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SUPPLEMENTARY INFORMATION

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Experimental test and refutation of a classic hypothesis of molecular adaptation in *Drosophila melanogaster*

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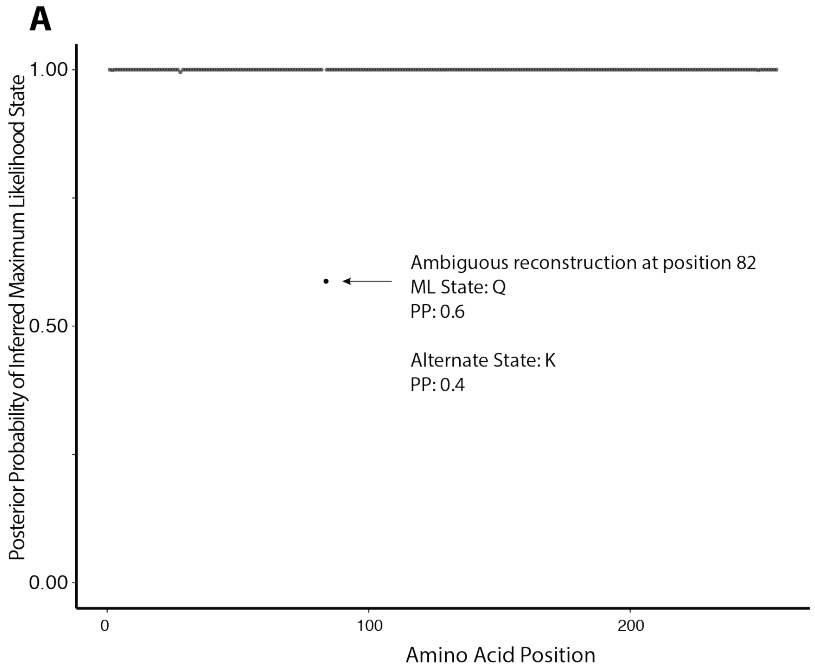
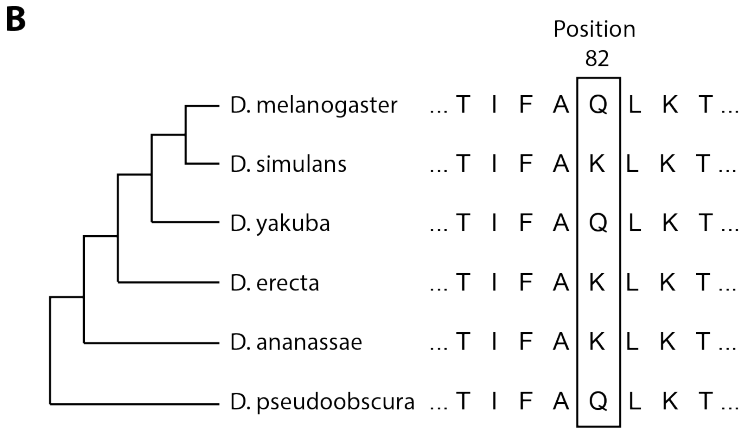
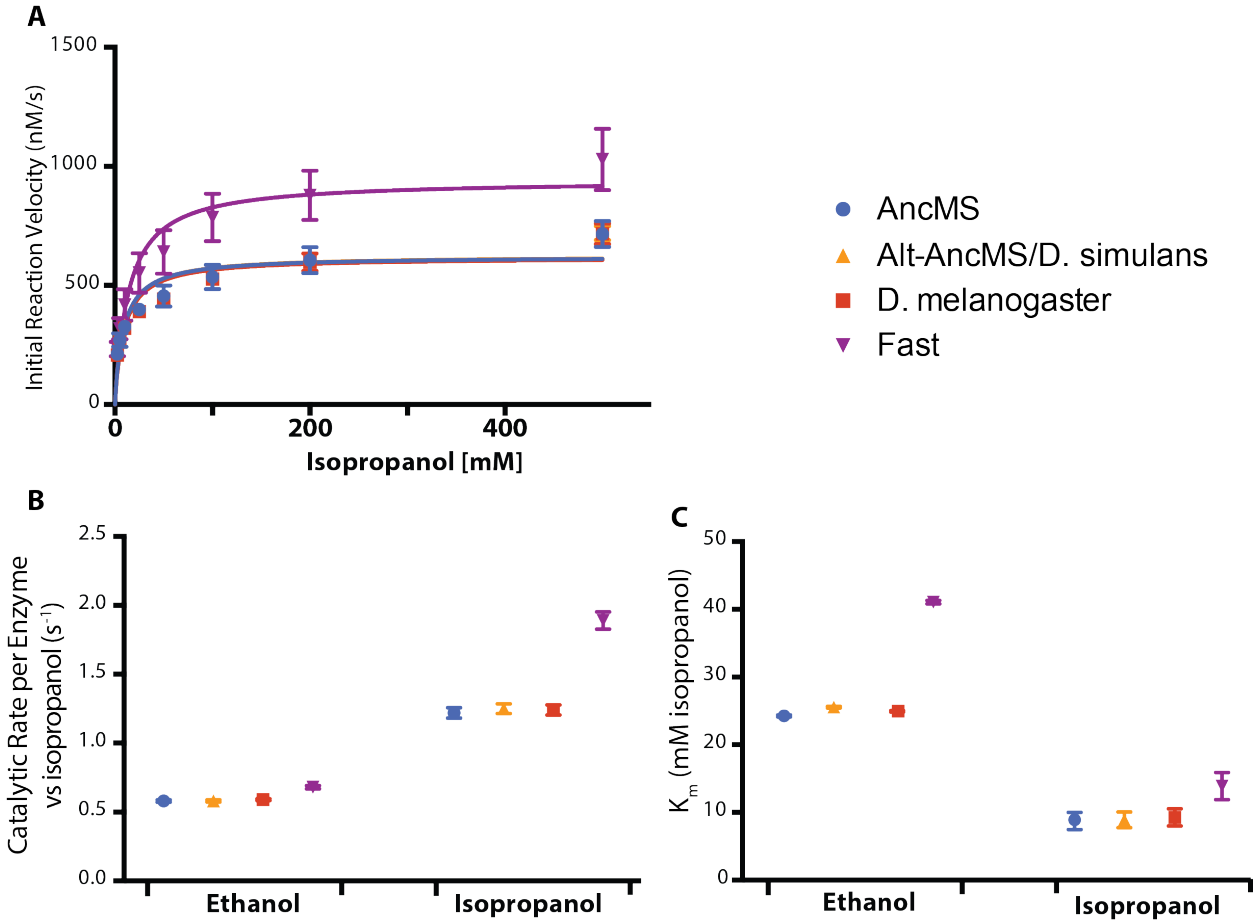
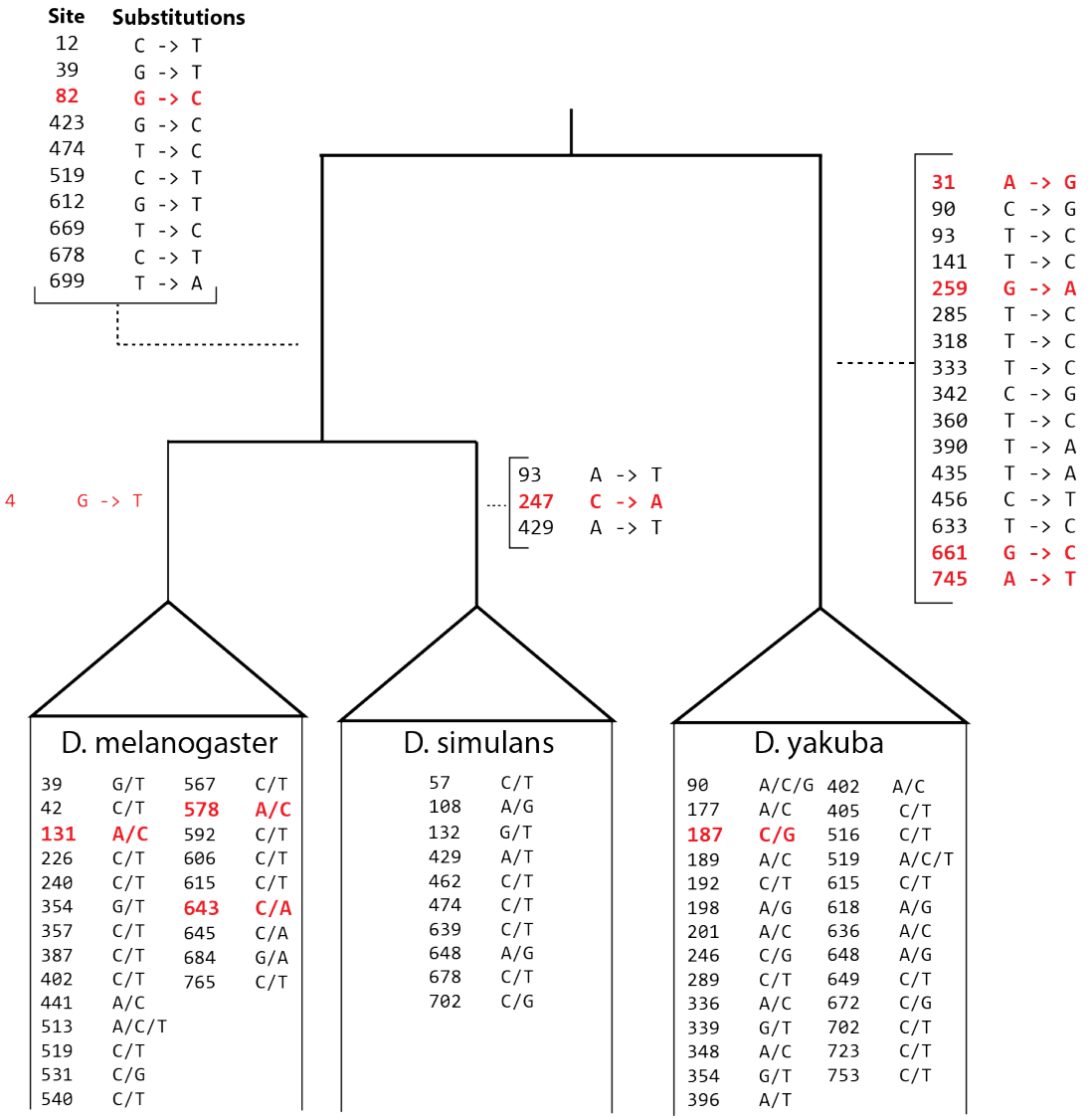




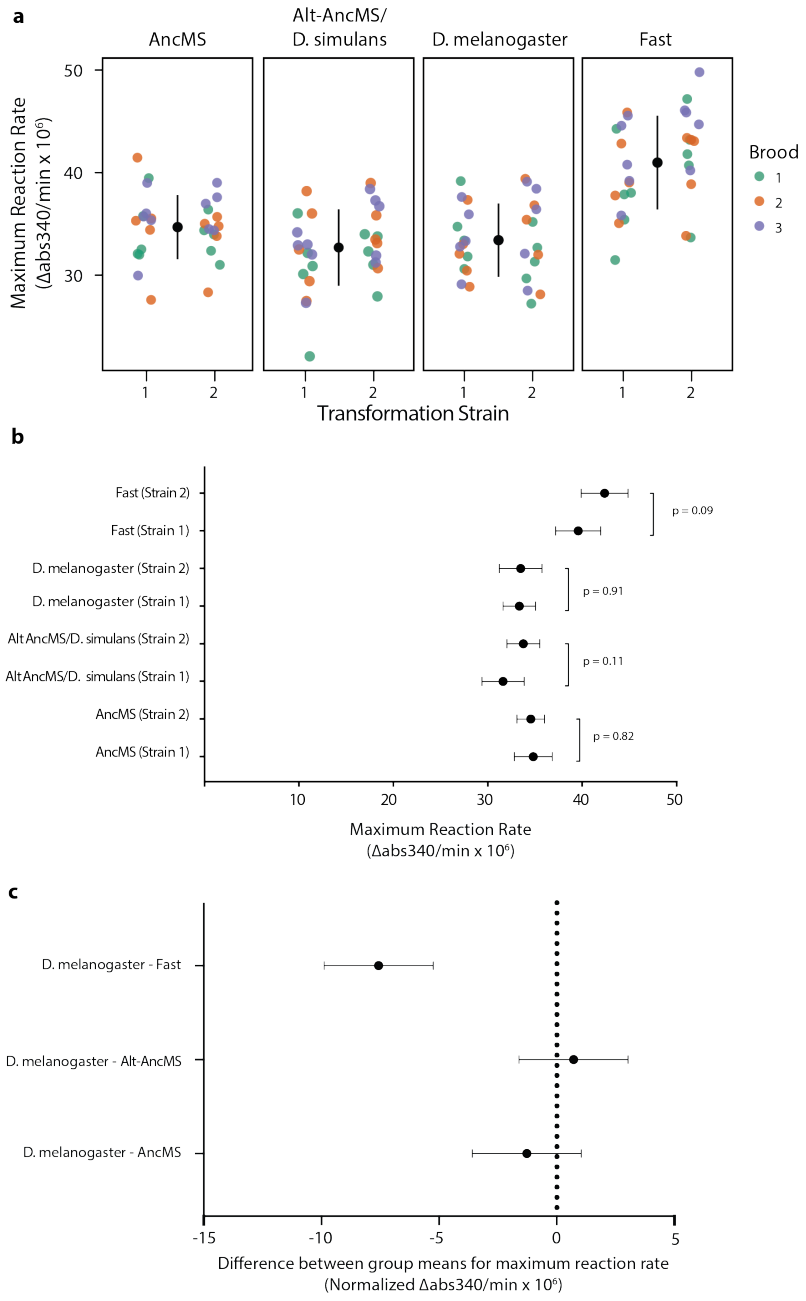
Supplemental Figure 1: The sequence of AncMS is reconstructed with high statistical confidence. **(A)** Posterior probability (PP) of the maximum PP site across sequence sites. Only one site (arrow) is ambiguously reconstructed in AncMS. (**B**) States observed in extant sequences at ambiguously reconstructed site 82.



Supplemental Figure 2: ADH activity using isopropanol as a substrate did not change between ancestral and *D. melanogaster* alleles. Reaction rates measured at different isopropanol concentrations were fit to a Michaelis-Menten model. **A)** Plot of initial reaction velocity across isopropanol concentrations for purified ADH proteins of different genotypes. Each point shows the mean and 95% confidence interval of six reactions at each dose. **B&C)** Estimated maximal catalytic turnover rates (k_{cat}) and Michaelis-Menten constant (K_m), each with 95% confidence interval inferred by nonlinear regression from the data in panel A. Activity for the same enzymes using ethanol as a substrate (data from Figure 2), are shown for comparison.



Supplemental Figure 3: Divergence and polymorphism data used for the McDonald-Kreitman tests. States at each variable sequence site (numbered by nucleotide) are shown. Nonsynonymous substitutions and polymorphisms are highlighted in red.



Supplemental Figure 4: Additional analysis of Vmax data from transgenic flies presented in Fig. 3A. **A)** Measurements of the maximum reaction rate, normalized by total protein in the homogenate. There were two transformation strains per genotype, shown on the horizontal axis; for each strain, three independent broods were initiated (colors), and flies from each brood were sampled on five different days. Each dot shows the Vmax of the homogenate of one sample of flies. The mean and standard deviation for each genotype is shown. **B)** No significant variation between Vmax from transformation strains within a genotype. The normalized maximum reaction rate for adults from different transgenic strains of each ADH construct is shown. For each strain, the mean and 95% confidence interval for 15 homogenates is shown. P-values are from unpaired t-tests. **C)** Comparisons of Vmax for transgenic animals of different ADH coding sequence assessed by ANOVA. Mean differences in activity between strains with the ancestral *D. melanogaster* ADH and other alleles were calculated from Dunnet's test to account for multiple comparisons. Error bars show 95% CI.

Supplemental Table 1: Kinetic parameters of ADH alleles expressed and purified from bacteria.

Genotype	Substrate	K_m (95% CI) mM	k_{cat} s^{-1} (95% CI)
AncMS	Ethanol	24.2 (20.7 – 27.7)	0.580 (0.558 – 0.602)
Alt-AncMS/D. simulans	Ethanol	25.5 (22.2 – 28.7)	0.582 (0.563 – 0.602)
D. melanogaster (Slow)	Ethanol	24.9 (22.2 – 27.7)	0.595 (0.577 – 0.612)
Fast	Ethanol	**41.0 (34.3 – 47.8)**	**0.678 (0.645 – 0.710)**
AncMS	Isopropanol	87.4 (63.3 – 111)	1.24 (1.17 – 1.32)
Alt-AncMS/D. simulans	Isopropanol	89.2 (67.4 – 111)	1.24 (1.16 – 1.31)
D. melanogaster (Slow)	Isopropanol	92.7 (68.1 – 117)	1.25 (1.18 – 1.31)
Fast	Isopropanol	**138 (100.2 – 177)**	**1.88 (1.76 – 2.00)**

The best-fit value of each parameter with its 95% confidence interval is shown. Only the Fast allele (bold) differs from the other genotypes.

Supplemental Table 2: McDonald-Kreitman test results for different sets of ADH sequence data.

Dataset	Dn/Ds	Pn/Ps	p-value
McDonald-Kreitman(1991)	**7/17**	**2/42**	**0.007**
MK + modern data	**7/23**	**5/59**	**0.049**
D. melanogaster	**1/0**	**3/21**	**0.16**
D. simulans	**1/2**	**0/10**	**0.231**
D. yakuba	**4/12**	**1/28**	**0.047**
D. yakuba + D. simulans	**6/23**	**1/38**	**0.037**
D. yakuba + D. melanogaster	**6/21**	**4/49**	**0.079**
D. melanogaster + D. simulans	**2/2**	**3/31**	**0.076**

McDonald-Kreitman test using different sets of ADH data, including the originally published 1991 alignment[24]; an expanded dataset with more new polymorphism data added; only those substitutions and polymorphisms from each branch leading to each of the three major species analyzed; and alignments from which the sequences of one of the species have been removed. The number of synonymous and nonsynonymous divergences between species (Dn and Ds) and polymorphisms (Pn and Ps) are shown, with the p-value associated with the null hypothesis of neutral evolution in each case.

Supplemental Table 3: ANOVA for effect of brood within transformaton lines on V_{max}

Genotype	F	p-value
AncMS—strain 1	0.58	0.94
AncMS—strain 2	2.48	0.12
Alt-AncMS/D. simulans—strain 1	0.44	0.65
Alt-AncMS/D. simulans—strain 2	1.69	0.23
D. melanogaster—strain 1	0.36	0.71
D. melanogaster—strain 2	1.21	0.33
Fast—strain 1	1.02	0.39
Fast—strain 2	1.89	0.19

The results of ANOVAs to analyze whether the three broods within each transformatioin strain differ in observed Vmax. There was no significant effect of brood in any of the transformation strains in any genotype.

Supplemental Table 4: Primers used for molecular cloning and transgenesis

Primer name	Sequence	Purpose
Adh_clone_S3aAF1	GGGCGAATTCGCCggcgcgccGCGAATAATCAAGACTCAGCACCA	Amplify D. melanogaster Adh (with pS3aG ends)
Adh_clone_S3aNR1	TATGATCTAGAGTCgcggccgcTGGTGACCAGGCACGTATTG	Amplify D. melanogaster Adh (with pS3aG ends)
melst-0408R_BspQI_R	cGAAGAGCgtgttcttGCTCTTCaggcGCCGATTAACAGGGACGAGT	Make vector pS3aCCBQ
melst+1662F_BspQI_F	tGAAGAGCaagaacacGCTCTTCgttcAGGGAGACGGGGAAGTTACTATC	Make vector pS3aCCBQ
melst_+1662R	GATAGTAACTTCCCCGTCTCCCT	Amplify within melanogaster Adh
melst_-0408F	ACTCGTCCCTGTTAATCGGC	Amplify within melanogaster Adh
pgem_-0408F	tggcggccgcgggaattcgatACTCGTCCCTGTTAATCGGC	Amplify within melanogaster Adh (with pGem-T-Easy ends)
pgem_+1662R	ccgcgaattcactagtgatGATAGTAACTTCCCCGTCTCCCT	Amplify within melanogaster Adh (with pGem-T-Easy ends)
adhF_F1	CACGTTCAACTCCTGGTTGGATGTTG	Replace mel-F amino acid
adhF_R1	GGCTCAACATCCAACCAGGAGTTGAAC	Replace mel-F amino acid
adhMS_aR1	GTTGGTCAAAGTAAACGCCATGGTGACTTC	replace ancMS amino acid
adhMS_bF1	TGGCGTTTACTTTGACCAACAAGAACGTG	replace ancMS amino acid
adhMS_bR1	CTTGGCGAAGATGGTCTTCAGCAGC	replace ancMS amino acid
adhMS_cF1	CCAAGCTGCTGAAGACCATCTTCGCC	replace ancMS amino acid
Adhseq_-3285F	GGCAAATGGGTCAAATGGGT	Sequence melanogaster Adh
Adhseq_-2834F	ATAAGGCGCACACTGTCCACT	Sequence melanogaster Adh
Adhseq_-2309F	GCAACCCGTTCGATTAGTGG	Sequence melanogaster Adh
Adhseq_-1857F	TCCTCCCACATCATATTTAACGC	Sequence melanogaster Adh
Adhseq_-1294F	ACACTTCTCCTTGCTATGCTTG	Sequence melanogaster Adh
Adhseq_-0773F	TCAGTGCAGTTGTCAGTTGC	Sequence melanogaster Adh
Adhseq_-0278F	TGCCGGTACAAAATCCCAAA	Sequence melanogaster Adh
Adhseq_+0224F	CAATCCAAAGGTGACCGTCA	Sequence melanogaster Adh
Adhseq_-0429F	TACTCGTCCCTGTTAATCGGC	Sequence melanogaster Adh
Adhseq_+0728F	TGGATGTTGAGCCTCAGGTT	Sequence melanogaster Adh
Adhseq_+1209F	AATGTTCGATTTGACGGGCA	Sequence melanogaster Adh
Adhseq_+1587F	CACCTATGAAGTCTATTTTAATCGCC	Sequence melanogaster Adh
Adhseq_+2219F	CTGTGATGGCGGTTTGTTGT	Sequence melanogaster Adh
Adhseq_+2807F	GTCAAAACCAGGTTAGGGAGG	Sequence melanogaster Adh
Adhseq_+3206F	CGAATGCATGTCGAAATTTTGC	Sequence melanogaster Adh
Adhseq_+3710F	TCATCGGACATTGAGGACCC	Sequence melanogaster Adh
Adhseq_+4203F	ATTCTGGGCCGGTAATTGAC	Sequence melanogaster Adh
Adhseq_-3409R	GCAAGACGGCTAACTCATCC	Sequence melanogaster Adh
Adhseq_-2900R	CGTACCTGCCAAAACCGATT	Sequence melanogaster Adh
Adhseq_-2391R	GGTCTGGCCTAGTCTGGG	Sequence melanogaster Adh
Adhseq_-1915R	AGCATGCGGAAGAAATAAGCT	Sequence melanogaster Adh
Adhseq_-1419R	TGGAGGCAGCGTAGAATCAA	Sequence melanogaster Adh
Adhseq_-0909R	CGCAAGCAAGTGATCTGCAA	Sequence melanogaster Adh

Adhseq_-0408R	GCCGATTAACAGGGACGAGT	Sequence melanogaster Adh
Adhseq_+0105R	AGTTACCTTCAGATCGCGCT	Sequence melanogaster Adh
Adhseq_+0585R	CCTTTGATCAACTTACCGCCA	Sequence melanogaster Adh
Adhseq_+1611R	GCGATTAAAATAGACTTCATAGGTGG	Sequence melanogaster Adh
Adhseq_+2092R	TATATGCGCAGAAAACCGGC	Sequence melanogaster Adh
Adhseq_+2937R	CTTAATGTACACCCCAACAGCCTA	Sequence melanogaster Adh
Adhseq_+3600R	CCACAACTCACCCCACCATA	Sequence melanogaster Adh
Adhseq_+4108R	GCACTGCCTCTCCCATTTTG	Sequence melanogaster Adh
AdhSeq_+2381R	TCCGATCTCTCGATGGCATT	Sequence melanogaster Adh
AdhSeq-2274R	CTTGGGGAATGCATGATTGG	Sequence melanogaster Adh
AdhSeq-2228R	TTGGGGGTCGCATCGAG	Sequence melanogaster Adh
AdhSeq-2483F	AGATGTTAGGCACGTCTGCTGAT	Sequence melanogaster Adh
AdhSeq-2626F	TAAATTCCTTTGACGCATGCAC	Sequence melanogaster Adh
S3a_F2	CACATGTGCAAGAGAACCCAGTG	Sequence from vector pS3aG
S3a_R5	GCATTCATTTTATGTTTCAGGTTCA	Sequence from vector pS3aG
S3a_R6	CTTTATTTGTAACCATTATAAGCTGCAA	Sequence from vector pS3aG