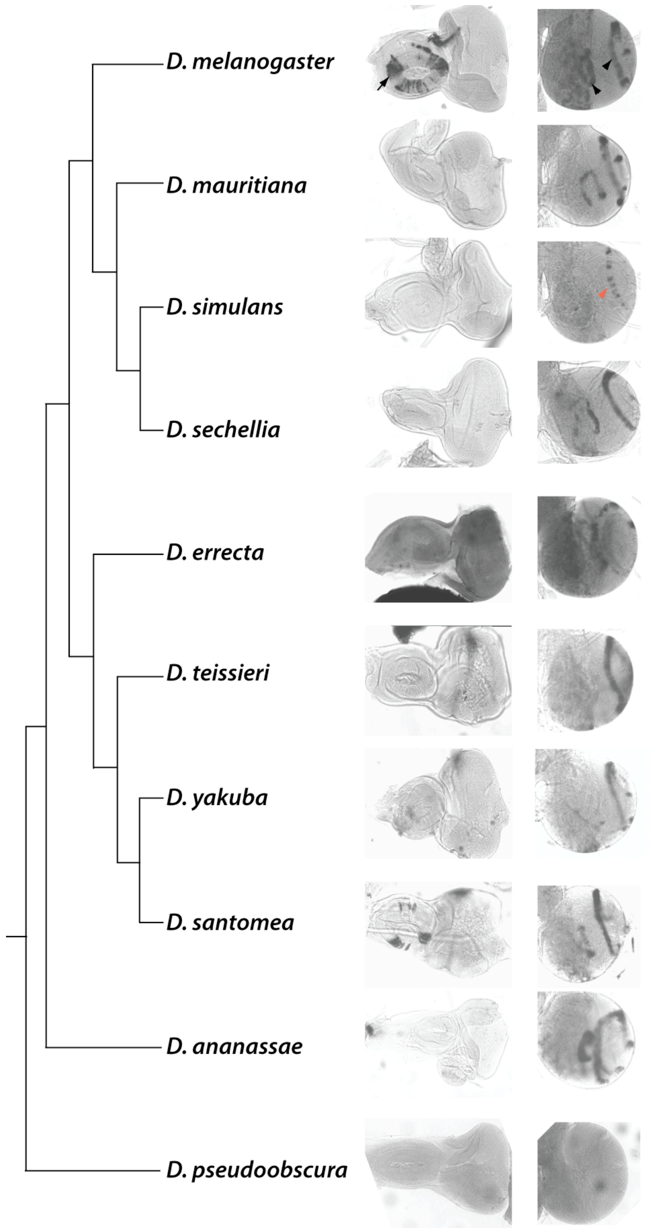


Figure S1. Rapid evolution of *Obp99a* expression patterns in the optic lobe and eye-antennal disc of *Drosophila species.* In the third larval instar antennal disc of *D. melanogater*, *Obp99a* is expressed in a species-specific manner in the Johnston's organ (black arrow). In the optic lobe, expression is observed in cells of both the outer and inner proliferation centers of most species (black arrowheads in *D. melanogaster*). However, in *D. simulans, Obp99a* is expressed in much less elaborate pattern in the optic lobe (red arrowhead).

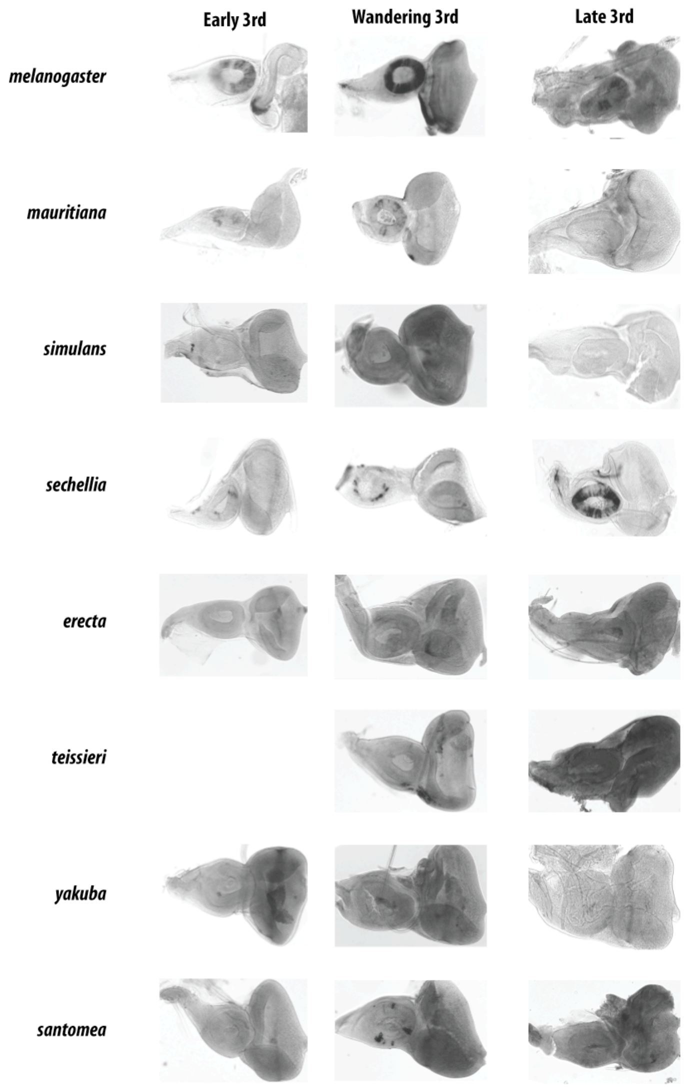


Figure S2. Timecourse of *Obp99a* expression during eye-antennal disc development. Eye-antennal discs were collected from early, mid (wandering), and late third instars, and hybridized to an *Obp99a* probe. The pattern observed in *D. melanogaster* wandering stage antenna was observed in late third instar for *D. sechellia*. A much weaker pattern was observed in in *D. mauritiana* starting in early third instar. A similar pattern of expression was observed in wandering third instar *D. santomea* eye-antennal discs. The detection of these patterns in more distant species, and at differing timepoints highlights the importance of temporal resolution and species coverage when seeking to identify novel patterns of expression.

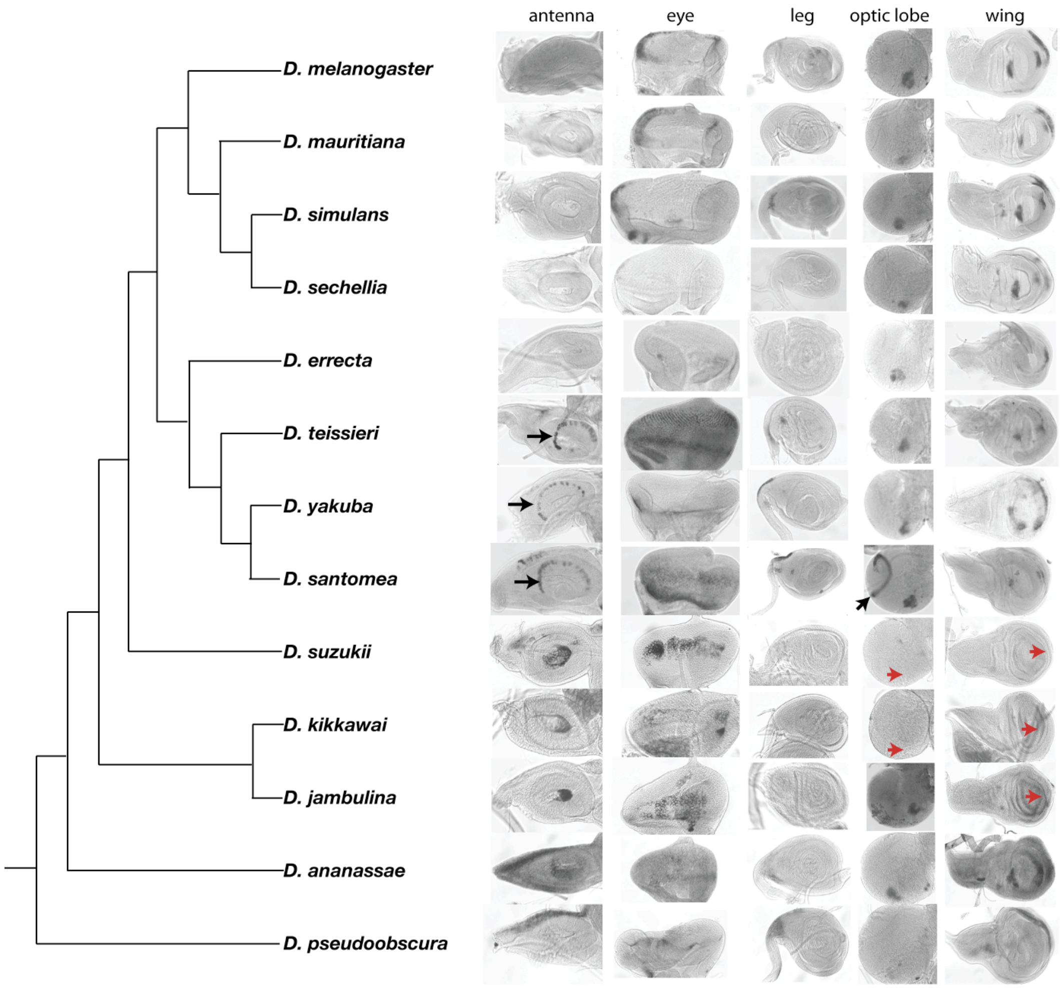


Figure S3. Rapid divergence in *Nep1* expression among *Drosophila* species. In a comparison of imaginal disc gene expression among thirteen *Drosophila* species, multiple cases of shifting patterns, pattern loss and apparent gains were observed. Patterns of expression were found to vary widely in spatial extent within the antennal, eye, leg and wing disc. Losses of expression were evident in the mushroom body of the CNS of *D. suzuki* and *D. kikkawai* (red arrows in the "optic lobes" column). These species also appear to have lost expression in the wing hinge region (red arrows in the "wing" column). In the *D. yakuba* clade, two apparent gains were observed. In the antennal discs of *D. teissieri, D. yakuba,* and *D. santomea,* expression was observed in sensory organ precursors of the third antennal segment (black arrows in "eye" column). In *D.* santomea, a novel pattern of expression was observed in laminar neuroblasts of the developing visual system (black arrow in "optic lobe" column).

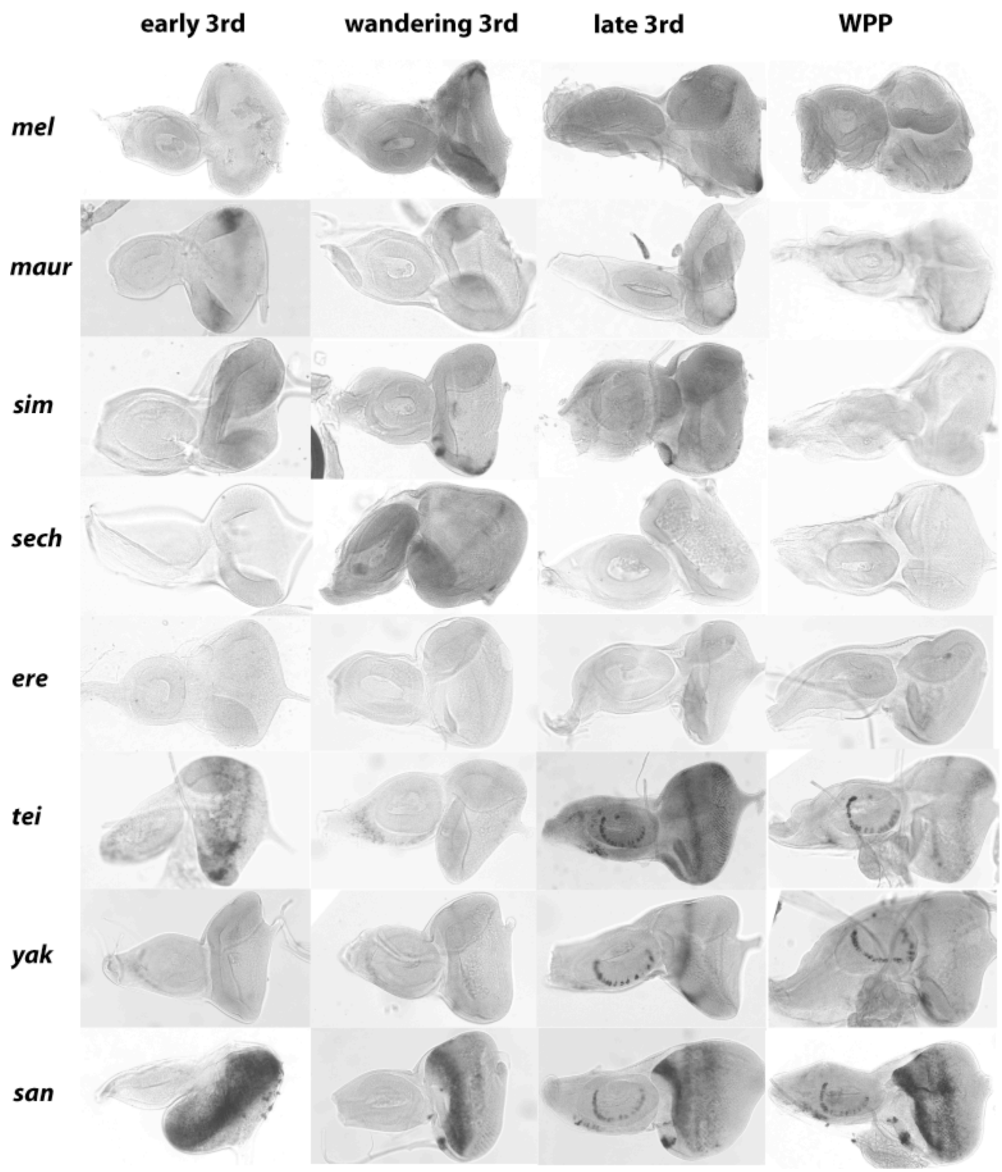


Figure S4. Time course of *Nep1* expression in eye-antennal discs. From early in the third instar, *D. santomea* exhibits robust expression of *Nep1* in the region anterior to the morphogenetic furrow fated to become the frons. In *D. yakuba, D. santomea,* and *D. teissieri*, a unique pattern of expression is observed in the sensory organ precursors of the third antennal segment, late in the third instar. None of the other species examined show this pattern in any of the stages tested.

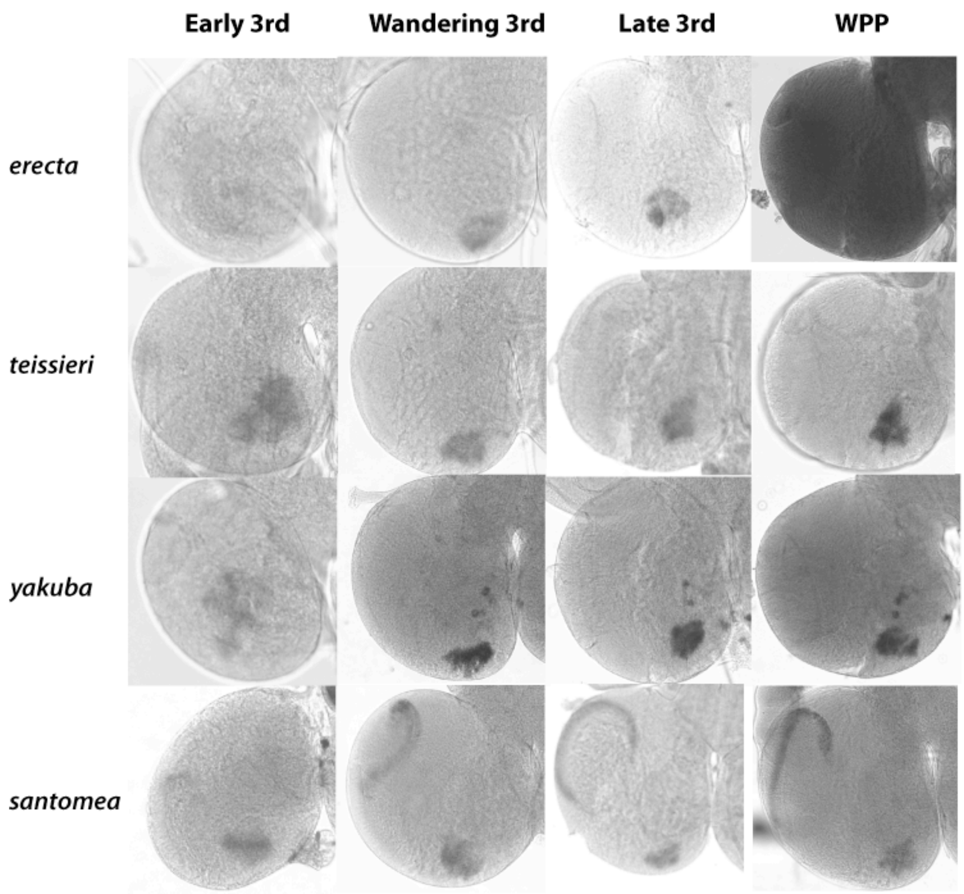


Figure S5. Timecourse of *Nep1* expression in larval optic lobes. Optic lobes from four developmental stages were tested for laminar neuroblast expression. From mid third instar, and into early pupal stages, only *D.santomea* expresses *Nep1* in the laminar neuroblasts.

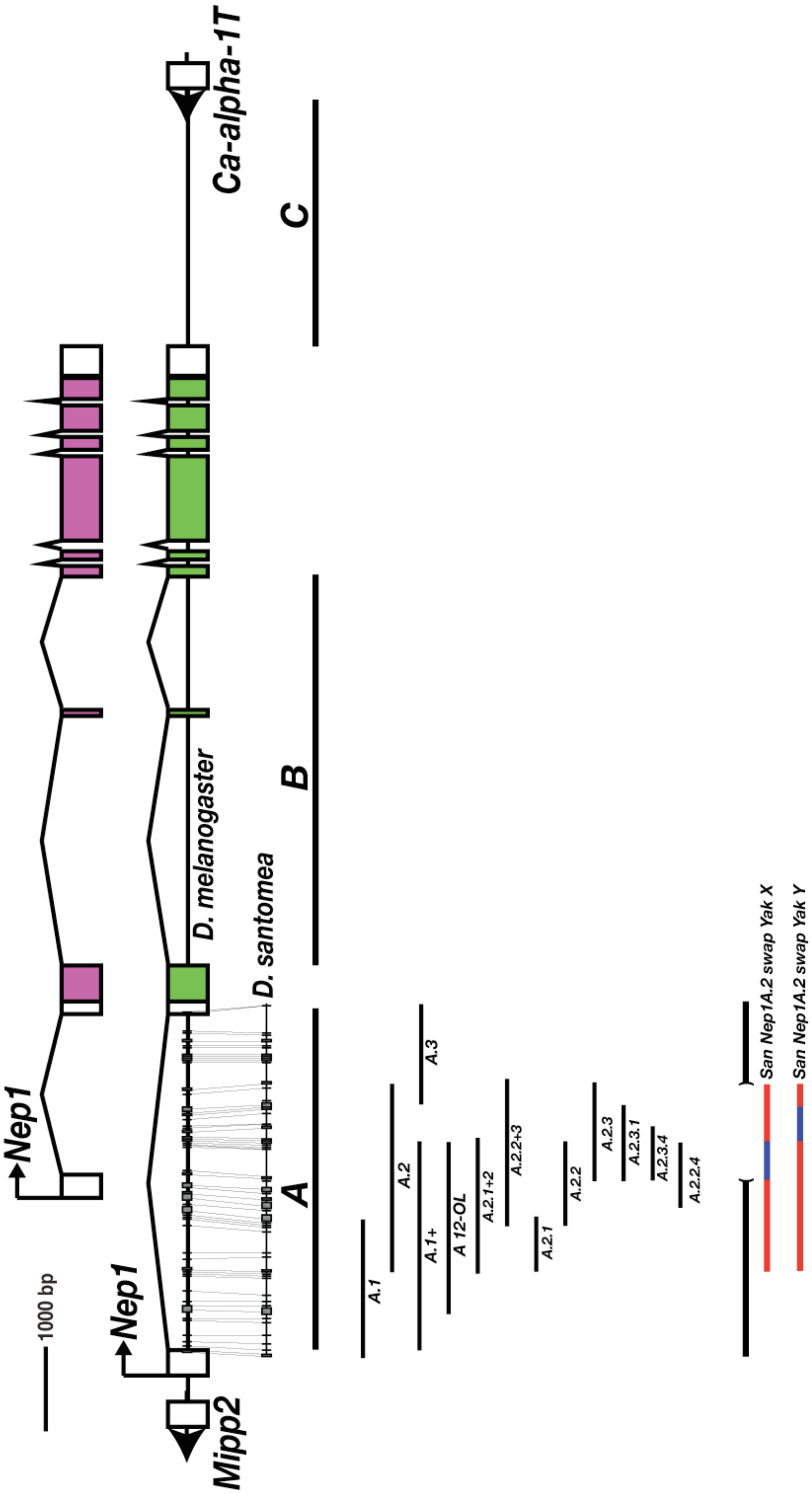
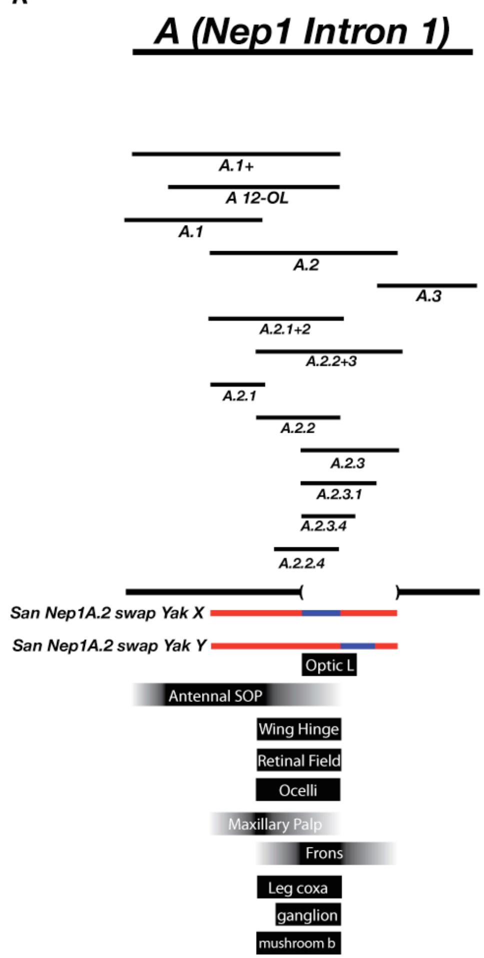

1000 bp
Nep1
Nep1
Mipp2
Ca-alpha-1T
D. melanogaster
D. santomea
A
B
C
A.1
A.2
A.3
A.1+
A 12-OL
A.2.1+2
A.2.2+3
A.2.1
A.2.2
A.2.3
A.2.3.1
A.2.3.4
A.2.2.4
San Nep1A.2 swap Yak X
San Nep1A.2 swap Yak Y

Figure S6. *Nep1* reporter constructs. Fragments were amplified by PCR using the primers listed for each construct in Table S3, and inserted into the pS3AG vector containing an Hsp70 TATA box and enhanced nuclear GFP reporter gene.

A



B

Construct	Optic Lobe (Laminar nb)	Antenna (SOPs)	Wing Hinge	Retinal Field	Ocelli	Maxillary Palp	Frons	Leg (cx)	CNS (vg)	CNS (mb)	Wing (ad, ectopic)	Leg joints (ectopic)
A	+	+(yak)	+	+	+	+	+	+	+	+	+	+
B	-	-	-	-	-	-	-	-	-	+/-	-	-
C	-	-	-	-	-	-	-	-	-	-	-	-
A.1+	-	+(yak)	ND	+	+	-	-	ND	ND	ND	ND	ND
A12-OL	-	-	-	+	+	-	-	+	+	+	+	+
A.1	-	-	-	-	-	-	-	-	-	-	-	-
A.2	+	-	-	+	+	+	+	+	+	+	+	+
A.3	-	-	-	-	-	-	-	-	-	-	-	-
A.2.1+2	-	-	-	+	+	+	-	ND	+	+	+/-	ND
A.2.2+3	+	-	ND	+	+	-	+	ND	ND	ND	+/-	ND
A.2.1	-	-	-	-	-	-	-	-	-	-	-	-
A.2.2	-	-	+	+	+	-	-	+	+	+	+/-	+/-
A.2.3	+	-	-	-	-	-	-	-	+/-	-	-	-
A.2.3.1	+	-	-	-	-	-	-	-	+/-	-	-	-
A.2.3.4	+	-	-	+/-	-	-	-	-	+/-	-	-	-
A.2.2.4	-	-	ND	-	-	-	-	ND	+	-	ND	ND

C

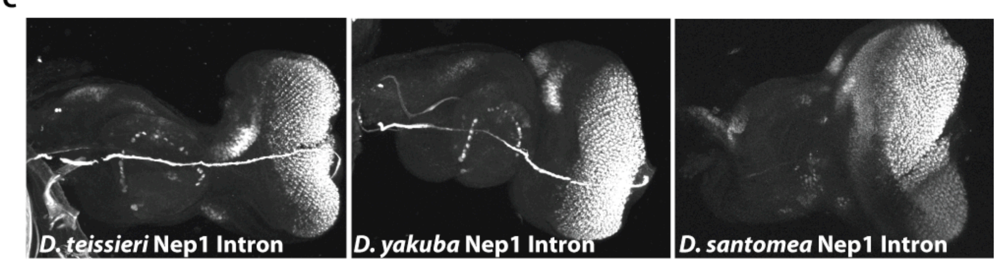


Fig S7. Nep1 Constructs and expression patterns. (A) constructs generated from the first intron of *Nep1.* (B) The presence of GFP in a in a variety of patterns is listed for each construct. (+) denotes the presence of high expression, (-) indicates the absence of detectable expression. (+/-) indicates weak/trace expression and (ND) indicates a pattern that was not determined. The antennal SOP pattern novel to *D. yakuba, D. santomea,* and *D. teissieri* was mapped in the context of the *D. yakuba* intron, indicated by "(yak)" in the table. (C) Eye-antennal discs of animals bearing a *Nep1* intron reporter from *D. yakuba, D. teissieri, and D. santomea*, showing that the *D. santomea* intron lacks antennal SOP activity, likely due to a shift in position of the enhancer.

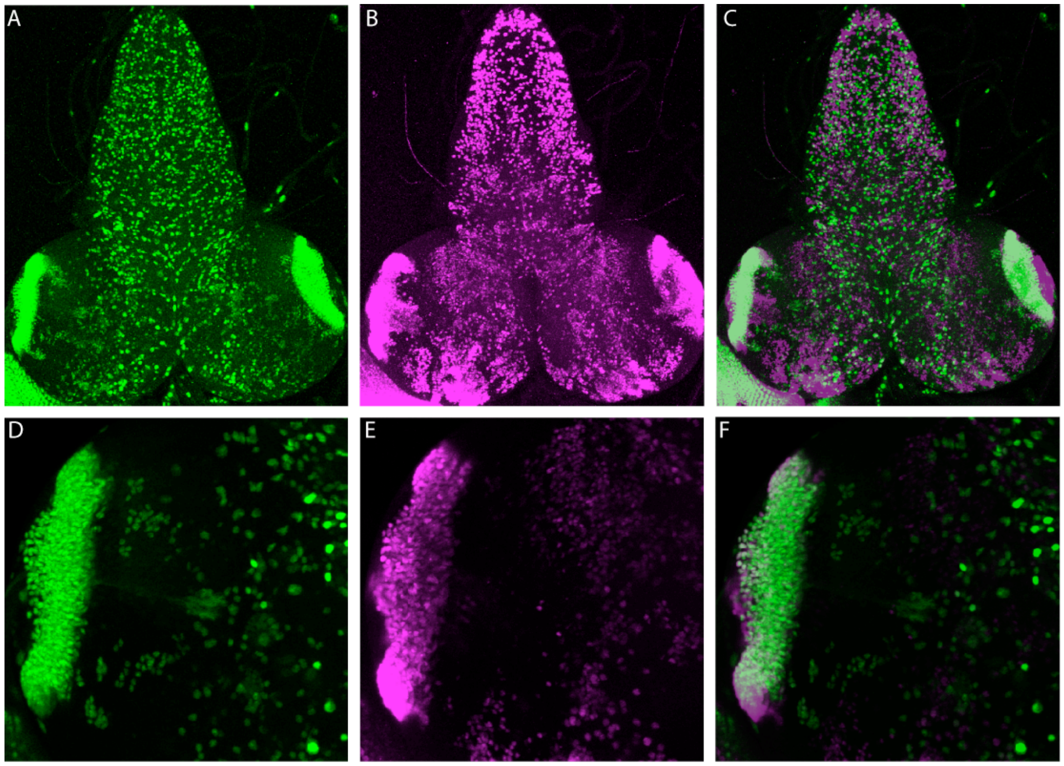


Figure S8. The optic lobe activity of *Nep1* is restricted to the Dachshund-positive laminar neuroblasts. Larvae expression GFP driven by a 2.2 kb region of the *D. santomea Nep1* intron (A, D) ("A.2" fragment), containing most of the regulatory activities of *Nep1* , were co-stained with the monoclonal antibody to the Dachshund protein (B, E). (C, F) Merged image.

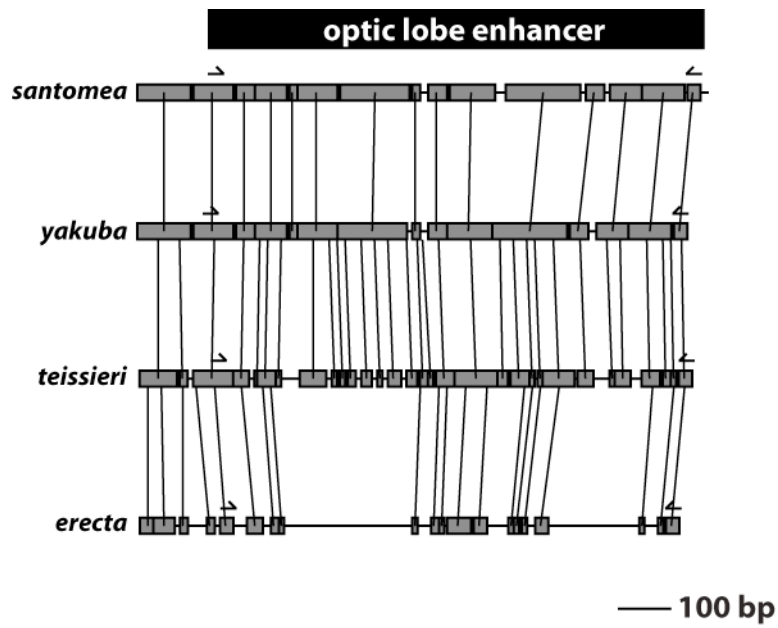
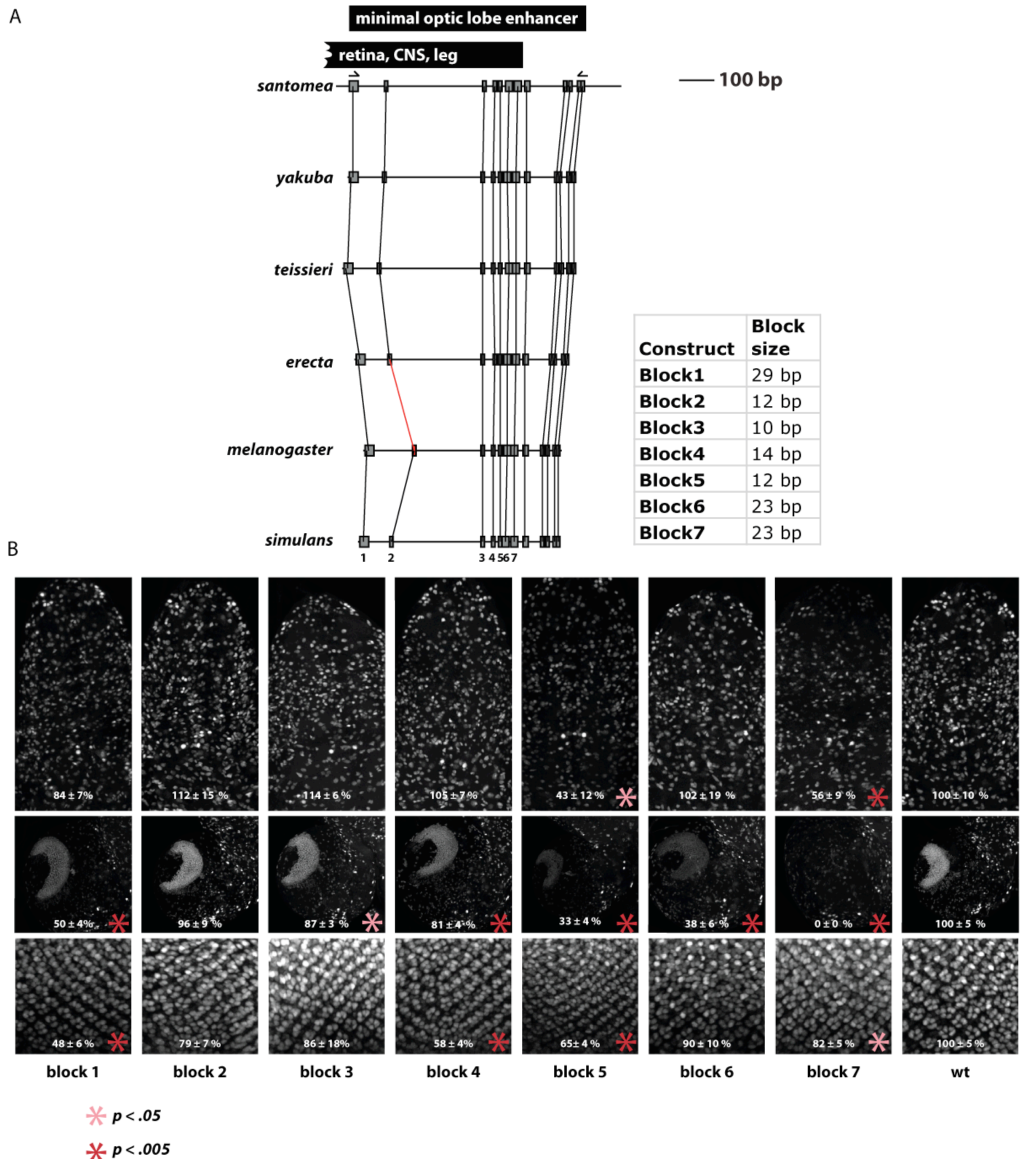
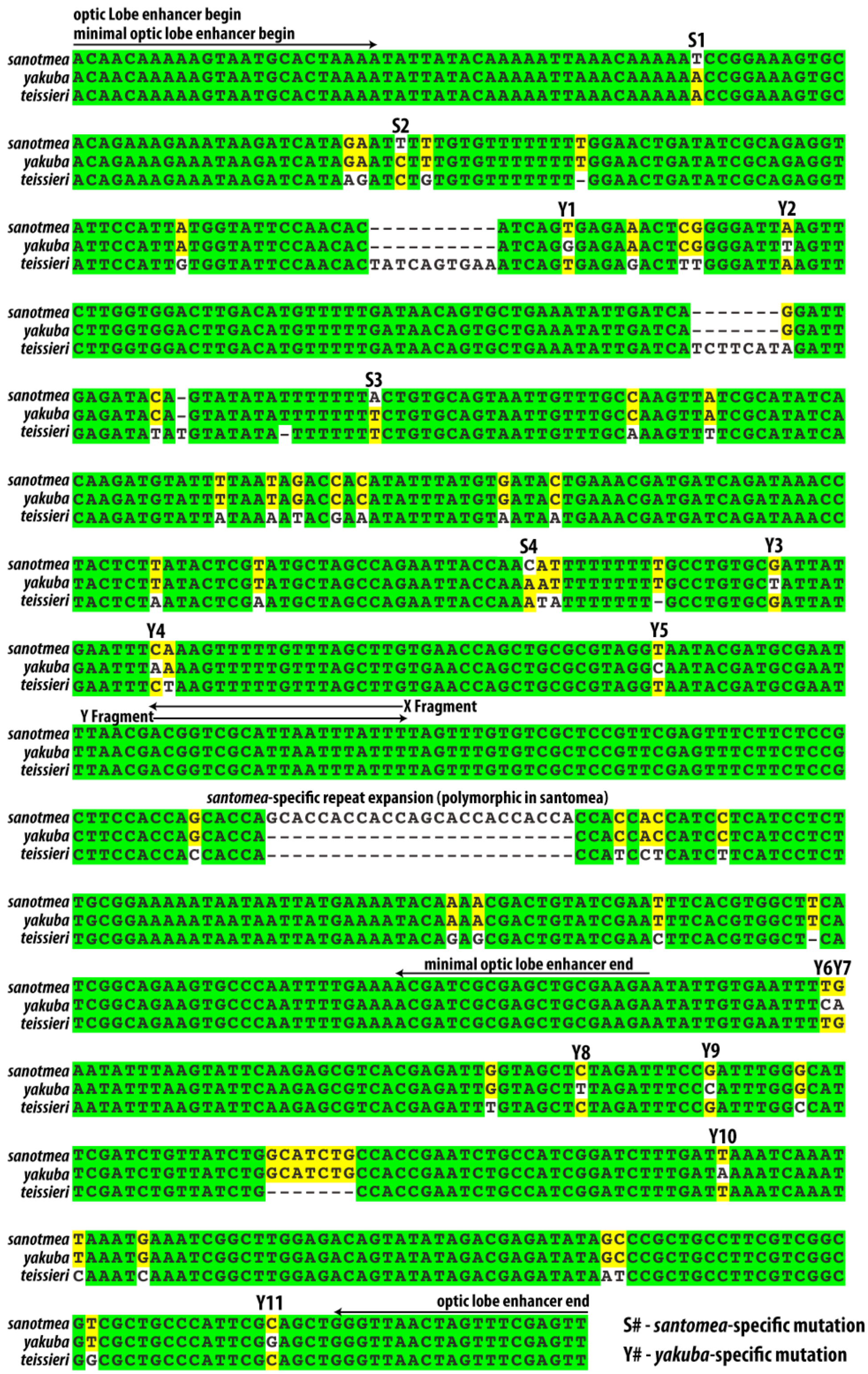


Figure S9. Visual alignment of the optic lobe enhancer. *Nep1* intron sequences containing the optic lobe enhancer were compared for regions of exact identity. Gray boxes denote regions of exact identity between the sequences connected by lines. Arrows mark the location of primers used to amplify the optic lobe enhancer from different species. *D. santomea* and *D. yakuba* sequences were compared to each other in the GenePalette tool, generating a pairwise plot of the sequences. *D. teissieri* and *D. erecta* DNA sequences were built into the alignment sequentially (thus, *D. teissieri* has more blocks of conservation than *D. erecta*).



Construct	Block size
Block1	29 bp
Block2	12 bp
Block3	10 bp
Block4	14 bp
Block5	12 bp
Block6	23 bp
Block7	23 bp

Figure S10. The optic lobe enhancer shares regulatory inputs with other enhancers. Mutations that scramble short blocks of sequence conserved from *D. santomea* to *D. melanogaster* in the context of a large fragment of the intron ("A.2" fragment, 2.2 kb) affect multiple enhancer activities residing within the region. (A) Schematic of the position of conserved blocks located in the region of overlap of optic lobe and retina/CNS/frons activities. (B-H) Enhancer activity in the larval CNS (top), optic lobe (middle) and retinal field (bottom) upon scrambling the sequence of each block, visualized by GFP expression. Asterix color indicates level of significance in as t-test.



optic Lobe enhancer begin
minimal optic lobe enhancer begin
sanotmea
yakuba
teissieri
S1
S2
Y1
Y2
S3
S4
Y3
Y4
Y5
Y Fragment
X Fragment
santomea-specific repeat expansion (polymorphic in santomea)
minimal optic lobe enhancer end
Y6Y7
Y8
Y9
Y10
Y11
optic lobe enhancer end
S# - santomea-specific mutation
Y# - yakuba-specific mutation

Figure S11. Alignment of the optic lobe enhancer from *D. santomea* with *D. yakuba* and *D. teissieri* sequences. Arrows denote primers used to amplify enhancers. *D. santomea*-specific mutations are marked with an S, and *D. yakuba*-specific mutations are marked with a Y.

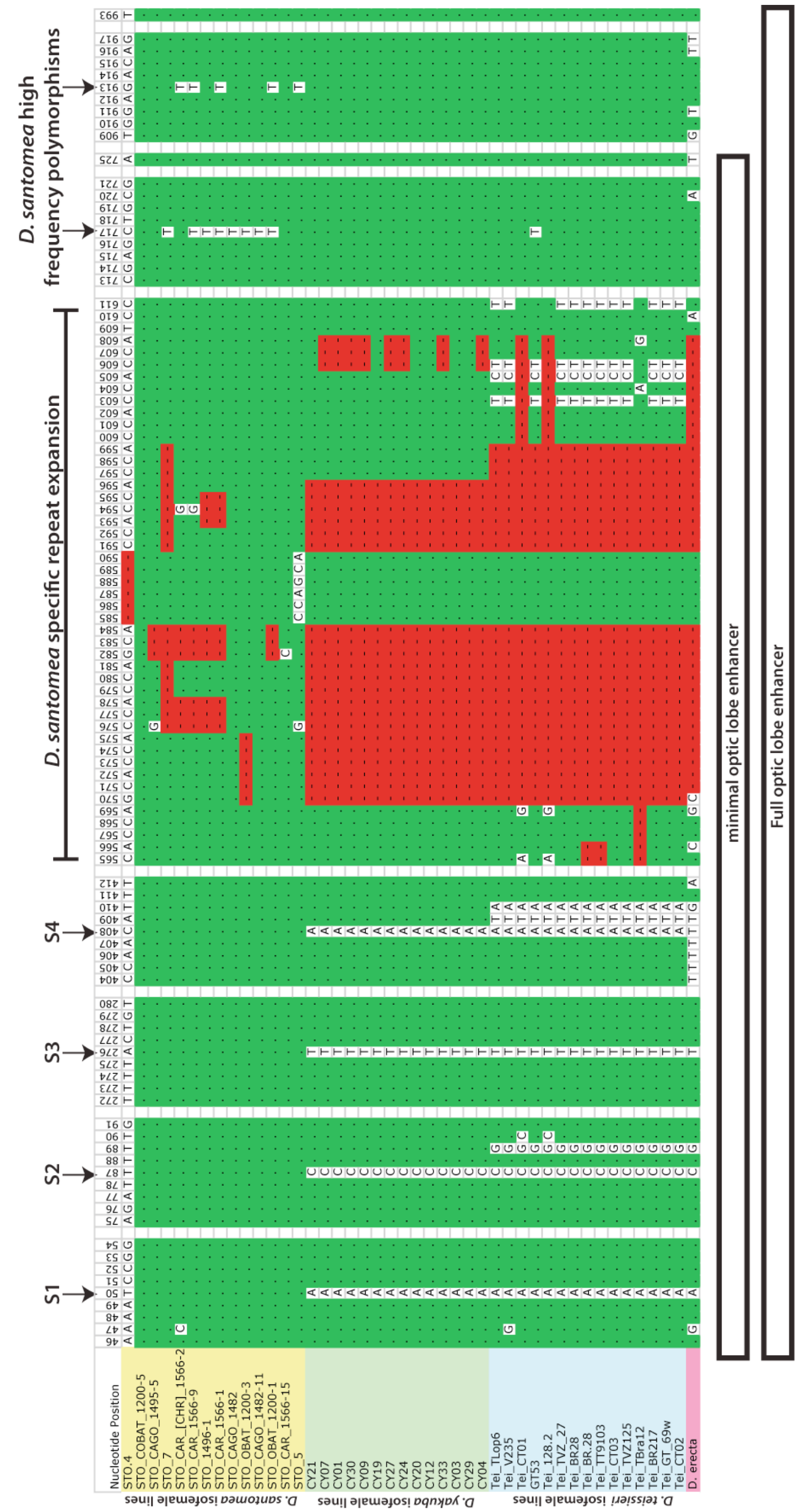

D. santomea high frequency polymorphisms
D. santomea specific repeat expansion
S1
S2
S3
S4
minimal optic lobe enhancer
Full optic lobe enhancer
Nucleotide Position
D. santomea isofemale lines
D. yakuba isofemale lines
D. teissieri isofemale lines
D. erecta

Figure S12. Alignment of isofemale lines. The flanking sequence surrounding each *D. santomea*-specific substitution (S1-S4), and other high frequency polymorphisms in *D.* santomea are given. Numbers indicate the position of each feature in the context of the *D.* santomea optic lobe enhancer shown in Fig. S11.

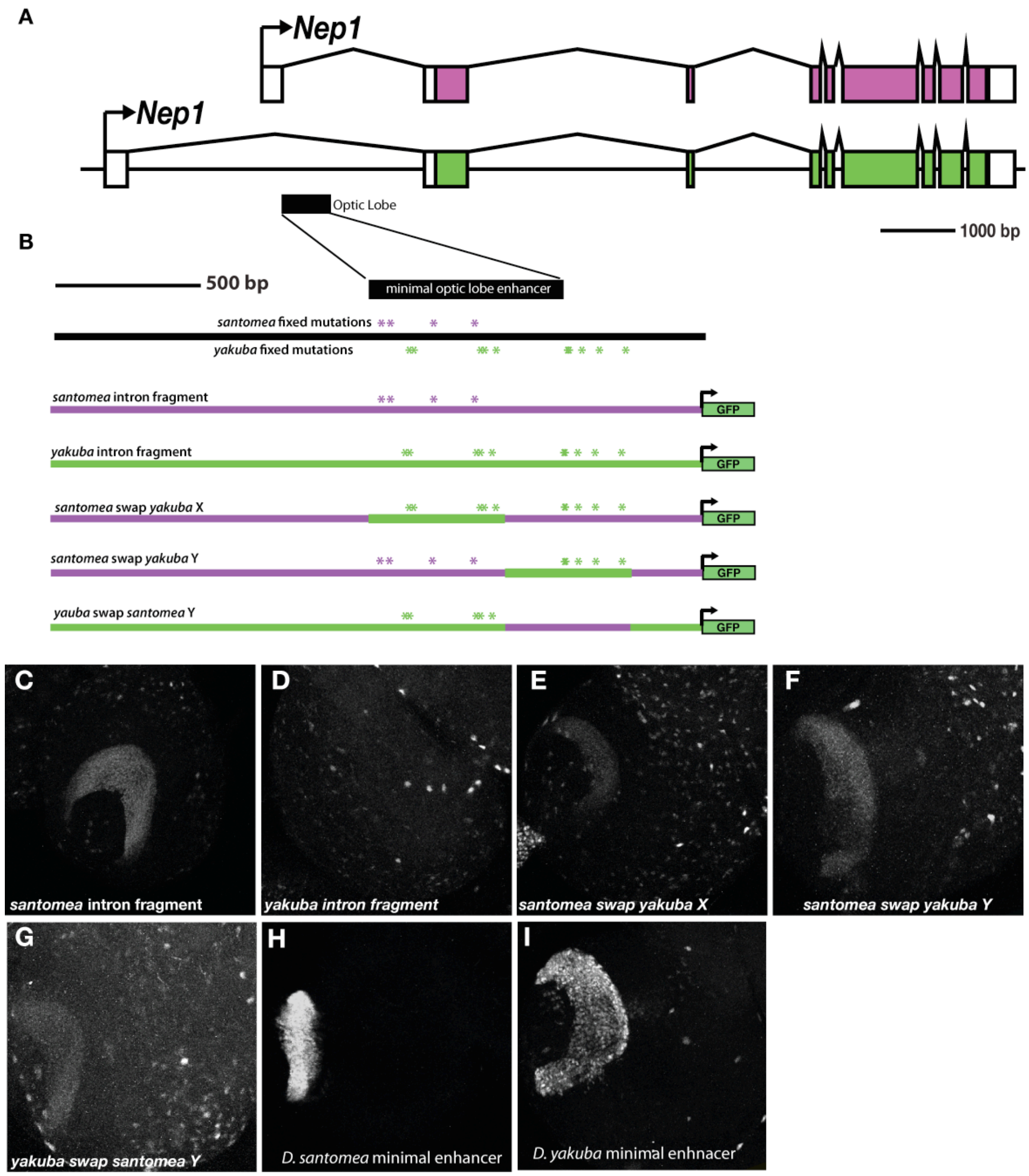

A
Nep1
Nep1
Optic Lobe
1000 bp
B
500 bp
minimal optic lobe enhancer
santomea fixed mutations
yakuba fixed mutations
santomea intron fragment
GFP
yakuba intron fragment
GFP
santomea swap yakuba X
GFP
santomea swap yakuba Y
GFP
yauba swap santomea Y
GFP
C
santomea intron fragment
D
yakuba intron fragment
E
santomea swap yakuba X
F
santomea swap yakuba Y
G
yakuba swap santomea Y
H
D. santomea minimal enhancer
I
D. yakuba minimal enhnacer

Figure S13. The loss of optic lobe activity in *D. yakuba* is due in part to the gain of repressive inputs. (A) Schematic of the Nep1 gene, indicating the position of the minimal optic lobe enhancer. (B) Schematic of chimeric reporter constructs that combine different *yakuba* and *santomea* intron regions, within and adjacent to the optic lobe enhancer. (C) The *D. santomea* intron fragment drives optic lobe activity, and yet the orthologous region from *D. yakuba* does not (D). Upon swapping in the "X" fragment, which contains all four santomea-specific mutations, the optic lobe activity is strongly reduced (E). Swapping the "Y" fragment from yakuba into the *santomea* intron fragment causes a slight reduction in activity (F). Surprisingly, the reciprocal construct, in which the santomea "Y" fragment (which contains no derived mutations) is swapped into the yakuba intron fragment exhibits weak optic lobe activity (G, detected with greatly increased gain), suggesting that mutations in this region in repress the activity of the minimal enhancer in *D. yakuba*. Consistent with this hypothesis, the minimal optic lobe enhancer of *D. yakuba* (I) is sufficient to drive optic lobe expression, though at reduced levels in comparison to the *D. santomea* minimal fragment (H).

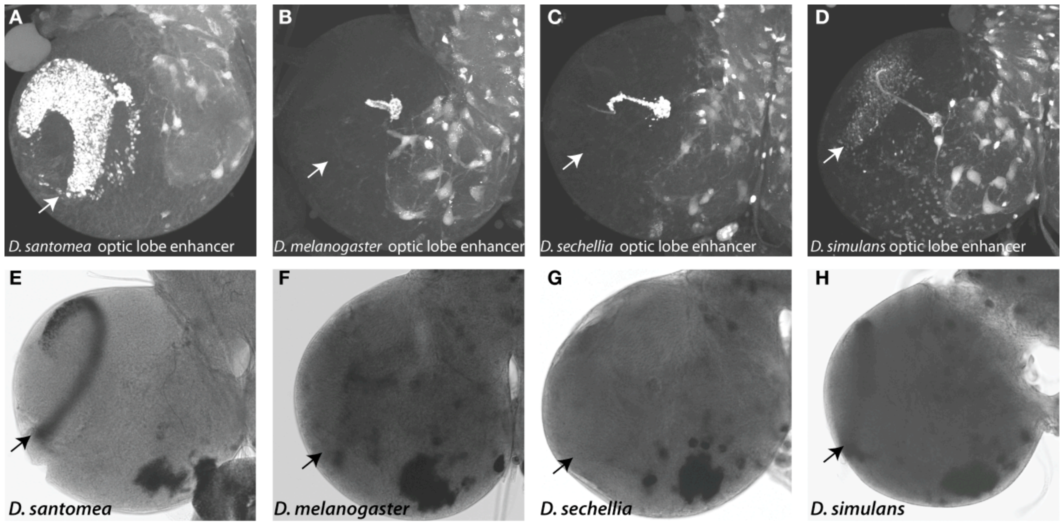


Figure S14. The potential to evolve optic lobe expression of *Nep1* has persisted for >10 million years. (A-D) Comparison of *D. santomea* optic lobe enhancer activity (A) to that of melanogaster complex species (B-D). (E-H) *Nep1* expression in optic lobes treated with high proteinase K to detect trace levels of expression. (A, E) *D. santomea* exhibits both strong optic lobe enhancer activity (A, arrow), and expression of *Nep1* in the optic lobes (E, arrow). *D. melanogaster* (B, F), and *D. sechellia* (C,G) have no detectible enhancer activity or optic lobe expression (arrows). The *D. simulans* optic lobe enhancer has weak activity in the laminar neuroblasts (D, arrow), a pattern that recapitulates the weak endogenous *Nep1* expression in the optic lobes (H, arrow).

Table S1. Summary of pattern changes

Gene	Leg	Wing	Antenna	Eye	Brain
CG14534			A, B	A, B	
Gld	B	A			
Nep-1	A, B	B	A, B, C	A, B	B, C
Obp56d		C		B	
Obp99a				A, B	A, B

A = domain/intensity shift (8 total)
B = heterochrony/loss (10 total)
C = apparent gain (3 total)

Table S2. Species used in the study

Species	UCSD Stock Number / Stock Origin
D. melanogaster	*yw* stock
D. simulans	14021-0251.165
D. mauritiana	14021-0241.01
D. sechellia	Cousin Island
D. yakuba	14021-0261.01
D. santomea	14021-0271.00
D. teissieri	14021-0257.00
D. erecta	14021-0224.01
D. suzukii	14023-0311.00
D. kikkawai	Gift from Artyom Kopp
D. jambulina	NH0115
D. ananassae	14024-0371.13
D. pseudoobscura	14011-0121.87

Table S3. Primers for generating riboprobes

Gene	Species	Forward Primer	Reverse Primer
CG17278	*D. melanogaster*	atttaggtgacactatagaTCGTGCGCGAATTAATCTACAACG	taatacgactcactataggACTGGCCAACAATACTGCTGAGAGC
CG5758	*D. melanogaster*	atttaggtgacactatagaTATGTAAAGCTTCTCTCGCCGAAAAC	taatacgactcactataggATCGTCGATGAACCGGTAGACCTGCT
Doc2	*D. melanogaster*	atttaggtgacactatagaGAGTCTGACATGTCGCCAACGAAAGG	taatacgactcactataggCCGATATGCTGAAGCCCTTGCTCCTT
CG10275	*D. melanogaster*	atttaggtgacactatagaGGGAGATTCTGCTGACCAGCGATGTA	taatacgactcactataggCTCGTGGATTCGGTATCGTCATGGCA
Chn	*D. melanogaster*	atttaggtgacactatagaAAATGGACGCCATTAGTCGCAACACC	taatacgactcactataggGCTGAACCTTTGGAGCAATGGTCAGG
CG8483	*D. melanogaster*	atttaggtgacactatagaTGGCAATGTGGTGGGCTACAATCCCT	taatacgactcactataggCAGGTTAGAAGGCTCCATCCACGACT
CG14534	*D. melanogaster*	atttaggtgacactatagaGCGATCCAGTCCAACCCGAATACCGA	taatacgactcactataggCGATAACTAAGGCTAGCTCGCAGTGA
Tsp	*D. melanogaster*	atttaggtgacactatagaTTCTTCCTGGACCGCAAACAGCAG	taatacgactcactataggTCCATCTGGGTGTACAGCTTGGCCAG
CG14301	*D. melanogaster*	TACTGCGACATCGATGGACGAC	TCAGGTGAAGATGTCCTCGACC
tup	*D. melanogaster*	ATCGAACCGAATCTAATCCAGC	AACATCCTTGGTACACTTCGTC
Pdm2	*D. melanogaster*	ACTGCGAGATGCCACAGAATCT	GGGCACAACAGATACACACGTA
cyp310a1	*D. melanogaster*	atttaggtgacactatagaCTCGGAAAAGGCCGGCATTACGTGGA	taatacgactcactataggACTTTCTGGACCTGCCCGAACATCTC
CG8780	*D. melanogaster*	CAGCAGCGTTGCCGTTTCGATG	CAGCGGTTGTGACTGGTGCGAA
CG9008	*D. melanogaster*	TGCTATCAGATTATCATCGAGGGCAATG	CTGTGAAATATTTGTAATCTGCAAAG
Obp56a	*D. melanogaster*	TCCCTTAATCTGAGCGACGAGCAG	AACTTCTTTAGGCCTTAGCCTCGG
Obp56d	*D. melanogaster*	atttaggtgacactatagaGAGGGAATCACCAAGGATCAGGCGAT	taatacgactcactataggGATGTGGGCGCGATTCTTGTAGTAGCAC
Obp56d	*D. yakuba*	atttaggtgacactatagaGAACTACAACTTTCGGATGAGCAG	taatacgactcactataggCACACAATAAAATAGCGTTCGTTGG
ana	*D. melanogaster*	GACATCTTCTTCAACGGTAGCA	CAAATGCACTTAGTAGGTATGG
ana	*D. yakuba*	CTCCATGAAGAGCACCTACAACAC	GCGGTTTGACTGGATGAACATGTG
Gld	*D. melanogaster*	TACAACGACGTGCTTCCGTTCT	CAACGCGGTGTGCCTAGAGTGA
Gld	*D. yakuba*	ACGGAATGATGTATATCCGCGGCA	AGATAACTTGGATGGAGATTGCGC
Obp99a	*D. melanogaster*	GCCGACTATGTGGTGAAGAACC	TTTTTCCCCACTGAATCGAGAA
Obp99a	*D. yakuba*	TGTGGTGAAGAACCGGAACGACAT	TATCAGTCCATGCGCCCAACTAAG
Obp99a	*D. ananassae*	GTTATAGGCCTCGGCTGATTACGT	GCCAGGCTCTTTTTGATCTGCTCC
Obp99a	*D. pseudoobscura*	TATCGCGACGAGTGCGTCAAGGAA	TGGGGGCCAGGCTCTTCTGGATCTT
Nep1	*D. melanogaster*	TCATCGAGCGGAACTGGAGTCC	ATTCACGTACTCCTGCTCCAGC
Nep1	*D. yakuba*	AAATCCGCAAAATCGGAACGGGACGA	GGATAGCCGATACGCTCGTTCAT
Nep1	*D. erecta*	AAATCCGCAAAATCGGAACGGGACGA	GGATAGCCGATACGCTCGTTCAT
Nep1	*D. ananassae*	AAGCAGGTCCTGAAGTCCCTCGGT	TCCAGTTCGGTGGCATTCGTCAG
Nep1	*D. pseudoobscura*	CGATCTCTCGGTGGAGAAACTGAT	CCAATCCTCTCGTTCATAGAGTCG
Nep1	*D. suzukii*	TACTACCTGAAGGAGAGCAGTG	taatacgactcactataggCTGCATCATNTTNCCCTCCTTGTCG
Nep1	*D. kikkawai*	TACTACCTGAAGGAGAGCAGTG	taatacgactcactataggCTGCATCATNTTNCCCTCCTTGTCG
Nep1	*D. jambulina*	TACTACCTGAAGGAGAGCAGTG	taatacgactcactataggCTGCATCATNTTNCCCTCCTTGTCG

Lowercase letters indicate promoter sequences used for *in vitro* transcription. (SP6 on forward primers, T7 on reverse primers). Primers pairs that vector.

Table S4. Primers for generating reporter constructs

Construct	Forward Primer	Reverse Primer
A	TTCCGggcgcgccTGAAACGCACATGCCAAAAGTTTTGCG	TTGCCcctgcaggCATCAGCCTCGATGGCACTGGAAAAAA
B	TTCCGggcgcgccGGTGGTGTCGCCACTCCTGGG	TTGCCcctgcaggGAGTTGAGGTCCATGGCCGAGAGCAG
C	TTCCGggcgcgccCGATATACACCATACGAGTACT	TTGCCcctgcaggACAGCGACTACAAGGAGCTGCT
A.1+	TTCCGggcgcgccTGAAACGCACATGCCAAAAGTTTTGCG	TTGCCcctgcaggACTAAAATAAATTAATGCGAC
A12-OL	TTCCGggcgcgccTGAAACGCACATGCCAAAAGTTTTGCG	TTGCCcctgcaggACTAAAATAAATTAATGCGAC
A.1	TTCCGggcgcgccTGAAACGCACATGCCAAAAGTTTTGCG	TTGCCcctgcaggCAAGTATTGCCAATGGAAGTGACACC
A.2	TTCCGggcgcgccGACACGATGATCACGCACTGATAA	TTGCCcctgcaggGAGACTAGTAAACCTGTCAAA
A.3	TTCCGggcgcgccAGCTGGGTTAACTAGTTTCGAGTT	TTGCCcctgcaggCATCAGCCTCGATGGCACTGGAAAAAA
A.2.1+2	TTCCGggcgcgccGACACGATGATCACGCACTGATAA	TTGCCcctgcaggACTAAAATAAATTAATGCGAC
A.2.2+3	TTCCGggcgcgccGACACGATGATCACGCACTGATAA	TTGCCcctgcaggCATCAGCCTCGATGGCACTGGAAAAAA
A.2.1	TTCCGggcgcgccGACACGATGATCACGCACTGATAA	TTGCCcctgcaggCAAGTATTGCCAATGGAAGTGACACC
A.2.2	TTCCGggcgcgccCATCTGGCCCCATATTATTT	TTGCCcctgcaggACTAAAATAAATTAATGCGAC
A.2.3	TTCCGggcgcgccACAACAAAAAGTAATGCACTAAA	TTGCCcctgcaggGAGACTAGTAAACCTGTCAAA
A.2.3.1	TTCCGggcgcgccACAACAAAAAGTAATGCACTAAA	TTGCCcctgcaggAACTCGAAACTAGTTAACCCAGCT
A.2.3.4	TTCCGggcgcgccACAACAAAAAGTAATGCACTAAA	TTGCCcctgcaggTCTTCGCAGCTCGCGATCGTTT
A.2.2.4	TTCCGggcgcgccAGAGAGAGAGCGCCACACTAGC	TTGCCcctgcaggACTAAAATAAATTAATGCGAC

Forward primers contain an *Asc I* site (lowercase), and reverse primers contain a site for *Sbf I* (lowercase) for cloning into the S3AG GFP reporter transformation vector.

Table S5. Primers for generating chimeras and introducing mutations to reporter constructs.

Construct	Forward Primer	Reverse Primer
X-fragment swap	CAACAAAAAGTAATGCACTAAA	GAAACTCGAACGGAGCGACACAAAC
Y-fragment swap	GTTTGTGTCGCTCCGTTCGAGTTTC	AACTCGAAACTAGTTAACCCAGCT
Optic Lobe enhancer Deletion	ATAAAACACTAAGTCAGCAGCAGGTAAACTAAAACTAATTCC	GGAATTAGTTTTAGTTTACCTGCTGCTGACTTAGTGTTTTAT
santomea polymorphic repeat removal	CTCCGCTTCCACCAGCACCACCACCACCATCCTCATCCTCTTGCGG	CCGCAAGAGGATGAGGATGGTGGTGGTGGTGCTGGTGGAAGCGGAG
santomea specific mut 1	ATTATACAAAAATTAAACAAAAAACCGGAAAGTGCACAGAAA	TTTCTGTGCACTTTCCGGTTTTTTGTTTAATTTTTGTATAAT
santomea specific mut 2	GAAAGAAATAAGATCATAGAATCTTTGTGTTTTTTTTGGAACTGAT	ATCAGTTCCAAAAAAAACACAAAGATTCTATGATCTTATTTCTTTC
santomea specific mut 3	GAGATACAGTATATATTTTTTTTCTGTGCAGTAATTGTTTGCCAAG	CTTGGCAAACAATTACTGCACAGAAAAAAAATATATACTGTATCTC
santomea specific mut 4	ATGCTAGCCAGAATTACCAAAATTTTTTTTGCCTGTGCGATTATG	CATAATCGCACAGGCAAAAAAAATTTTGGTAATTCTGGCTAGCAT
Block 1 mutation	CAGCAGCAGA cCcAaAcAcAtTcAgGaAaTcAcAgAgTc TACAAAAATT	AATTTTTGTAGACTCTGTGATTTCCTGAATGTGTTTGGGTCTGCTGCTG
Block 2 mutation	TTTGTGTTTTTTTTG tAcCgGcTcTaG CAGAGGTATTCC	GGAATACCTCTGCTAGAGCCGGTACAAAAAAAACACAAA
Block 3 mutation	TTACCAACATT gTgTgTgGaC TGTGCGATTATG	CATAATCGCACAGTCCACACACAATGTTGGTAA
Block 4 mutation	GGTTCACAAGCGACAAACACAATGTGAAATTCAT	GGTTCACAAGCGACAAACACAATGTGAAATTCAT
Block 5 mutation	TTTAGCTTGT tAcCaAtCgGaG CGTAGGTAAT	ATTACCTACGCTCCGATTGGTAACAAGCTAAA
Block 6 mutation	GCGCGTAGGT cAgAaGcTtCtAcTgTcAaGcC GGTCGCATTA	TAATGCGACCGGCTTGACAGTAGAAGCTTCTGACCTACGCGC
Block 7 mutation	TTTAACGACG tTaGaAgTcAgTgAgTgTcGgTg GTGTCGCTCC	GGAGCGACACCACCGACACTCACTGACTTCTAACGTCGTTAAA

Lowercase letters in forward primers indicate the nucleotides that were mutated to scramble conserved block sequences.