959
	Thorax	0.558	0.644	0.61987	0.74585	0.96329
tan	Abdomen	4.85	0.00285	0.12649	0.07594	0.00205
	Scutellum	0.935	0.425	0.89283	0.4431	0.91896
	Thorax	0.177	0.912	0.99757	0.99912	0.99423
Dat	Abdomen	0.162	0.922	0.93054	0.98801	0.97699
	Scutellum	0.17	0.916	0.94041	0.89818	0.99981
	Thorax	0.692	0.558	0.66698	0.99866	0.55199

References:

Schaeffer, S. W. *et al*., 2008 Polytene Chromosomal Maps of 11 *Drosophila* Species: The Order of Genomic Scaffolds Inferred From Genetic and Physical Maps. Genetics 179:1601-1655.

File S1

Nucleotide alignment in FASTA format.