

1 **Multiple loci linked to inversions are associated with eye** 2 **size variation in species of the *Drosophila virilis* phylad**

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34 Abstract

35

36 The size and shape of organs is tightly controlled to achieve optimal function. Natural
37 morphological variations often represent functional adaptations to an ever-changing
38 environment. For instance, variation in head morphology is pervasive in insects and the
39 underlying molecular basis is starting to be revealed in the *Drosophila* genus for species of the
40 *melanogaster* group. However, it remains unclear whether similar diversifications are governed
41 by similar or different molecular mechanisms over longer timescales. To address this issue, we
42 used species of the *virilis* phylad because they have been diverging from *D. melanogaster* for
43 at least 40 million years. Our comprehensive morphological survey revealed remarkable
44 differences in eye size and head shape among these species with *D. novamexicana* having the
45 smallest eyes and southern *D. americana* populations having the largest eyes. We show that
46 the genetic architecture underlying eye size variation is complex with multiple associated
47 genetic variants located on most chromosomes. Our genome wide association study (GWAS)
48 strongly suggests that some of the putative causative variants are associated with the presence
49 of inversions. Indeed, northern populations of *D. americana* share derived inversions with *D.*
50 *novamexicana* and they show smaller eyes compared to southern ones. Intriguingly, we
51 observed a significant enrichment of genes involved in eye development on the 4th chromosome
52 after intersecting chromosomal regions associated with phenotypic differences with those
53 showing high differentiation among *D. americana* populations. We propose that variants
54 associated with chromosomal inversions contribute to both intra- and inter-specific variation
55 in eye size among species of the *virilis* phylad.

56

57 Introduction

58

59 One of the most important goals of biological research is to understand the mechanisms
60 underlying morphological diversification. The molecular basis of simple morphological traits,
61 such as pelvic reduction in sticklebacks (Shapiro et al. 2004), presence or absence of trichomes
62 in *Drosophila* (Sucena and Stern 2000), and pigmentation variation in flies (Wittkopp et al.
63 2003; Wittkopp et al. 2009) and mice (Hoekstra 2006), have been determined and are usually
64 caused by a small number of large effect loci. However, the molecular basis of variation in
65 complex traits remains largely elusive.

66 The insect head represents a great model to study complex trait evolution, since it
67 harbors major sensory organs, which facilitate fundamental processes like feeding and
68 reproduction. Natural variation in insect head size and shape is pervasive in insects and it is
69 often driven by a functional trade-off between visual and olfactory sensory investment
70 (Balkenius et al. 2006; Stieb et al. 2011; Montgomery and Ott 2015; Keesey et al. 2019;
71 Ramaekers et al. 2019; Sheehan et al. 2019; Özer and Carle 2020), suggesting that it is likely
72 caused by functional adaptations to an ever-changing environment. Externally, this trade-off is
73 often observed by extensive head shape variation if compound eye size increases at the expense
74 of the cuticle between the eyes (i.e. interstitial head cuticle) (Norry et al. 2000; Posnien et al.
75 2012; Keesey et al. 2019; Gaspar et al. 2020). The compound eyes are the most noticeable
76 sensory structures in the insect head and differences in eye size have been reported between
77 species, as well as between populations of the same species across the *Drosophila* genus (Norry
78 et al. 2000; Hämmerle and Ferrús 2003; Posnien et al. 2012; Arif et al. 2013; Keesey et al.
79 2019; Ramaekers et al. 2019; Gaspar et al. 2020). Interestingly, eye size can vary due to
80 variation in facet size or due to changes in ommatidia number (Posnien et al. 2012; Arif et al.
81 2013; Hilbrant et al. 2014; Gaspar et al. 2020), suggesting that different functional needs
82 influence final eye size.

83 Quantitative genetics approaches have revealed multiple loci associated with variation
84 in eye size between *D. simulans* and *D. mauritiana* supporting the complex genetic architecture
85 of this trait (Arif et al. 2013). Similar observations were made for intra-specific variation in *D.*
86 *melanogaster* (Norry and Gomez 2017; Ramaekers et al. 2019) and *D. simulans* (Gaspar et al.
87 2020). However, Ramaekers et al. (2019) have shown that a single mutation affecting the
88 regulation of the *eyeless/Pax6* gene can explain up to 50% of variation in eye size between two
89 *D. melanogaster* strains. Although, the genetic architecture underlying eye size variation is
90 starting to be revealed for species of the *melanogaster* group, it remains unclear whether similar
91 independent morphological diversifications identified in *Drosophila* (Norry et al. 2000; Keeseey
92 et al. 2019) share the same molecular basis over longer timescales.

93 Chromosomal inversions are an interesting genetic variant because suppression of
94 recombination is thought to maintain linkage of favorable alleles which are protected from
95 immigrant alleles carrying variants which decrease fitness (Kirkpatrick and Barton 2006;
96 Kirkpatrick 2010). Therefore, chromosomal inversions can act as super genes influencing a
97 myriad of phenotypes that can have a large adaptive value. The impact of chromosomal
98 inversions on many life-history and physiological traits is well established and is often
99 associated with local adaptation (Huang et al. 2014; Durmaz et al. 2018; Fuller et al. 2019;
100 Kapun and Flatt 2019). Additionally, chromosomal inversions are associated with differences
101 in rather simple morphological traits. For instance, natural variation in chromosomal inversions
102 affect wing, thorax and head phenotypes in *D. buzzatii* (Norry et al. 1995; Fernández Iriarte et
103 al. 2003) and wing size and shape in *D. mediopunctata* (Hatadani and Klaczko 2008) and in *D.*
104 *melanogaster* (Rako et al. 2006). Chromosomal inversions are commonly associated with
105 population structure and hinder the distinction between correlated and causative variants
106 (Wellenreuther and Bernatchez 2018). Therefore, the impact of inversions on the diversity of
107 complex morphological traits remains largely elusive.

108 Species of the *virilis* phylad of *Drosophila* are diverging from *D. melanogaster* for at
109 least 40 million years (Morales-Hojas and Vieira 2012; Russo et al. 2013) and they have been
110 extensively used in comparative genomics studies of important ecological traits, such as body
111 color (Wittkopp et al. 2009; Wittkopp et al. 2011), cold resistance (Reis et al. 2011), life span
112 (Fonseca et al. 2013) and developmental time (Reis et al. 2014). *D. virilis* is a cosmopolitan
113 species of Asian origin while *D. americana* and *D. novamexicana* are endemic to the USA
114 (Throckmorton 1982) and constitute the *americana* complex. *D. americana* shows a wide
115 geographical distribution along the eastern part of the USA while *D. novamexicana* has a
116 smaller distribution in the south-central part of the USA (Patterson and Stone 1949). Several
117 chromosomal inversions segregate in *D. americana* populations showing latitudinal and
118 longitudinal gradients (Hsu 1952; Throckmorton 1982). Some of these inversions create highly
119 differentiated genomic regions between northern and southern *D. americana* populations and
120 they are shared between northern *D. americana* populations and *D. novamexicana* (Reis et al.
121 2018). Since species of the *virilis* phylad, and in particular the *americana* complex, have
122 multiple well characterized chromosomal inversions and extensive phenotypic variability,
123 these species are a prime model to link variation in phenotypes to the presence of chromosomal
124 inversions and simultaneously understand whether natural variation in organ morphology is
125 due to the same molecular basis in divergent *Drosophila* lineages.

126 In this work we provide a comprehensive morphological and genetic characterization
127 of eye size variation among species of the *virilis* phylad. We show that eye size differences are
128 most pronounced between *D. novamexicana* and a southern strain of *D. americana*. Applying
129 quantitative genetics approaches we establish that eye size differences are caused by multiple
130 genes located in multiple chromosomes. Additionally, we found an association between the
131 presence of chromosomal inversions and eye size. A thorough integration of population
132 genetics, GWAS and phylogenetic datasets revealed a significant enrichment for eye

133 developmental genes among genes located on the 4th chromosome (Muller B). We argue that
134 some of these variants can explain both intra- and interspecific variation in eye size.

135

136 Results

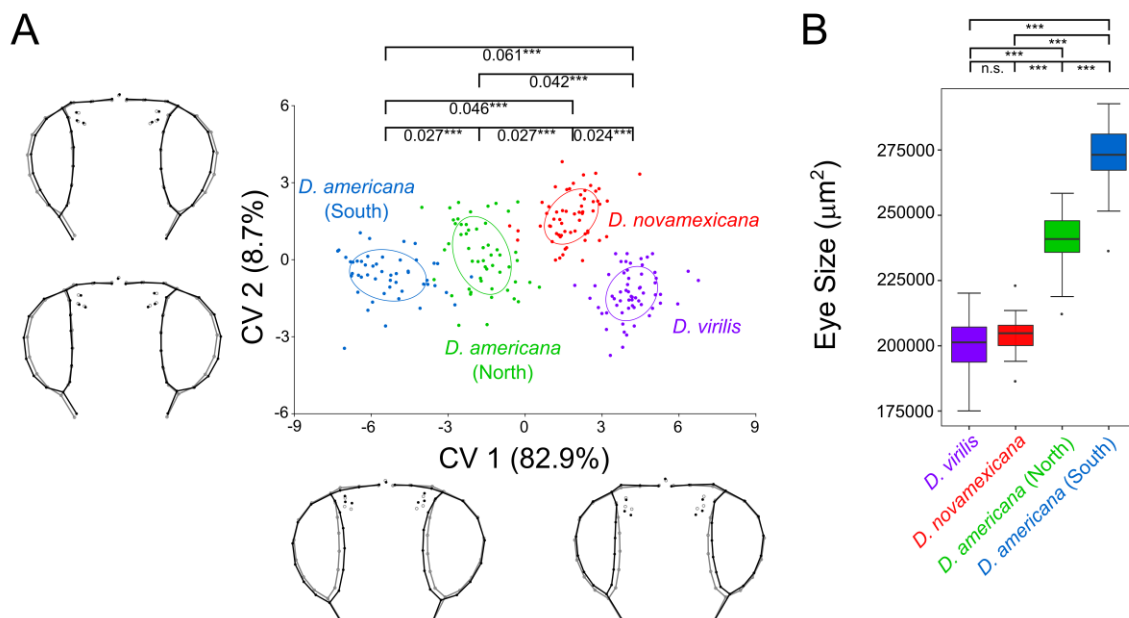
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138 Head shape and eye size is remarkably variable in species of the *virilis* phylad

139

140 To evaluate the extent of variation in overall head shape in the *virilis* phylad, we
141 performed a geometric morphometrics analysis to quantify shape differences in females of two
142 strains of *D. virilis*, *D. novamexicana*, a northern and a southern population of *D. americana*,
143 respectively. The mean shapes were significantly different for all possible pair-wise
144 comparisons among species/populations (Fig 1A). We found that bigger eyes were associated
145 with reduced interstitial cuticle and this effect was more pronounced in the ventral part of the
146 head (Fig. 1A).

147



148

149 **Fig. 1. Eye size and head shape are remarkably variable among species of the *virilis* phylad.**

150 **A.** Head shape variation among species/populations (Canonical variate analysis of the procrustes
151 coordinates obtained from the first 12 principal components (90.8% of the total variation); procrustes
152 distances are provided with *** = $p < 0.0001$ after a permutation test with 10,000 iterations; equal

153 frequency ellipses are given with probability of 0.5). The wireframes depict changes in shape along the
154 two main canonical variates (CV1 and CV2) (black - the maximum and minimum values on the axis
155 (Mahalanobis distances); grey – mean shape for each axis). The amount of variation explained by each
156 CV is shown in brackets. **B.** Eye size variation (after accounting to variation in body size) among
157 species/populations (Kruskal-Wallis test, followed by post-hoc Dunn’s test and Holm correction for
158 multiple testing: * $p < 0.05$, *** $p < 0.001$, n.s. $p > 0.05$).
159

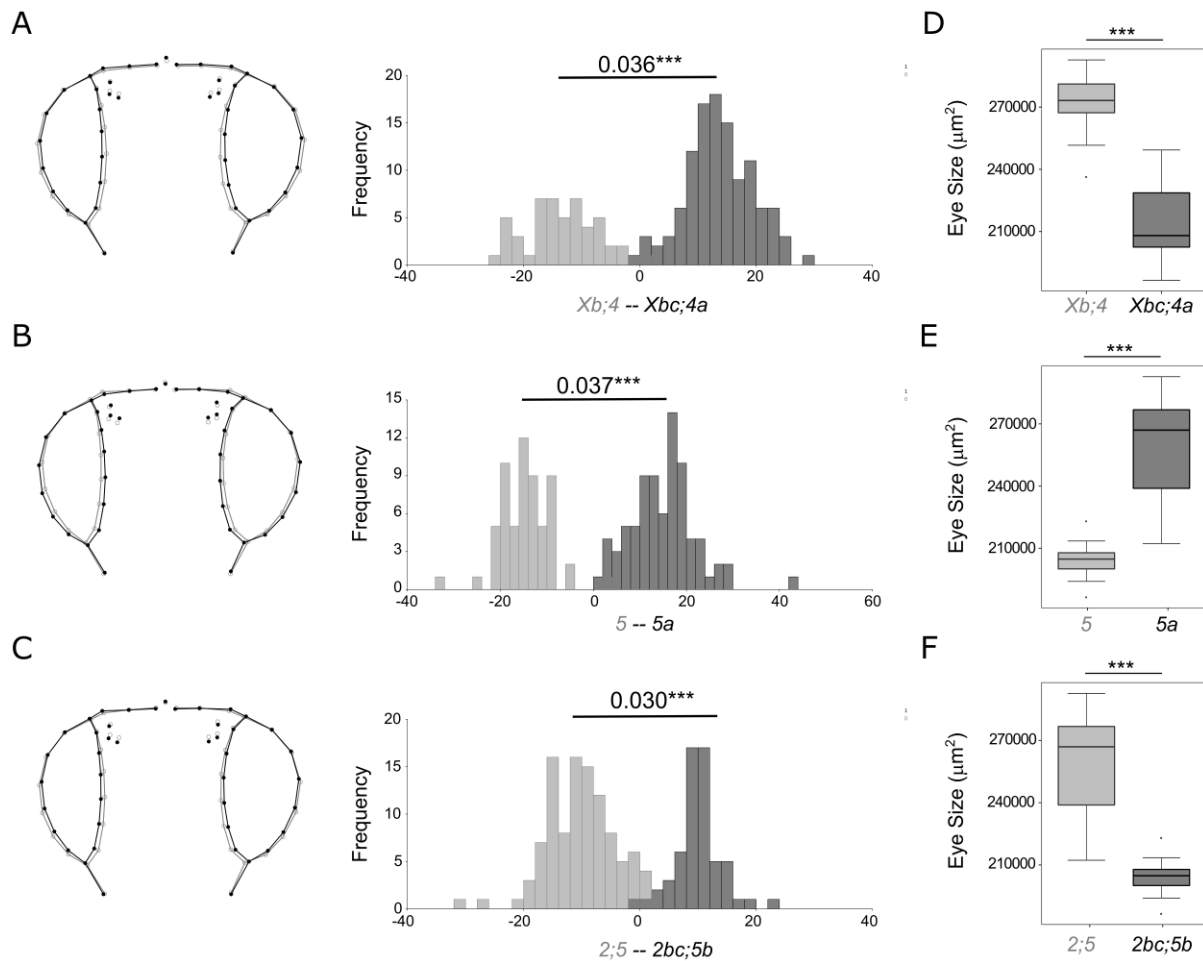
160 To confirm the observed variation in eye size, we measured eye area in females of each
161 species/population. We observed that southern *D. americana* strains had the largest eyes while
162 *D. virilis* and *D. novamexicana* had the smallest (Fig. 1B). Differences in eye size reached
163 36.2% when southern *D. americana* strains were compared to *D. virilis* and 13.7% between *D.*
164 *americana* populations (File S1). Therefore, differences in head shape are accompanied by eye
165 size (after accounting for variation in body size) variation and this association was further
166 confirmed by the significant correlation between the former trait and CV1 (Pearson’s $r =$
167 -0.925 , $p < 2.2e-16$). Overall, these results show that eye size and head shape differ remarkably
168 between species of the *virilis* phylad and among *D. americana* populations.
169

170 Variation in head shape and eye size is associated with chromosomal inversions in 171 strains of the *americana* complex 172

173 Chromosomal inversions are pervasive in the *virilis* phylad (Hsu 1952; Throckmorton
174 1982). Therefore, the observed variation in eye size and head shape provides an excellent
175 model to test whether inversions are associated with differences in complex morphological
176 traits. We developed new molecular markers for each chromosomal inversion and genotyped
177 all analyzed strains (see Material and Methods). Our results were largely compatible with
178 previous observations (File S2, (Hsu 1952; Throckmorton 1982)). Inversions *Xa* (Muller A)
179 and *2a* (Muller B) were exclusive of *D. virilis*, while inversion *Xb* (Muller A) was present in
180 all *D. novamexicana* and *D. americana* strains. Inversions *2b* (Muller E) and *5b* (Muller C)
181 were exclusive of *D. novamexicana*, while inversion *5a* (Muller C) was exclusive of *D.*

182 *americana*. Inversions *Xc* (Muller A) and *4a* (Muller B) were present in *D. novamexicana* and
183 *D. americana* (O43, O53). For inversion *5a* (Muller C) we found evidence for heterozygosity
184 in *D. americana* (O43) (*5a/5*). Surprisingly, we could not find evidence for the presence of
185 inversion *5b* (Muller C) in northern *D. americana* strains, which was previously described to
186 be fixed in northern populations (Hsu 1952).

187 Since most of the inversions, except *Xa* and *2a*, are derived in the lineage leading to *D.*
188 *americana* and *D. novamexicana* (Throckmorton 1982; Reis et al. 2018), we excluded *D.*
189 *virilis*, to address the impact of inversions on head shape and eye size (after accounting for
190 variation in body size) in the *americana* complex. We found significant associations between
191 the presence of inversions and head shape variation, mostly affecting the ratio between eye size
192 and the head cuticle (Fig. 2A-C). Accordingly, we also found significant associations between
193 the presence of inversions and eye size among strains (*Xbc,4a* v. *Xb,4* (Muller A, B) ($W =$
194 5489 , $p < 2.2e-16$); *2bc,5b* v. *2,5,5a* (Muller E, C) ($W = 6113$, $p < 2.2e-16$) (Fig. 2D-F). The
195 presence of inversions *Xc,4a* (*D. novamexicana* and northern *D. americana*) and *2b,5b* (*D.*
196 *novamexicana*) resulted in a 19.1% and 20.3% reduction in eye size, respectively. Inversion *5a*
197 (*D. americana*) led to a significant increase of 26.8% (*5a* v. *5b* ($W=7$, $p < 2.2e-16$). These
198 results suggest that at least part of the causative variants underlying variation in eye size and
199 head shape must be located in chromosomal inversions.



200

201 **Fig. 2. Variation in eye size and head shape is strongly associated with the presence of**
 202 **chromosomal inversions among species of the *americana* complex.** A-C. Head shape variation
 203 among species/populations with (dark grey) or without (light grey) chromosomal inversions $Xc,4a$ (D),
 204 $5a$ (E), and $2bc,5b$ (F) (Discriminant function analysis based on the Procrustes coordinates obtained
 205 from the first 12 principal components explaining 90.8% of the total variation; procrustes distances are
 206 provided with *** = $p < 0.0001$ after a permutation test with 10,000 iterations). The wireframes depict
 207 changes in the mean shape (grey – without inversions; black – with inversions). D-F. Eye size variation
 208 (after accounting to variation in body size) between species/populations with (dark grey) or without
 209 (light grey) chromosomal inversions $Xc,4a$ (A), $5a$ (B), and $2bc,5b$ (C) (Wilcoxon rank-sum test and
 210 Holm correction for multiple testing: *** $p < 2.2e-6$).
 211

212 Eye size is an incomplete dominant trait between *D. americana* and *D. novamexicana*

213

214 The strains showing the largest differences in eye size (after accounting for body size)

215 were *D. americana* (SF12) and *D. novamexicana* (15010-1031.00) (File S1). Additionally, with

216 respect to chromosomal inversions they showed the most divergent karyotypes, supporting the

217 association between inversions and eye size. Therefore, we selected those two strains to

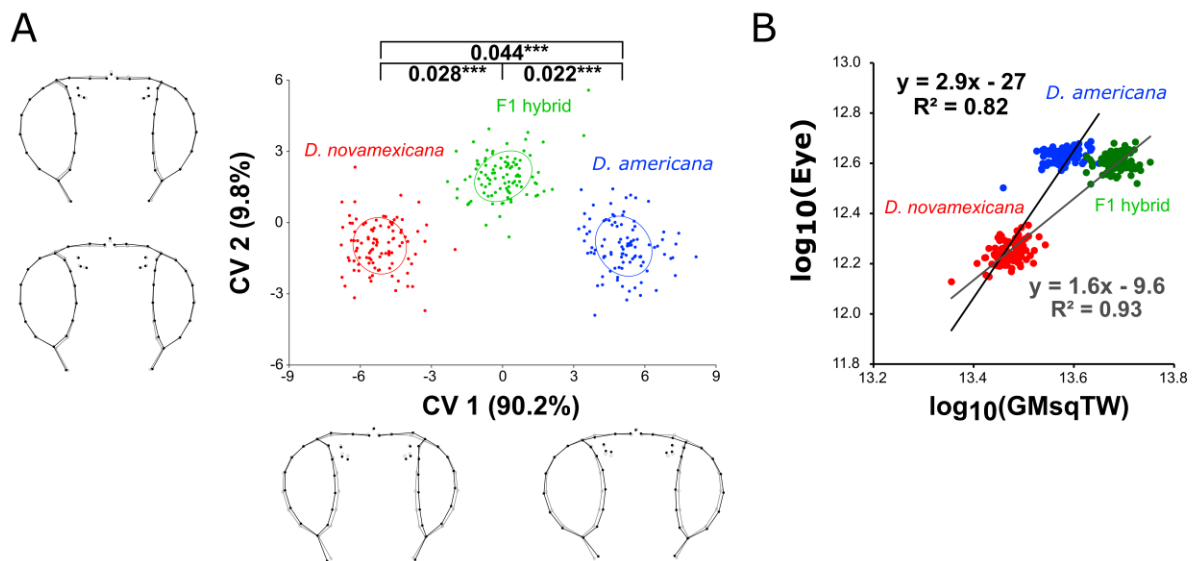
218 characterize head shape and eye size variation and dominance relationship more
219 comprehensively.

220 We performed a geometric morphometrics analysis to evaluate differences in head
221 shape between both parental strains and their F1 hybrids. We found that head shapes were
222 significantly different for all comparisons (Fig. 3A). The main differences between the species
223 and their hybrid was explained by CV1 with the hybrid showing an intermediate head shape.
224 CV1 captured an expansion of the eye that was accompanied by a contraction of the interstitial
225 cuticle. This effect was more pronounced in the ventral region for *D. americana* (Fig. 3A).

226 To evaluate the dominance relationships for eye size, we compared eye areas of F1
227 hybrids to the parental strains and found for all comparisons statistically significant differences
228 (Kruskal-Wallis test followed by Dunn's post-hoc test, $p < 0.001$; File S1), with *D. americana*
229 females having 46.4% larger eyes than *D. novamexicana* females. The eyes of hybrids were
230 slightly, but significantly smaller (2.4%) than *D. americana* female eyes (File S1). Since the
231 F1 hybrids showed almost the same size as *D. americana*, apparently the larger eyes are
232 dominant over smaller eyes. Ommatidia counting revealed that the eye size differences were
233 exclusively caused by variation in ommatidia number (Fig. S1).

234 To test whether body size influenced the observed eye size differences, we measured
235 wing areas as well as tibiae lengths in both parental strains and in hybrids. All comparisons
236 between the two strains and their inter-specific hybrids were statistically significant (Kruskal-
237 Wallis test followed by Dunn's post-hoc test, $p < 0.001$; File S1). Interestingly, while eye area
238 was apparently dominant, the other organs were larger in F1 hybrids (Fig. 3B; File S1) leading
239 to significantly larger allometric coefficients between *D. novamexicana* and *D. americana*
240 when compared to *D. novamexicana* and F1 hybrids ($B=1.30$, $p < 0.001$; Fig. 3B). This result
241 suggests that part of the difference in eye size between the F1 hybrids and *D. novamexicana*
242 may be caused by pronounced changes in total body size. In summary, our results show that

243 eye size is an incomplete dominant trait between *D. novamexicana* and *D. americana* that is
 244 largely affected by overall body size.



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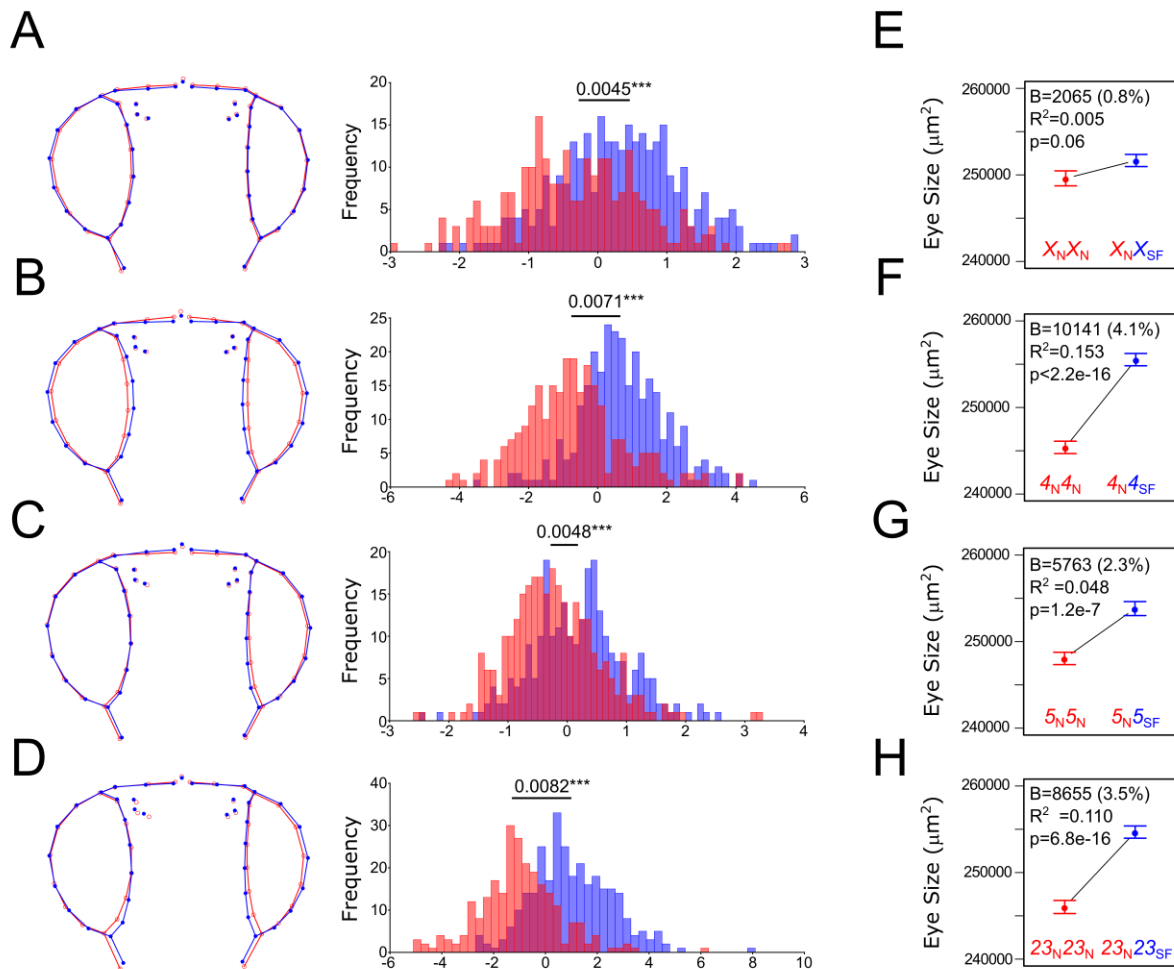
246 **Fig. 3. Eye size and head shape are co-dominant between *D. americana* and *D. novamexicana*.**
 247 Head shape variation between parental strains (*D. novamexicana*, N=99, *D. americana*, N=100) and
 248 their inter-specific hybrids (N=100). Canonical variate analysis was applied to the procrustes distances
 249 obtained from the first nine principal components (90.5% of the total variation); procrustes distances
 250 are provided with *** = $p < 0.0001$ after a permutation test with 10,000 iterations; equal frequency
 251 ellipses are given with probability of 0.5. The wireframes depict changes in shape along the two main
 252 canonical variates (CV1 and CV2) (black - the maximum and minimum values on the axis; grey – mean
 253 shape for each axis). The amount of variation explained by each CV is shown between brackets. **B.**
 254 Scaling relationships between females of *D. novamexicana* (15010-1031.00) (N=99) and *D. americana*
 255 (SF12) (N=98), as well as between females of *D. novamexicana* and F1 inter-specific hybrids (progeny
 256 of crosses between *D. novamexicana* males and *D. americana* females; N=98) for eye area relatively to
 257 the GMsqTW. The slopes of the equations represent the allometric coefficients between *D.*
 258 *novamexicana* and F1 hybrids (grey) as well as between *D. novamexicana* and *D. americana* (black).
 259

260 Normalized eye size and head shape is affected by variation on multiple chromosomes
 261

262 To reveal genetic variants associated with head shape and eye size differences between
 263 *D. americana* and *D. novamexicana*, we performed a backcross study (see Materials and
 264 Methods for details). Most chromosomes were associated with the size of multiple adult organs
 265 simultaneously with a pronounced effect of the 5th chromosome (Muller C) (Fig. S2),
 266 suggesting that variants in general factors affecting overall body size are segregating in these
 267 crosses. Therefore, we evaluated the effect of individual chromosomes on the non-allometric
 268 component of shape (Fig 4A-D; for the effect of each chromosome on the allometric shape see

269 Fig. S3). We found significant associations between every chromosome and head shape
270 variation, with smaller effects of the *X* or *5th* chromosomes (Muller A or C) compared to the
271 *2nd*, *3rd*, and *4th* chromosomes (Muller E, D, and B) (Fig. 4A-D). The differences in mean
272 shape caused by the *D. americana* fused *2nd* and *3rd* chromosomes (Muller E and D) and the *4th*
273 chromosome (Muller B) were compatible with a trade-off between the eyes and the interstitial
274 cuticle (Fig. 4B, D). This effect was only observed in the ventral region of the head for *2nd* and
275 *3rd* chromosomes (Muller E and D) (Fig. 4D) and the presence of the *4th* chromosome (Muller
276 B) additionally caused an expansion of the eye area in the lateral part of the head (Fig 4B).
277 Hence, genes located on all chromosomes contribute to head shape variation between *D.*
278 *americana* and *D. novamexicana*.

279 Next, we assessed the effect of each chromosome on eye size. Since variants affecting
280 overall body size segregated in our crosses, we determined which chromosomes affect eye size
281 exclusively. To this end, we adopted a very conservative approach for normalization to account
282 for variation in overall body size (see Materials and Methods for details). After accounting for
283 differences in total body size, we observed that the *X* chromosome (Muller A) had no
284 significant effect, while all other chromosomes showed a strong association with the
285 normalized eye size (Fig. 4E-H). The main effects were caused by the presence of the *4th*
286 chromosome (Muller B) (Fig. 4F) and the fused *2nd* and *3rd* chromosomes (Muller E and D)
287 (Fig. 4H), which explained 15.3% and 11.0% of the variation and resulted in an increase of
288 4.1% and 3.5% in normalized eye size, respectively. We also found a slight contribution of the
289 *5th* chromosome (Muller C) (Fig 4G) which explained 4.8% of the variation and led to an
290 increase of 2.3% in normalized eye size.



291

292 **Fig. 4. Variation in normalized eye size and head non-allometric shape is mainly explained by**
 293 **the 2nd and 3rd fused chromosomes (Muller E and D) as well as by the 4th chromosome (Muller B).**
 294 **A-D.** Variation in mean head shape among the female progeny of the backcross between F1 hybrid
 295 females and *D. novamexicana* males (Discriminant function analysis of the procrustes coordinates
 296 obtained from the first 19 principal components (90.8% of the total variation); procrustes distances are
 297 provided with *** = $p<0.0001$ after a permutation test with 10,000 iterations). The wireframes depict
 298 changes in the mean shape multiplied by a factor of 5 along the axis of Mahalanobis distances
 299 (homozygous *D. novamexicana* (red) or heterozygous *D. novamexicana/D. americana* (blue)). **E-H**
 300 Distributions of normalized eye size (plots of means \pm SEM) for females, progeny of the backcross,
 301 which were homozygous for a given *D. novamexicana* chromosome (red) or heterozygous *D.*
 302 *novamexicana/D. americana* (blue) for the respective chromosome. Information about the magnitude
 303 of change in eye size, the significance values as well as the percentage of variation explained obtained
 304 using linear regression models is shown inside the graphs.
 305

306 We did not find evidence for epistasis between the chromosomes showing significant
 307 associations with normalized eye size (NormE ~ Ch23 x Ch4; NormE ~ Ch23 x Ch 5; NormE
 308 ~ Ch4 x Ch5; and NormE ~ Ch23 x Ch4 x Ch5, $p > 0.05$ for all interactions), suggesting that
 309 the contribution of the different chromosomes was additive. This result is further supported by

310 the observation that the presence of the fused 2nd and 3rd chromosomes (Muller E and D), the
311 4th chromosome (Muller B) or the 5th chromosome (Muller C) by themselves contributed very
312 little to an increase in normalized eye size (Fig. S4). Indeed, the genotypic class showing the
313 highest values of normalized eye size was the one heterozygous for all chromosomes except
314 the X chromosome (Muller A) (11.8% bigger than the class having *D. novamexicana*
315 chromosomes only, File S1). We conclude that genes located on the *D. americana* 2nd, 3rd, 4th
316 and 5th chromosomes (Muller E, D, B, and C) when present simultaneously on a *D.*
317 *novamexicana* background contribute additively to the highest increase in normalized eye size.

318 To increase the mapping resolution and to reveal SNPs associated with normalized eye
319 size we performed a Genome-Wide Association Study (GWAS) using pools of individuals after
320 17 generations of recombination between hybrids (see Materials and Methods for details). The
321 results obtained were highly compatible with our backcross study. We found clear regions with
322 major differentiation between extreme quartiles on the 2nd and 3rd chromosomes (Muller E and
323 D), as well as on the 4th chromosome (Muller B) and the 5th chromosome (Muller C) (Fig. S5).
324 Additionally, we confirmed that the chromosomal inversions segregating in our crosses largely
325 suppress recombination even after 17 generations. Further analysis of intermediate quartiles
326 showed that the frequencies of the reference variants increased for different chromosomes
327 between adjacent quartiles (Fig. S5B-D). Increased normalized eye size is, thus, caused by
328 combinations of different chromosomes and it is largest when the frequencies of *D. americana*
329 variants are highest across the genome. Overall, these results represent compelling evidence
330 for the role of multiple genes located in different chromosomes in normalized eye size
331 determination.

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333

334 Variants located in genes involved in eye development can explain both intra- and
335 interspecific variation in normalized eye size
336

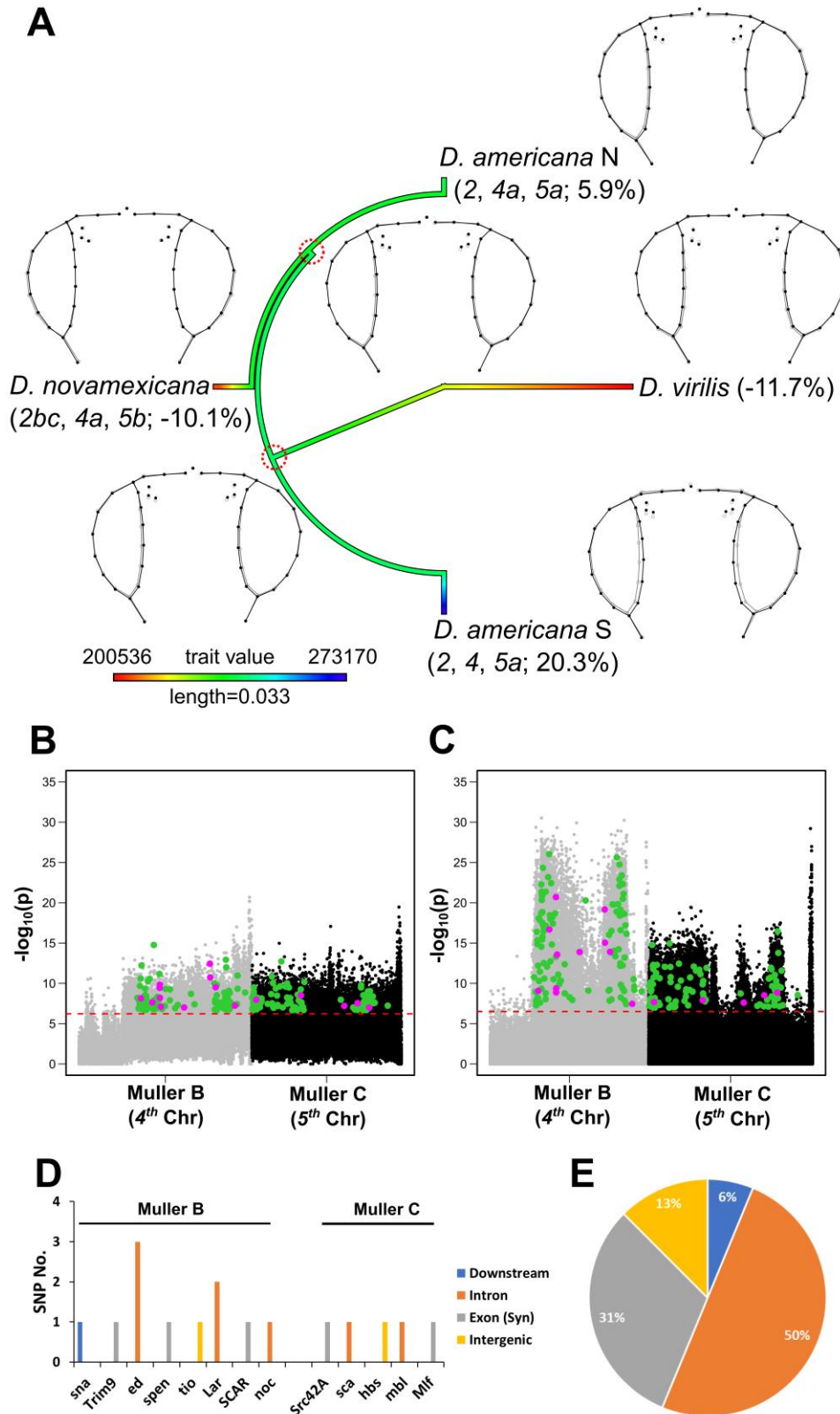
337 To narrow down the high number of potential variants (SNPs) obtained from our
338 GWAS approach, we integrated phylogenetic and population genetics data. Under a simple
339 additive model, the sum of the effects of the different chromosomes lead to the overall effect
340 observed. In southern *D. americana* populations (e.g. SF12), this leads to bigger eyes while in
341 *D. novamexicana* this leads to smaller eyes (Fig. 5A and Fig. 1B). The highly differentiated
342 regions between northern and southern *D. americana* populations (Reis et al. 2018) should be
343 at least partly shared between northern populations and *D. novamexicana*, because they share
344 inversions *Xc* (Muller A), *4a* (Muller B) and *5b* (Muller C) (Fig. 5A, Fig. S6A, but see previous
345 results). In contrast, chromosomes not showing differentiation (*2nd* and *3rd*, Muller E and D)
346 are shared between northern and southern populations (Fig. S6B) and when combined with *Xc*,
347 *4a* and *5b* chromosomes resulted in an intermediate eye size in northern *D. americana*
348 populations (Fig. 5A and Fig. 1B). However, the *2nd* and *3rd* chromosomes show extensive
349 differentiation between *D. novamexicana* and *D. americana* alleles after 17 generations of
350 recombination (Fig. S5), due to the presence of inversions *2b*, *2c* and *3a*, which are fixed in *D.*
351 *novamexicana* (Hsu 1952; Throckmorton 1982) and contributed to a smaller eye size (Fig. 5A
352 and Fig. 1B). Therefore, we raised the hypothesis that regions showing high differentiation due
353 to the presence of inversions will contain the variants associated with differences in eye size in
354 the *americana* complex.

355 Since *D. virilis* also showed smaller eye size, we reconstructed the ancestral state of
356 size traits to understand how these phenotypes evolved in this group of species. We observed
357 that the ancestral eye size was intermediate (Fig. 5A) and this result was supported by the
358 ancestral reconstruction of size phenotypes of 59 *Drosophila* species reported in (Keesey et al.
359 2019) (Fig. S7). Given the high levels of phenotypic and nucleotide variation characteristic of

360 *D. americana* (Fonseca et al. 2013), it is likely that the intermediate ancestral eye size
361 represented the mean of a quantitative trait including smaller and bigger eyes. Thus, we
362 assumed that the ancestral population of the *virilis* phylad was highly polymorphic for eye size,
363 and both bigger and smaller eye size were selected for in specific lineages from a pool of
364 standing genetic variation (Fig 5A). Under this hypothesis, the variants responsible for
365 increased eye size should be fixed in the *D. americana* (SF12) reference genome and at higher
366 frequencies in southern populations, while the alternative variants should be fixed between *D.*
367 *novamexicana* and *D. virilis*. The SNPs matching these conditions and showing significant
368 differences in frequency between extreme quartiles of the GWAS between *D. americana* and
369 *D. novamexicana* represent prime candidates to explain eye size variation in species of the
370 *virilis* phylad.

371

372



373

374 **Fig. 5. Phylogenetic and population genetics approaches revealed loci linked with inversions that**
 375 **can explain variation in eye size in species of the *virilis* phylad. A.** Ancestral reconstruction of the
 376 ancestral eye size (after accounting for variation in body size) of species of the *virilis* phylad. The

377 karyotypes, the wireframes (black – mean head shape of each species/population, grey – mean head
378 shape of the estimated ancestral), and the percentage of variation in eye size compared to the grand
379 mean are shown for each species/population. **B-C.** Manhattan plots of the data obtained between Q4
380 and Q1 (F18 pool seq) and between southern and northern *D. americana* populations, respectively for
381 Muller B and C. The green dots depict the SNPs showing significant differences in frequencies in both
382 data sets after Bonferroni correction; the purple dots depict the subset of significant SNPs located inside
383 or nearby candidate genes for eye development **D.** Regions of eye development candidate genes where
384 the SNPs depicted in purple in B-C were located. **E.** Frequency of the SNPs located in different gene
385 regions.
386

387 To obtain a list of candidate SNPs (see Fig. S8 and Materials and Methods for details),
388 we intersected the SNPs showing significantly higher frequency of the reference variant (*D.*
389 *americana* (SF12)) in Q4 compared to Q1 with those showing the reference variants at higher
390 frequencies in southern *D. americana* populations (Reis et al. 2018). From these SNPs, we kept
391 only those that showed the alternative variant common between *D. novamexicana* and *D.*
392 *virilis*, and we annotated the SNPs to genes using the information available for *D. virilis*. We
393 obtained a total of 6,670 SNPs within or close to 3,006 unique genes. However, only 2,627
394 unique genes of *D. virilis* have recognizable orthologs in *D. melanogaster* (1,254; 534; 366 and
395 473 on the 2nd, 3rd, 4th and 5th chromosomes (Muller E, D, B and C), respectively; File S3).
396 Next, we identified the orthologs of these 2,627 *D. virilis* genes in *D. melanogaster* (2,701
397 genes due to some ambiguities, File S3) and intersected them with the 397 genes associated
398 with eye development (see Methods). We obtained 446 SNPs located within or close to 126
399 unique genes (59, 22, 21 and 24 on the 2nd, 3rd, 4th and 5th chromosomes (Muller E, D, B, and
400 C), respectively File S3). These results showed that one fifth of the total number of genes
401 estimated to be present in *D. melanogaster* (2,701/13,767) had SNPs with significant frequency
402 differences in the GWAS and the same variant in *D. novamexicana* and *D. virilis*, but a different
403 variant in *D. americana* (SF12). Almost one third of the total number of candidate genes for
404 eye development (126/397) were present among those genes. Thus, in our dataset we observed
405 a significant over-representation of eye developmental genes (126 out of 2,701 vs. 397 out of

406 13,701, Chi-square statistics with Yates correction = 37.25, $p < 1.00e-5$) that may explain
407 variation in normalized eye size among species of the *virilis* phylad.

408 We also postulated that the combination of different variants shared either with *D.*
409 *novamexicana* or with southern *D. americana* populations leads to an intermediate phenotype
410 in northern *D. americana* populations (Fig. 5A). Thus, the overlap between the chromosomal
411 regions associated with phenotypic differences with those showing high differentiation among
412 *D. americana* populations (Reis et al. 2018) may explain both inter- and intra-specific variation
413 in eye size. The intersection of both datasets resulted in a total of 119 SNPs in 102 genes on
414 the 4th chromosome (Muller B) and 101 SNPs in 102 genes on the 5th chromosomes (Muller C)
415 (File S3). After intersecting these 204 genes with the 397 candidates for eye development (see
416 methods), we obtained 11 SNPs in 8 out of 102 genes on the 4th chromosome, and 5 SNPs on
417 5 out of 102 genes in the 5th (Fig 5B-C). There was a significant over-representation of genes
418 involved in eye development on the 4th chromosome (8 out of 102 v. 397 out of 13,701; Chi-
419 square statistics with Yates correction = 7.13, $p = 7.6e-3$), but this was not the case for the 5th
420 (5 out of 102 v. 397 out of 13,701; Chi-square statistics with Yates correction = 0.84, $p = 0.36$)
421 (Fig 5 D). The majority of the identified SNPs were in non-coding regions (69%), suggesting
422 that regulatory sequences and thus associated gene expression may be predominantly affected
423 (Fig. 5E). As expected, most of the SNPs that can explain both intra- and interspecific variation
424 in normalized eye size were located on the 4th and 5th chromosomes (Muller B and C).
425 However, we also observed two SNPs in two genes on the 2nd chromosome (Muller E) and four
426 SNPs in four genes on the 3rd chromosome (Muller D). One of the SNPs on the 3rd chromosome
427 was a synonymous mutation located in one candidate gene for eye development) (File S3). In
428 summary, we revealed a significant enrichment in genes involved in eye development on the
429 4th chromosome carrying variants strongly associated with normalized eye size variation
430 between species of the *virilis* phylad and within *D. americana*.

431 Discussion

432

433 We provide the most comprehensive morphological and molecular characterization of
434 head shape and eye size variation in species outside the *melanogaster* group. We observed
435 remarkable differences in these two traits among species of the *virilis* phylad. Our shape
436 analysis revealed that increased eye size was accompanied by a contraction of the interstitial
437 head cuticle. A similar trade-off has been observed in other *Drosophila* species (Norry et al.
438 2000; Posnien et al. 2012; Arif et al. 2013; Keesey et al. 2019; Ramaekers et al. 2019; Gaspar
439 et al. 2020) and it may be associated with different investment in visual or olfactory sensory
440 perception (Keesey et al. 2019; Ramaekers et al. 2019). Indeed, it has been shown that a
441 northern *D. americana* strain is more “visual” because it has significantly bigger eyes
442 compared to the antennae, while *D. virilis* is a more “olfactory” species with smaller eyes and
443 bigger antennae (Keesey et al. 2019). Following this rationale, *D. novamexicana* may be a more
444 “olfactory” species compared to the southern *D. americana* strain studied in detail here.

445 While differential investment in visual or olfactory sensory information is a common
446 phenomenon in animals (Balkenius et al. 2006; Stieb et al. 2011; Montgomery and Ott 2015;
447 Keesey et al. 2019; Ramaekers et al. 2019; Sheehan et al. 2019; Özer and Carle 2020), it
448 remains to be established whether the genetic underpinnings are the same or not. Since the
449 compound eyes (i.e. vision) and the antennae (i.e. olfaction) originate from the same imaginal
450 disc during larval development (Haynie and Bryant 1986), the pervasive variation in head
451 shape and eye size in *Drosophila* is an excellent model to test this. Variation in eye size between
452 *D. melanogaster* strains is highly associated with one SNP in the Cut transcription factor
453 binding site in the *eyeless/Pax6* regulatory region (Ramaekers et al. 2019). However, neither
454 *eyeless/pax6* nor *cut* can explain the natural variation in eye size observed among species of
455 the *virilis* phylad, because they are located on chromosomes that are not associated with
456 differences in eye size among these species in our study. Also, data obtained for *D. mauritiana*

457 and *D. simulans* showed that differences in eye size are due to variation in facet size (Posnien
458 et al. 2012), while eye size differences between *D. novamexicana* and *D. americana* were
459 exclusively caused by differences in ommatidia number. Since facet size and ommatidia
460 number are specified by different developmental processes (Şahin and Çelik 2013; Treisman
461 2013), it is likely that different developmental mechanisms underly natural variation in eye
462 size. Indeed, the most significant QTL explaining eye size variation in *D. mauritiana* and *D.*
463 *simulans* mapped to the X chromosome (Arif et al. 2013). In contrast, our data showed that
464 genetic variants affecting exclusively eye size were located in all chromosomes, but not in the
465 X and 6th chromosomes (Muller A and F). Therefore, our comparative morphological and
466 mapping data strongly suggest an independent evolution of eye size in different lineages. This
467 observation is supported by similar data obtained for two species of the *melanogaster* group
468 (Gaspar et al. 2020).

469 The imaginal disc that gives rise to the *Drosophila* head is a modular structure that
470 contributes cells to almost all organs of the head (Haynie and Bryant 1986). Since we observed
471 an additive effect of *D. americana* chromosomes in a *D. novamexicana* background on eye
472 size, it is conceivable that each chromosome or chromosomal region might influence different
473 developmental processes and different organ anlagen within the imaginal disc. This hypothesis
474 is supported by our observation that the *D. americana* 5th chromosome (Muller C) had a major
475 impact on the size of all organs in our study, while the 2nd, 3rd and 4th chromosomes (Muller E,
476 D and B) were associated with variation in eye size after accounting for body size. Please note
477 that we cannot rule out the presence of variants located on the 5th chromosome (Muller C)
478 affecting exclusively eye size that were masked by the conservative approach for body size
479 correction used in this work. Our shape analysis further revealed that only the 4th chromosome
480 (Muller B) was associated with variation in lateral eye regions, supporting a modular impact
481 of different chromosomes on overall head shape variation. For species of the *melanogaster*

482 group it has also been shown that the evolution of eye size and the size of the interstitial cuticle
483 is uncoupled (Arif et al. 2013; Gaspar et al. 2020). In contrast, the SNP in the *eyeless/Pax6*
484 locus associated with intra-specific variation in *D. melanogaster* influences the early
485 subdivision of the imaginal disc into the retinal and the antennal part of the imaginal disc
486 (Ramaekers et al. 2019). Therefore, this SNP may affect eye size and head cuticle/antennal size
487 simultaneously. Although more comparative developmental analyses are necessary, the picture
488 emerges that the modular nature of the imaginal disc with its different interconnected
489 developmental programs may facilitate the independent evolution of head shape because it
490 provides multiple targets for evolutionary changes.

491 In our survey we observed the most pronounced differences in eye size between *D.*
492 *novamexicana* showing the smallest eyes and southern *D. americana* strains showing the
493 largest eyes. Interestingly, compatible with studies using species of the *melanogaster* group
494 (Norry et al. 2000; Posnien et al. 2012; Norry and Gomez 2017; Ramaekers et al. 2019; Gaspar
495 et al. 2020), we also identified intra-specific differences among *D. americana* populations.
496 There are multiple chromosomal inversions segregating in *D. americana* populations and some
497 of them are shared with *D. novamexicana* (Hsu 1952; Throckmorton 1982). These inversions
498 largely affected the patterns of differentiation along chromosomes among *D. americana* natural
499 populations (Reis et al. 2018). Although the genomic structure caused by the presence of
500 inversions hampers the identification of causative variants, it has been proposed that they may
501 keep together favorable combinations of alleles (Kirkpatrick and Barton 2006; Kirkpatrick
502 2010). Interestingly, we observed a clear association between the presence of shared inversions
503 and eye size. Additionally, we found a significant enrichment of genes involved in eye
504 development among those genes containing SNPs that could explain both intra- and inter-
505 specific differences in eye size for the 4th chromosome (Muller B) only. These genes represent
506 prime candidates for future functional validation tests.

507 Since these inversions show latitudinal and longitudinal gradients in the *americana*
508 complex, it is likely that they carry the targets of selection associated with local adaptation in
509 natural populations. For instance, chromosomal inversions were found to be associated with
510 life-history and physiological traits likely involved in adaptation (Huang et al. 2014; Durmaz
511 et al. 2018; Kapun and Flatt 2019) as well as with morphological traits (Norry et al. 1995;
512 Fernández Iriarte et al. 2003; Rako et al. 2006; Hatadani and Klaczko 2008). Additionally, a
513 previous study found that the fixed variant explaining pigmentation differences between *D.*
514 *novamexicana* and *D. americana* was polymorphic in *D. americana* and explained the least
515 pronounced variation in pigmentation observed along a longitudinal transect in *D. americana*
516 populations (Wittkopp et al 2009). Therefore, it is conceivable that inter-specific differences
517 affecting exclusively eye size between *D. novamexicana* and *D. americana* can also explain
518 intra-specific differences in this trait among *D. americana* natural populations. The
519 longitudinal gradient for pigmentation in *D. americana* populations was further confirmed
520 (Wittkopp et al. 2011), and solar radiation and the diurnal temperature range has been shown
521 to be the best predictors of this gradient (Clusella-Trullas and Terblanche 2011). Interestingly,
522 *D. americana* populations showing darker pigmentation were more often found in geographical
523 regions with lower sun radiation and mean diurnal temperature ranges than lighter populations
524 (Clusella-Trullas and Terblanche 2011). According to these geographical parameters, we
525 observed here that flies showing bigger eye size, likely more sensitive to light than flies with
526 smaller eyes, came from regions with low sun radiation and possibly with less light. Therefore,
527 eye size variation in the *americana* complex may be associated with local adaptation as well.

528 In conclusion, natural variation in head morphology is common in *Drosophila* and has
529 a strong genetic component. We provide for the first time a comprehensive morphological
530 comparison of eye size and head shape between *D. novamexicana* and *D. americana* and
531 revealed a complex underlying genetic architecture. Our data strongly suggests that the

532 presence of inversions in these two species contributed to nucleotide diversity patterns that may
533 affect the regulation and function of multiple genes during head and eye development and thus
534 facilitating natural variation in this complex morphological trait.

535

536 Materials and Methods

537

538 Fly strains

539

540 The following isofemale fly strains were used in this work: *D. virilis* (15010-1051.47,
541 Hangzhou, China; 15010-1051.49, Chaco, Argentina), *D. novamexicana* (15010-1031.00,
542 Grand Junction, Colorado, USA; 15010-1031.04, Moab, Utah, USA) and *D. americana* (O43,
543 O53, SF12 and SF15). The *D. virilis* and *D. novamexicana* strains were obtained from the
544 Tucson stock center in 1995 and were kept in the laboratory since then. *D. americana* strains
545 were established with single inseminated females collected from the wild in different locations
546 of the USA (Omaha, Nebraska (O), 2008 and Saint Francisville (SF), Louisiana, 2010) (Reis
547 et al. 2011; Fonseca et al. 2013; Reis et al. 2015). All strains were kept at 25°C under 12h/12h
548 light/dark cycles.

549

550 Dissection and phenotyping

551

552 To study size variation, we dissected heads, wings and tibiae of 20-30 females between
553 4 and 7 days after eclosion for each strain. To avoid crowding effects on adult organ size, we
554 controlled for density by transferring 30 first instar larvae into single vials containing standard
555 food. The heads were mounted facing upwards on a slide with sticky tape, while the three legs
556 (one of each pair) and wings were randomly dissected from the left or right side and mounted
557 on a slide with Hoyer's medium. Pictures were taken using a stereomicroscope Leica M205

558 FA with a magnification of 50x for wings and 60x for the other structures. We also took a
559 picture of a ruler to be able to convert the measurements from pixels to μm or μm^2 . The
560 resulting JPG files were saved with a resolution of 2560x1920 pixels, and we used ImageJ
561 (Schneider et al. 2012) to measure eye areas as well as tibiae lengths and wing areas (Fig. S9A,
562 File S4). We calculated the geometric mean of squared tibiae (GMsqT) as a proxy for tibiae
563 size. The geometric mean of squared tibiae and wing area (GMsqTW) was used to estimate
564 overall body size. We then used the residuals of the linear regression between eye size and
565 GMsqTW to account for differences in overall body size between the strains. Since the
566 measurements were not normally distributed (Shapiro-Wilk, $p < 0.05$), we used Kruskal-Wallis
567 test followed by Dunn's post-hoc test with Holm correction to determine which comparisons
568 were significantly different between strains.

569

570 Geometric morphometrics

571

572 Frontal head images of every strain were used to generate tps files in which all images
573 were randomized with tpsUtil (version 1.60; (Rohlf 2015)). These tps files were used to place
574 43 landmarks and semilandmarks (see Fig. S9B) using tpsDig2 (version 2.18; (Rohlf 2015)).
575 A sliders file that contains information about the semilandmarks was generated with the "Make
576 sliders file" function in tpsUtil (version 1.60; (Rohlf 2015)). Using tpsRelw (version 1.57, 64
577 bit; (Rohlf 2015)) the semilandmarks were slid along a curve using an option to minimize the
578 bending energy required for a deformation of the consensus to the selected specimen (Slide
579 method = Chord min BE) allowing up to three iterations during the superimposition process
580 (Slide max iters = 3). The slid landmarks were treated as fixed landmarks and were
581 superimposed using Procrustes fit as implemented in MorphoJ (version 1.06d; (Klingenberg
582 2011)). Since we used 2D pictures of 3D structures, after a principal component analysis (PCA)
583 we observed that artificial pitch (up/down rotation) and yaw (left/right rotation) were partly

584 associated with shape variation along principal component (PC) 1 and PC3 axes, respectively
585 (Fig. S10). Additionally, we observed that part of the within-strain variation captured pitch and
586 yaw. To dissociate and remove the error from the true components of shape, we used a two-
587 step approach: First, we calculated the residuals of the within-strain pooled-regression between
588 Procrustes coordinates and PC1. Second, we used the new coordinates to repeat the above-
589 mentioned procedure to remove the effect of yaw (new PC2). To avoid inflation of the number
590 of the variables when compared to the number of samples in the statistical analysis, we used
591 the final Procrustes coordinates to determine and keep the number of PCs explaining about
592 90% of the total shape variation. The new dataset was rotated back into the original Procrustes
593 coordinates by transposing the orthogonal matrix. Differences in mean shape among
594 species/strains were evaluated using a T-square parametric test followed by 10,000
595 permutations (leave-one-out cross-validation) as implemented in the “Canonical Variate
596 Analysis” option in MorphoJ. The error correction and wireframes generation were performed
597 with MorphoJ while the PC removal and rotation to the original coordinates was done using a
598 custom R script.

599

600 [Impact of chromosomal inversions on eye size](#)

601

602 To test whether the presence of inversions was associated with differences in eye size
603 (after accounting for body size) and head shape, we screened *D. americana*, *D. novamexicana*
604 and *D. virilis* strains for the presence/absence of eight different inversions known to be fixed
605 or polymorphic within the *virilis* phylad of *Drosophila* (Hsu 1952; Throckmorton 1982) and
606 for which the breakpoint locations have been identified (*Xa*, *Xb*, *Xc* (Muller A), *2a*, *2b* (Muller
607 E), *4a* (Muller B), *5a* and *5b* (Muller C), (Evans et al. 2007; Fonseca et al. 2012; Reis et al.
608 2018)). Primers were developed for one breakpoint and its corresponding ancestral state for
609 each of the eight inversions (File S2) based on the *D. virilis* (Clark et al. 2007), *D. americana*

610 (H5, W11, (Fonseca et al. 2013) and SF12, (Reis et al. 2018)), and *D. novamexicana* (15010-
611 1031.00, (Reis et al. 2018)) genome sequences to avoid polymorphism at the primer binding
612 sites. Genomic DNA was extracted from pools of 20 females for each strain using a standard
613 phenol:chloroform protocol, and the concentration was normalized based on concentration
614 measurements using Nanodrop® prior to PCR amplification (File S2). The amplification
615 products were visualized on a UV transilluminator after electrophoresis using TAE buffer in
616 2% agarose gels stained with a 1:10 dilution of SERVA® stain. Associations between the
617 presence of inversions and eye size (after accounting for body size as described above) were
618 tested using Wilcoxon rank-sum test followed by Holm correction for multiple testing.
619 Associations between mean head shape variation and the presence of inversions were tested
620 using a parametric T-square test on the group mean shapes followed by 10,000 permutations
621 (leave-one-out cross-validation) as implemented in the “Discriminant Function Analysis”
622 option in MorphoJ.

623

624 Parental strains selection and dominance relationships

625

626 *D. americana* (SF12) and *D. novamexicana* (15010-1031.00) strains were selected as
627 representatives of both species, because they had the largest differences in eye size (see
628 Results), they show the most divergent karyotypes regarding chromosomal inversions and their
629 genomes are available (Reis et al. 2018). We established several crosses with 10 males and 10
630 females for each of both parental strains, and 10 *D. novamexicana* males and 10 *D. americana*
631 females to obtain F1 hybrids. Since, *D. americana* females and males take at least four to six
632 days to reach full maturity (Pitnick et al. 1995), we transferred the flies into new vials after
633 seven days. The flies were then allowed to lay eggs for 24h only, to avoid crowding effects on
634 adult size before dissection. Newly eclosed flies were sexed and collected into new vials and
635 were kept under the same conditions described above. Next, 100 females of each parental

636 strain, as well as 100 F1 females were dissected between 4 and 7 days after eclosion, as
637 described above. We used females only to avoid potential confounding effects caused by sex
638 differences (e.g (Siomava et al. 2016)) and to avoid hemizyosity for the *X* chromosome.
639 Pictures were taken using a stereomicroscope Nikon ZMS 1500 H with a magnification of 40x
640 for wings and 50x for the other structures. We also took a picture of a ruler to be able to convert
641 the measurements to μm or μm^2 . The resulting JPG files were saved with a resolution of
642 1600x1200 pixels. All pictures were treated using ImageJ (Schneider et al. 2012) as mentioned
643 before. After phenotyping, six individuals were excluded from the analysis because they
644 showed highly damaged wings (File S4). We used Kruskal-Wallis test followed by Dunn's
645 post-hoc test with Holm correction to determine which comparisons were significantly
646 different between strains and hybrids. The scaling relationships between parental strains, as
647 well as between *D. novamexicana* and F1 hybrids were evaluated using the regression of log-
648 transformed eye area (non-corrected for body size) on log-transformed GMsqTW. The slope
649 of the resulting curves is the allometric coefficient (Huxley 1924; Huxley and Teissier 1936).
650 We tested for the significance of differences in allometric coefficients using a linear model
651 with interaction terms.

652 The geometric morphometric analysis of head shape including removal of artificial
653 pitch and yaw was done as described above (geometric morphometrics section).

654 For ommatidia counting, the heads of 10 females for each parental strain and hybrids
655 were dissected 4-7 days after eclosion and cut in half longitudinally with a razor blade. One of
656 the eyes of every individual was mounted on sticky tape facing upwards. Serial stack pictures
657 (N=25) were taken using a microscope Zeiss Axioplan 2 with external light sources from the
658 sides and 160X magnification to capture the reflection of every ommatidia (Fig. S1A-B). We
659 also took a picture of a ruler to be able to convert the measurements from pixels to μm or μm^2 .
660 Images were saved with 1360 x 1036 resolution. Stack projection with maximum intensity was

661 achieved using ImageJ (Schneider et al. 2012). The area of the eye was outlined and measured.
662 The images were transformed into 8-bit (gray scale) and the area outside the selected region
663 was cleared. Next, we used the Fast_Morphology.jar plug-in with the following settings:
664 morphological filters, white Tophat – octagon – radius = 2. The images were inverted and the
665 ommatidia numbers were estimated using the ITCN_1_6.jar plug-in with the following
666 settings: width=7px; Minimum distance = 10, threshold=2.0 and detect dark peaks. To estimate
667 the average ommatidia size, the eye area was divided by the number of ommatidia.

668

669 [Genotype-phenotype association study using a backcross approach](#)

670

671 To determine the effect of the major chromosomes on size variation, we established
672 backcrosses between F1 females (progeny of crosses between *D. novamexicana* males and *D.*
673 *americana* females) and *D. novamexicana* males. A total of 570 females were dissected and
674 phenotyped for eye, face and wing areas, as well as for tibiae lengths as described above. After
675 phenotyping, 11 females were excluded from the analysis because they showed highly
676 damaged wings (File S4). The remaining 559 females were genotyped using the molecular
677 markers A6 (Muller A, X chromosome), B3 (Muller B, 4th chromosome), C3 and C5 (Muller
678 C, 5th chromosome), D7 (Muller D, 3rd chromosome) and E7 (Muller E, 2nd chromosome) (see
679 (Reis et al. 2014) and File S5 for more details). PCR reactions were done using Phire Plant
680 Direct PCR kit[®] (Thermo Scientific[®]) and gDNA from wings according to the manufacturer's
681 instructions. We found some unspecific amplification with molecular markers A6 and B3.
682 Based on the recently published *D. americana* (SF12) and *D. americana* (15010-1031.00)
683 genomes (Reis et al. 2018), we were able to slightly modify these primers to account for
684 polymorphisms and improve PCR amplification (File S4). No recombinants were found
685 between molecular markers C3 and C5 on the 5th chromosome (Muller C). The 2nd and 3rd
686 chromosomes (Muller E and D, respectively) are fused in *D. americana*. These are, thus,
687 transmitted as a single chromosome and we found only four recombinants between the

688 molecular markers D7 and E7 (File S4). We excluded the four recombinants and also three
689 individuals that showed the amplification product of *D. americana* only for marker A6 or E7
690 (File S4). This cleaned data set of 552 females was used to evaluate the effect of each
691 chromosome in eye and wing areas, as well as in GMSqT with the Wilcoxon-rank test followed
692 by Holm correction for multiple testing. Since we observed a strong effect of the 5th
693 chromosome (Muller C) on the size of all analyzed organs (see Results), we decided to use the
694 residuals of the multiple linear regression of eye size on tibiae and wing sizes to account for
695 body size variation. With this conservative approach we removed all the variation in eye size
696 that could be explained by variation in the other structures. We have summed the grand mean
697 of eye size to the residuals to get normalized eye size. Since, normalized eye size is normally
698 distributed (Shapiro-Wilk test, $p > 0.05$), we used linear models with each chromosome as fixed
699 effect to test for significant associations and to estimate the amount of variation explained. We
700 have further included interaction terms between different chromosomes to evaluate epistasis.

701 The geometric morphometric analysis of head shape was mainly done as described
702 above. In this analysis, pitch and yaw were partly associated with variation along PC1 and PC2,
703 respectively. The error was removed prior to the analysis using the method described before.
704 The impact of the different chromosomes on mean shape variation was evaluated using the
705 “Discriminant Function Analysis” option in MorphoJ. We further used the residuals of the
706 regression of the Procrustes coordinates on centroid size to estimate the impact of the different
707 chromosomes on the non-allometric component of shape. These analyses were also conducted
708 in MorphoJ.

709

710

711

712 [Genome-wide association study using a pool-seq approach](#)

713 Crosses, sample preparation and sequencing

714

715 To identify single nucleotide polymorphisms (SNPs) associated with normalized eye
716 size, we performed a genotype-phenotype association study using F18 individuals resulting
717 from brother sister mating for 17 generations starting from crosses between *D. novamexicana*
718 (15010-1031.00) males and *D. americana* (SF12) females. We phenotyped a total of 157
719 females, which represented the entire F18 female progeny of two independent vials, for eye
720 and wing areas, as well as tibiae lengths of one of each pair of legs. Wings and legs were
721 randomly dissected from left or right sides. Missing values for individuals ID=125 and 150
722 showing highly damaged wings were estimated based on the equation of the multiple linear
723 regression between wing area and tibiae length. The residuals of the multiple regression
724 between eye area and both tibiae lengths and wing areas were used to remove the variation in
725 eye area that could be explained by variation in total body size. The grand mean was summed
726 to the residuals and these new values were sorted in ascending order to divide the females into
727 four quartiles (Q1(n=40; $238,913 \pm 7,479 \mu\text{m}^2$); Q2(n=39; $252,838 \pm 2,352 \mu\text{m}^2$), Q3(n=38;
728 $263,652 \pm 3,777 \mu\text{m}^2$) and Q4(n=40; $278,609 \pm 9,736 \mu\text{m}^2$) (File S4). gDNA for pooled carcasses
729 was extracted for each quartile using a standard phenol:chloroform procedure. DNA quantity
730 and integrity were checked by agarose gel electrophoresis. The good quality samples were used
731 to prepare gDNA libraries for all four pooled samples with TruSeq® Nano DNA Library Prep
732 from Illumina (Catalog#FC-121-9010DOC). The libraries were further used for paired-end
733 sequencing with HiSeq2000 (Illumina) at the Transcriptome Analysis Laboratory (TAL) in
734 Göttingen.

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738 Quality checks and analysis

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740 After sequencing using HiSeq2000, a total of 88,783,338; 75,693,540; 82,724,476 and
741 108,310,437 paired-end reads with 100 bp were obtained for Q1, Q2, Q3 and Q4, respectively.
742 The reads are available at ENAXXXXX. The quality of the reads was assessed with FASTQC
743 v0.11.1 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). There was no need to
744 trim or mask positions, since all positions had quality above 20. As mapping reference the *D.*
745 *americana* (SF12) genome was used after it was reordered based on the hypothetical
746 chromosomes of the ancestral state between *D. virilis*, *D. americana* and *D. novamexicana*
747 (Reis et al. 2018). Read mapping, ambiguously mapped reads and optical duplicates removal,
748 as well as SNP calling and depth of coverage determination were done as described in (Reis et
749 al. 2018). The overall alignment rates using Bowtie2 v2.2.5 (Langmead and Salzberg 2012)
750 with default settings for Q1, Q2, Q3, and Q4 were 77%, 79%, 79% and 81%, respectively. The
751 distributions of coverage obtained with GATK DepthOfCoverage v3.4.46 (Van der Auwera et
752 al. 2013) were close to normal and the average values for Q1, Q2, Q3, and Q4 were 84X, 73X,
753 81X and 107X, respectively. For all quartiles, more than 94% of the sites showed coverage
754 values above 20X. To avoid bias in frequency estimations, we used a coverage interval which
755 included 68.2% of the total amount of sites around the mean for each quartile ([62-101X], [54-
756 89X], [60-97X] and [84-127X] for Q1, Q2, Q3 and Q4, respectively). The data was treated and
757 analyzed as described in (Reis et al. 2018). Briefly, we used the frequencies values for SNPs
758 identified using both Bowtie2 v2.2.5 (Langmead and Salzberg 2012) and BWA v0.7.12 (Li and
759 Durbin 2009) to determine which ones showed significant differences between the quartiles
760 using Fisher exact test followed by Bonferroni correction.

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762

763 Ancestral reconstruction of phenotypic traits

764

765 We used the phylogeny of 59 *Drosophila* species obtained by (Keeseey et al. 2019) to
766 reconstruct the ancestral state for body size, eye surface, as well as the ratio between eye and
767 head width using a Maximum Likelihood method as implemented in the function fastAnc in
768 the R package Phytools (v. 0.4.98, <http://www.phytools.org/eqg2015/asr.html>, (Revell 2012)).
769 The details about the strains and the phenotypes can be found in (Keeseey et al. 2019).

770 We also used Phytools to reconstruct the ancestral state of different traits for the species
771 of the *virilis* phylad. The traits considered were the following: GMsqTW as a proxy for body
772 size, eye area, the ratio between eye and head area, and eye area after accounting for body size
773 (residuals of the linear regression between eye area and GMsqTW plus the grand mean of eye
774 area). To obtain phylogenies representative of the *virilis* phylad, we started by downloading all
775 the *D. virilis* coding sequences (CDS) available at FlyBase
776 (ftp://ftp.flybase.net/genomes/Drosophila_virilis/current/fasta/). We further used SEDA
777 (López-Fernández et al. 2019) to retrieve only those CDS of genes located on scaffolds
778 anchored to chromosomes (Muller E: scaffolds 12,822; 13,047; 12,855 and 12,954; Muller B:
779 scaffolds 13,246; 12,963 and 12,723 and Muller C: scaffolds 12,823; 10,324; 12,875 and
780 13,324). When more than one isoform was available for the same gene only the longest one
781 was retrieved. Next, we used Splign-Compartment (as implemented in BDBM; (Vázquez et al.
782 2019), and the *D. virilis* CDS obtained above as references to annotate CDS in *D. americana*
783 (H5 and W11 (Fonseca et al. 2013); SF12, Northern, Central and Southern populations (Reis
784 et al. 2018)), as well as in *D. novamexicana* (15010-1031.00) contigs (Reis et al. 2018). To
785 obtain the CDS for the *D. americana* populations, we used Coral 1.4 (Salmela and Schroder
786 2011) with default parameters to reconstruct the gene sequences showing the major frequent
787 variant at polymorphic sites in the pool-seq reads of each population (Reis et al. 2018), prior
788 to contig assembly using Abyss 2.0 (Jackman et al. 2017) with K=25 and default parameters.

789 For each genome, we used SEDA to filter the datasets for complete CDS (those with annotated
790 start and stop codons) and obtain one file per gene with the orthologous CDS. Sequences were
791 aligned using Clustal Omega (Sievers et al. 2011) and concatenated, resulting in alignments
792 with 372,546 bp (379 genes), 335,931 bp (379 genes) and 298,233 (334 genes) for the 2nd, 4th
793 and 5th chromosomes (Muller E, B, and C), respectively. FASTA files were converted to
794 NEXUS format using ALTER (Glez-Peña et al. 2010). The phylogenies were obtained with
795 MrBayes (Ronquist et al. 2012) using the GTR model of sequence evolution allowing for
796 among-site rate variation and a proportion of invariable sites. Third codon positions were
797 allowed to have a different gamma distribution shape parameter than those for first and second
798 codon positions. Two independent runs of 1,000,000 generations with four chains each (one
799 cold and three heated chains) were used. Trees were sampled every 100th generation and the
800 first 2500 samples were discarded (burn-in) (Fig. S11 A-C). The Docker images used for
801 running the above software applications are available at the pegi3s Bioinformatics Docker
802 Images Project (<https://pegi3s.github.io/dockerfiles/>). Since we have no phenotypic data for *D.*
803 *americana* H5, W11, and the Central population, their branches were manually removed from
804 the phylogenies. The distances between nodes were accounted for to re-estimate the new
805 branch lengths when applicable (Fig. S11 D-F). We kept the Northern population branch as a
806 phylogenetic proxy for O43 and O53 phenotypes and we decided to remove the Southern
807 population branch because we have genomic data for SF12. The phylogenies were rooted by
808 the *D. virilis* branch prior to ancestral state reconstruction of the size phenotypes using Phytools
809 as described above and ancestral state reconstruction of shape using squared-changed
810 Parsimony (Maddison 1991) as implemented in the option “Map Onto Phylogeny” in MorphoJ.
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814 Intersection with previous results and SNP annotation

815

816 To identify variants associated with normalized eye size and linked with chromosomal
817 inversions, we intersected the SNPs obtained for the F18 pool-seq described above with those
818 obtained in a previously published pool-seq of *D. americana* populations (Reis et al. 2018)
819 (Fig. S8). We started by re-mapping the raw reads obtained for the genome sequencing of *D.*
820 *americana* (SF12) and *D. novamexicana* (15010-1031.00) against the reference *D. americana*
821 (SF12) genome. Read quality was assessed with FastQC v0.11.1
822 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>), and every position showing a
823 quality score under 20 was masked using fastq_masker implemented in the FASTX Tool kit
824 v.0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/index.html). Read mapping, alignment
825 filtering and SNP calling was done as described above. The mean coverage and standard
826 deviation (s.d.) were determined for both samples and all SNPs showing lower or higher
827 frequency than mean ± 3 s.d. were considered either as errors or originating from highly
828 repetitive regions and were discarded. Next, every SNP showing coverage higher than zero for
829 the reference variant in *D. novamexicana* (15010-1031.00) and the alternative one in *D.*
830 *americana* (SF12) was excluded. These were variants that might be shared between the two
831 strains, and they would cause biased frequencies. Then, these tables were intersected with those
832 obtained for Q1 and Q4 of the F18 pool-seq described above. Fisher exact test followed by
833 Bonferroni correction was used to determine which SNPs show significant frequency
834 differences between Q1 and Q4. We kept those SNPs which show the reference allele at higher
835 frequencies for Q4. Since, *D. virilis* and *D. novamexicana* show small eye size (see Results),
836 we considered that the variants causing this phenotype would be fixed in those species. The
837 alternative variant should be fixed in *D. americana* (SF12) and at higher frequencies in Q4 and
838 southern populations.

839 To annotate the SNPs to genes, we started by aligning the *D. americana* (SF12)
840 reference genome to the *D. virilis* genome using Mauve v2.4.0 (Rissman et al. 2009). The raw
841 SNPs coordinates were extracted and were converted according to the position and orientation
842 of the different *D. virilis* scaffolds to match the coordinates provided on the gtf file
843 (ftp://ftp.flybase.net/genomes/Drosophila_virilis/dvir_r1.07_FB2018_05/gtf/). The gtf file
844 was converted to genePred format using gtfToGenePred tools
845 (http://hgdownload.soe.ucsc.edu/admin/exe/linux.x86_64/). A file containing all the
846 transcripts of *D. virilis* was generated using the genePred file and the FASTA file (dvir-all-
847 predicted-r1.07.fasta.gz) with all predicted annotations available for *D. virilis*
848 (ftp://ftp.flybase.net/genomes/Drosophila_virilis/dvir_r1.07_FB2018_05/fasta/) using the
849 script retrieve_seq_from_fasta.pl provided in ANNOVAR v. (Mon, 16 Apr 2018),
850 (http://www.openbioinformatics.org/annovar/annovar_download_form.php, (Wang et al.
851 2010). The SNPs were then annotated using the script ./table_annovar.pl provided in
852 ANNOVAR.

853 To determine whether some of the identified SNPs were located in genes associated
854 with eye development, we intersected the list of genes with those described as being involved
855 in “eye development” (GO: 001654; <http://flybase.org> FB2018_03). We observed that the *D.*
856 *virilis* orthologs of three genes (*PQBPI*, *CkIIalpha*, and *hyd*) are located on scaffold 12,958.
857 Since this scaffold is not anchored to any Muller element in *D. virilis*, we excluded those genes
858 from the analysis. We also decided to include the manually curated genes *ewg* and *pnr* which
859 have been reported to be involved in eye development (FlyOde; <http://flyode.boun.edu.tr/>;
860 (Koestler et al. 2015), but are not present in the GO term: “eye development”.

861 To identify those regions that can explain both intra- and inter-specific variation in
862 normalized eye size, we have further filtered the SNPs which showed significant frequency
863 differences between northern and southern *D. americana* populations after Bonferroni

864 correction. Gene enrichment was tested using Chi-square test with Yates correction. To obtain
865 an estimation of the total number of genes in *D. melanogaster*, we obtained the list of genes in
866 the Geneontology Panther Classification system (<http://pantherdb.org/>). This list was used to
867 retrieve the chromosomal location in Flybase (<http://flybase.org/batchdownload>). Only those
868 genes located on Muller A, B, C, D, E and F were considered (File S3).

869

870 Data availability and statistical analysis

871

872 All pictures can be found at DRYAD/FIGSHARE (doi, tba), and raw measurements in
873 File S4. All statistical analyses presented in this work were done using R (R Core Team 2018)
874 and the R package Rcmdr (Fox 2005; Fox 2017; Fox and Bouchet-Valat 2018). The plots were
875 prepared using ggplot2 (Wickham 2016) and Microsoft Office. All custom scripts and analysis
876 pipelines are available on the DRYAD/FIGSHARE (doi, tba) repository.

877

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883 Authors contribution

884 **MR:** Conceptualization, planning and execution of the experiments (fly husbandry, handling
885 and crossing, dissections, mounting and pictures capturing, gDNA extraction for PoolSeq);
886 data analysis: size (script writing for statistical analysis using R), shape (handle tps files, create
887 sliding LM, R scripting to remove error, and statistical analysis in MorphoJ), ancestral
888 reconstruction (phytools and MorphoJ); Poolseq analysis (script writing for read quality
889 checking, and mapping, applying the scripts written by CR and HNT for SNP calling and
890 statistical analysis, script writing for table intersection to obtain relevant SNPs); Writing –
891 original draft, Writing – review and editing

892 **GW:** development of molecular markers for chromosomal inversions, DNA extraction and
893 genotyping

894 **JC:** support with the geometric morphometrics analysis, reviewing and editing the manuscript

895 **RL:** manual measurements of heads, eyes, tibiae and wings

896 **BH:** placing LM for geometric morphometrics analysis

897 **CR:** script writing for variant calling of the Poolseq GWAS data with GATK
898 **NTH:** script writing for statistical analysis (Fisher exact test) of the Poolseq GWAS data and
899 data visualization (Manhattan plots)
900 **CPV:** Conceptualization, review and editing manuscript
901 **JV:** Conceptualization, Funding acquisition, Phylogeny reconstruction of the virilis phylad,
902 review and editing of the manuscript
903 **NP:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision,
904 Visualization, Writing – original draft, Writing – review and editing.
905

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1096 [List of supplementary figures](#)

1097

1098 **Fig. S1. Differences in eye size, ommatidia number, and ommatidia size between**
1099 **parental strains and their interspecific hybrid.**

1100

1101 **Fig. S2. Variation in organ size in the genotype-phenotype associations using the**
1102 **backcross approach.**

1103

1104 **Fig. S3. Variation in eye size and head shape in the genotype-phenotype associations**
1105 **using the backcross approach.**

1106

1107 **Fig. S4. Normalized eye size variation for the 16 genotypic classes present in the**
1108 **backcross between F1 hybrid females and *D. novamexicana* males.**

1109

1110 **Fig. S5. Manhattan plots between adjacent quartiles of the pool-seq experiment**
1111 **involving F18 females.**

1112

1113 **Fig. S6. Phylogeny and ancestral reconstruction of the strains used in this study.**

1114

1115 **Fig. S7. Comparison between the ancestral reconstruction of phenotypic traits across**
1116 **the *Drosophila* genus and the strains used in this study.**

1117

1118 **Fig. S8. Flowchart summarizing the procedure used to obtain the list of relevant SNPs.**

1119

1120 **Fig. S9. Schematic representation of the procedure used for phenotyping.**

1121

1122 **Fig. S10. Sequential removal of error associated with head tilting.**

1123

1124 **Fig. S11. Phylogenies of species of the *virilis* phylad.**

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1126 [List of files](#)

1127

1128 **File S1. Descriptive statistics for all datasets.**

1129

1130 **File S2. List of primers used as molecular markers for chromosomal inversions and**
1131 **genotyping results.**

1132

1133 **File S3. SNP tables after intersecting the datasets obtained for the GWAS and *D.***
1134 ***americana* populations and candidate genes for eye development.**

1135

1136 **File S4. Raw measurements.**

1137

1138 **File S5. List of primers used as indel markers for the different chromosomes and**
1139 **genotyping of the progeny of the backcross between hybrid females and *D.***

1140 ***novamexicana* males.**