Title

Divergence of olfactory receptors associated with the evolution of assortative mating and reproductive isolation in mice

Authors

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Abstract

Deciphering the genetic bases of behavioural traits is essential to understanding how they evolve and contribute to adaptation and biological diversification, but it remains a substantial challenge, especially for behavioural traits with polygenic architectures. In this study, we developed a population genomics approach coupled with functional predictions to address the evolution and genetic basis of olfactory-based assortative mate preferences in the house mouse, suspected to have evolved as a response to selection against hybridisation. We used whole genome resequencing data and the C_2 statistic of the program BAYPASS, which contrasts allele frequencies corrected for population structure, to characterize genetic differentiation between sets of populations with strong contrast in behaviour (expressing or not assortative mate preferences) and we identified some regions of the genome showing the expected significant and consistent association with behavioural divergence. A series of Olfactory and Vomeronasal Receptor genes, among the most differentiated genomic regions and in line with functional predictions, stand out as the prime candidates underlying this olfactory-based behavioural divergence. These genes form large gene clusters in the genome, with two main candidate clusters extending up to 1.8 Mb. Variant analyses indicate a potential dual role of regulatory and protein-coding changes in the evolution of choosiness. This study shows that combining expectations on the genomic patterns of divergence with functional expectations represents a promising route to unravelling the genetic architecture of complex trait variation and provides novel insights into the role of olfactory and vomeronasal receptors in mammal adaptation and speciation.

Keywords: behaviour, genomics, olfaction, mate choice, speciation, reinforcement, *Mus musculus*, mammals

Introduction

The evolution of behaviours plays a fundamental role in biological diversification, adaptation and speciation, but it remains a central challenge in biology to understand how behavioural traits evolve and the genetic basis of behavioural differences (Arguello and Benton 2017; Niepoth and Bendesky 2020; Jourjine and Hoekstra 2021). Behaviours involved in mate choice play a key role during the evolution of new species, as they act as premating components of reproductive isolation and may diverge rapidly through the action of direct and indirect selective effects (natural selection, sexual selection, reinforcing selection (Servedio and Noor 2003; Smadja and Butlin 2011; Servedio and Boughman 2017; Kopp et al. 2018; Mendelson and Safran 2021). Some progress has been made, often by using quantitative genetics approaches, in characterising genes underlying assortative mate preferences in some biological systems (e.g. in *Drosophila* flies: Laturney & Moehring 2012, Pardy et al. 2021; sticklebacks: Bay et al. 2017; *Heliconius* butterflies: Merrill et al. 2019; European corn borer moths: Unbehend et al. 2021), but they represent cases where the genetic basis of behavioural divergence is relatively simple. Determining the genetic basis of behavioural divergence for more polygenic traits and identifying the signature of divergent selection acting on these underlying genomic regions remain more challenging.

In this study, we developed a population genomics approach based on the analysis of genetic differentiation between sets of populations with contrasted behaviours and selective pressures to address the evolution and genetic basis of olfactory-based assortative mate preferences in the house mouse. Population genomics has been successful in identifying targets of selection in the genome (Hohenlohe et al. 2010; Haasl and Payseur 2015) but its application to characterise the genetics of behavioural divergence remains largely unexplored (Kent et al. 2019; Niepoth and Bendesky 2020). With information on phenotype divergence among natural populations, on the proximal basis of the behavioural response, and on populations where these behaviours should be under selection, the house mouse system provides a predictive framework to address the evolution and genetics of behavioural divergence involved in reproductive isolation.

The two European subspecies of the house mouse (*Mus musculus musculus* and *M. m. domesticus*) diverged in allopatry ~350-500 kya from Central Asia/Middle East regions before coming into secondary contact in Europe ~5 kya (Boursot et al. 1996; Geraldes et al. 2011). Some degree of postzygotic isolation has been demonstrated in this hybrid zone, mostly in the form of reduced hybrid fertility (Britton-Davidan et al. 2005; Albrechtova et al. 2012; Turner et al. 2012). However, the two subspecies also display some degree of sexual isolation where they meet and form a hybrid zone: assortative mate preferences (accompanied by a propensity to mate with the

81 preferred partner) have been evidenced in both sexes of both subspecies, though preference is
82 stronger in *M. m. musculus* (Smadja and Ganem 2002; Smadja et al. 2004; Smadja and Ganem
83 2005; Ganem et al. 2008; Bimova et al. 2011; Latour et al. 2014; Smadja et al. 2015).

84 Several arguments suggest that these assortative mate preferences have evolved under selection,
85 as a result of a reinforcement process (Servedio and Noor 2003; Butlin and Smadja 2018). Within
86 each subspecies, assortative mate preferences are only displayed in populations from the hybrid
87 zone ('Choosy' populations) where selection against hybridization is known to act, while
88 populations from allopatry do not display any significant directional preferences ('Non-Choosy'
89 populations) (Smadja and Ganem 2005; Smadja and Ganem 2008; Bimova et al. 2011; Latour et al.
90 2014; Smadja et al. 2015). This pattern of reproductive character displacement suggests a
91 mechanism of reinforcement, by which assortative mate preferences would have evolved as an
92 adaptive response to the existing postzygotic barriers in this hybrid zone. In this study, we used
93 this information to identify the genes underlying the evolution of assortative mate preferences by
94 articulating and testing predictions on the patterns of genomic divergence expected for loci
95 experiencing reinforcing selection. One key prediction is that genetic divergence will be
96 significantly elevated at loci underlying assortative mate preferences when sympatric (Choosy) and
97 allopatric (Non-Choosy) individuals of the same species are compared (Garner et al. 2018).

98 To identify loci showing this expected pattern of divergence, we leveraged the high-quality
99 reference genome available for this species (Waterston et al. 2002), produced whole-genome
100 resequencing data for the same individuals and populations that had been behaviourally typed
101 previously (Smadja et al. 2015), and finely characterised genomic divergence between Choosy and
102 Non-Choosy populations within *M. m. musculus*, the subspecies where the contrast in behaviour is
103 the strongest between these two types of populations (Smadja et al. 2004; Smadja and Ganem
104 2005; Ganem et al. 2008; Latour et al. 2014). We opted for a pool-sequencing strategy as a cost-
105 effective way to assess genetic differentiation among populations with contrasted behaviours
106 (Bastide et al. 2013; Yang et al. 2015), particularly relevant in our case where mate preferences are
107 statistically assessed in wild mice at the population level and show limited variability among
108 individuals within populations (Smadja et al. 2004; Smadja and Ganem 2005; Smadja et al. 2015).
109 To ensure the identification of genetic variants specifically divergent between Choosy and Non-
110 Choosy populations, we combined multiple population comparisons (focal and control
111 comparisons between groups of populations with or without contrasted behaviours) and a
112 replicated design within each group compared. We used the program BAYPASS and its recently
113 implemented C_2 contrast statistic to identify outlier loci under selection between Choosy and Non-
114 Choosy populations and associated with the behavioural contrast (Gautier 2015; Olazcuaga et al.

115 2021) (Figure 1 and Methods). The Bayesian hierarchical model implemented in BAYPASS
116 represents a flexible and powerful framework to carry out association analyses and scans for
117 selection since it efficiently accounts for the neutral correlation structure among allele frequencies
118 in the sampled populations. In addition, it allows handling properly Pool-Seq data and accounts for
119 the additional level of variation they introduce in the estimation of the parameter of interest. .

120 We applied this combination of population comparisons for two types of outlier analyses at
121 different genomic scales: SNP-based differentiation across the whole genome (based on the
122 estimation of SNP-based C_2 contrast statistic), and gene-based differentiation across all annotated
123 genes. While most existing statistical methods for detecting outliers focus on analysing SNPs one-
124 by-one, SNP-based analyses may have some limitations, among which a loss of power due to
125 stringent correction applied to limit false positives in this large number of comparisons (millions of
126 SNPs in a genome) and weak sensitivity to detecting multiple causal variants effects. Leveraging
127 methods developed in the context of GWAS in humans (Neale and Sham 2004; Huang et al. 2011;
128 Chung et al. 2019), we complemented SNP-based analyses with gene-based analyses to gain
129 power in detecting outliers by aggregating disparate signals within a gene as well as to make more
130 direct link of the outlier analyses with the phenotype of interest. To capture different potential
131 underlying genetic architectures of adaptive divergence (single causal variant versus multiple
132 weakly selected variants within a gene), we analysed two commonly used gene-based statistics
133 representing the highest SNP-based differentiation value of a gene and the average differentiation
134 value of a gene (Wang et al. 2017; Dickson et al. 2020), called respectively C_{2_max} and C_{2_mean} in our
135 study.

136 Not all differentiated genomic regions between Choosy and Non-Choosy populations are expected
137 to be associated with the behavioural trait of interest, since other traits and factors can differ
138 between this set of populations. To further interpret genomic patterns of divergence and link
139 phenotypic and genomic variation, we therefore combined this population genomics approach
140 with functional expectations (Haasl and Payseur 2015; Ravinet et al. 2017; Wolf and Ellegren 2017),
141 by leveraging existing knowledge on the implication of olfaction in the expression of these
142 assortative mate preferences in the house mouse. Previous behavioural assays and chemical
143 analyses have shown that subspecies recognition and associated assortative mate preferences
144 could be driven (at least partly) by olfactory cues present in the mouse urine (Smadja and Ganem
145 2007; Hurst et al. 2017) and that urinary cues are sufficient to elicit mate choice between the two
146 subspecies (Smadja and Ganem 2002; Smadja et al. 2004; Smadja and Ganem 2005; Ganem et al.
147 2008; Bimova et al. 2011). The olfactory system thus plays a major role in the expression of
148 assortative mate choice between the two subspecies, and we expect some genes involved in

odorant recognition to be associated with the evolution of sexual isolation in the house mouse hybrid zone. This includes olfactory receptor (OR) genes expressed in the main olfactory epithelium and primarily involved in the recognition of airborne chemicals present in the environment (odorants and pheromones), and vomeronasal receptor (VR) genes expressed in the vomeronasal organ and known to play a key role in both volatile and non-volatile kairomone and pheromone recognition (Isogai et al. 2011; Chamero et al. 2012; Hayden and Teeling 2014; Liberles 2014; Bear et al. 2016). These genes are organized in very large multi-gene families in the mouse genome (more than 1,200 ORs and 530 VRs (Zhang and Firestein 2002; Ibarra-Soria, M. Levitin, et al. 2014; Ibarra-Soria, M.O. Levitin, et al. 2014)). Here, we considered those genes as key candidates among the possible genetic determinants of assortative mate preferences and used this list of candidate genes to interrogate outlier regions and narrow down regions of interest.

Patterns of genomic differentiation between Choosy and Non-Choosy populations confirmed that some regions of the genome show the patterns of divergence expected under selection. Among those, a handful of olfactory and vomeronasal receptors showed genetic divergence associated with the observed behavioural divergence between hybrid zone and allopatric populations. Our study thus provides new insights into the role of olfactory and vomeronasal receptors in mammal adaptation and speciation. It also offers a novel experimental framework to identify loci showing the expected patterns of divergence under reinforcement, and confirms that combining expectations on the genomic patterns of divergence with functional expectations is a promising route to decipher the genetic architecture of behavioural trait variation.

170 Results

171 **Sequence divergence between Choosy and Non-Choosy populations**

172 Whole-genome resequencing of pools of individuals representing two Choosy and two Non-
173 Choosy populations of *M. m. musculus* (see Figure 1a and Table S1 for a detailed sample
174 description) identified a total of 28,500,265 SNPs in the dataset after stringent filtering. Genetic
175 dissimilarity indexes estimated from the scaled genome-wide covariance matrix of the population
176 allele frequencies (Ω matrix, produced by the program BAYPASS) indicated that the two Choosy
177 populations do not cluster together (Figure 1b). This result could be explained by the dense river
178 network in Jutland acting as geographic barriers to migration and hence influencing population
179 structure (Raufaste et al. 2005). Interestingly, this pattern of population relatedness makes the
180 identification of outlier divergent loci in the Focal Test comparing Choosy and Non-Choosy
181 populations (Figure 1c) conservative. A Control Test was performed to remove outlier loci from
182 the Focal Test representing population-specific divergence and therefore not a consistent
183 divergence between both Choosy versus both Non-Choosy populations (Figure 1c and Methods).

184 ***Outlier SNPs***

185 We confirmed, in both Focal and Control Tests, that the SNP-based C_2 contrast statistic follows a χ^2
186 distribution with one degree of freedom as expected under the null hypothesis (Figure S1). We
187 thus calculated χ^2-associated *p*-values and FDR to identify outlier SNPs showing extreme genetic
188 differentiation in each population comparison (Focal and Control). We identified 1,612,787 outlier
189 SNPs ($P < 0.05$) in the Focal Test before multiple testing corrections, reduced to 6546 outlier SNPs
190 after applying FDR correction. In the Control Test, 1,111,516 SNPs were identified as outliers
191 before multiple testing corrections. We retained as the final list of 'behavioural' outlier SNPs those
192 4818 SNPs showing both significant differentiation between Choosy and Non-Choosy populations
193 after FDR correction in the Focal Test and no significant differentiation in the Control Test (Table
194 S3).

195 ***Outlier genes***

196 In the Focal Test, at the $P < 0.05$ threshold, we identified 999 C_{2_max} outlier genes (over 41,702
197 annotated genes included in this permutation test) (Table S4a) and 1756 C_{2_mean} outlier genes (over
198 39,598 annotated genes included in this analysis) (Table S4b). In the Control Test, comparable
199 numbers of outlier genes were found: 1108 C_{2_max} outlier genes (Table S4c) and 1612 C_{2_mean} outlier
200 genes (over 39,447 tested genes, among which 1472 genes with no permutation results due to
201 insufficient SNP number or too large gene size) (Table S4d). However, the large majority of genes

202 found significant in the Focal Test were not found significant in the Control Test: 77 genes had
203 significant C_{2_max} in both the Focal and the Control Tests (considered as non-consistently
204 associated with the behavioural contrast); as a result, from the 999 C_{2_max} initial outlier genes in
205 the Focal Test, 922 were retained as the final C_{2_max} outlier genes; similarly, out of the 1756 initial
206 C_{2_mean} outlier genes, 1622 were retained as the final C_{2_mean} outlier genes after removing 134
207 outliers not consistently associated with the behavioural contrast. The union of these two cured
208 outlier gene sets, composing the final and complete list of outlier genes, included 2118 genes,
209 among which 496 were C_{2_max} specific outliers, 1196 were C_{2_mean} specific outliers and 426 were
210 C_{2_max} and C_{2_mean} outlier genes (Table S5).

211 Among those 2118 outlier genes, we categorised the most strongly differentiated ones between
212 Choosy and Non-Choosy populations according to (1) their level of differentiation at one of the
213 two gene-based contrast statistics (*p*-value $C_{2_max} < 0.01$ or *p*-value $C_{2_mean} < 0.01$), hereafter called
214 highly significant outlier genes or (2) their level of differentiation at both gene-based statistics (see
215 Methods and Table S5 for details), hereafter called the 'top' outlier genes. We found a total of 479
216 highly significant outlier genes, among which 111 C_{2_max} specific outliers, 142 C_{2_mean} specific
217 outliers and 226 C_{2_max}+C_{2_mean} outlier genes (Table S5, bold gene_IDs). Among these highly
218 significant outlier genes, we further identified 95 outlier genes showing consistent signals of
219 strong differentiation at both C_{2_max} and C_{2_mean} gene-based contrast statistics, that were
220 consequently classified as 'top outlier genes' (Table S5, bold and underlined gene_IDs).

221 Functional impact and nature of candidate genomic regions associated with behavioural divergence

222 *Genic and regulatory regions primarily associated with behavioural divergence*

223 Using the ENSEMBL Variant Effect Predictor (VEP), we found that the 4818 outlier SNPs identified as
224 significantly differentiated between Choosy and Non-Choosy populations include 1390 novel
225 variants and 3428 already annotated variants in the ENSEMBL database (71.1%). Those outlier SNPs
226 overlap 977 annotated genes, 2878 transcripts and 412 regulatory features (Table S6). Genic
227 variants represent a majority of those outlier SNPs (54.03%), with 50.76% of intronic variants and
228 the rest corresponding to exonic (1.31%) and UTRs (1.96%) variants (Table S6, Figure 2a). Protein-
229 coding variants include 64% of synonymous variants, 32% of missense variants and 3% of stop-
230 gained variants (respectively 0.48%, 0.24% and 0.02% of the total, Figure 2a). Non-genic variants
231 are slightly less represented (45.97%) but include a large number of variants (32.91% of all outlier
232 SNPs) potentially involved in gene regulation: down- and upstream variants as well as already
233 known regulatory region variants (Table S6, Figure 2a). Only 13% of all outlier SNPs fall in
234 intergenic regions. In comparison, predictions made on all SNPs (and not only outlier SNPs) in the

235 dataset show fewer variants potentially involved in gene regulation (24%), fewer UTR variants
236 (0.70%), fewer synonymous and missense variants (0.37% and 0.19% respectively) and 10 times
237 less stop-gained variants (0.0022%). In contrast, they show more intergenic and intronic variants
238 (16.15% and 57.62%, respectively).

239 ***Olfactory and vomeronasal receptor genes as prime candidates***

240 Functional enrichment analyses using the program DAVID were performed on four gene datasets:
241 genes within 5 kb of outlier SNPs (977 initial genes, 656 *Ensembl* gene IDs passing DAVID database
242 criteria), the complete list of outlier genes (2118 initial genes, 1181 DAVID gene IDs), the list of
243 highly significant outlier genes (479 initial genes, 291 DAVID gene IDs) and the list of 'top' outlier
244 genes (95 initial genes, 64 DAVID gene IDs) (see Table S5 for a complete list of outlier genes, with
245 their gene names and gene IDs). For the gene set within 5 kb of outlier SNPs, we found 14 highly
246 significant functional annotation terms (Table S7a) and 17 significant functional annotation
247 clusters (Table S7b). The three most enriched functional annotation clusters were membrane
248 proteins, trans-membrane region proteins and olfactory receptor activity; Golgi apparatus, SH3
249 domain, glycoproteins, reduction-oxidation process and synapse were also part of enriched
250 annotation clusters (Figure 2b). For outlier gene sets, olfactory receptor types of function were
251 systematically found the most significantly enriched and enrichment score increased as the set of
252 outlier genes is narrowed down to the most significant outlier genes (Figure 2b, Table S7c-h). For
253 the gene sets corresponding to the most promising candidates, we found a very highly significant
254 enrichment of only one group of functional categories corresponding to olfactory receptor
255 functions (olfactory receptor/ G-protein-coupled receptor/ GPCR family3 vomeronasal receptor
256 type2) (Figure 2b & Table S7e-f: highly significant outlier genes $p < 0.01$, Table S7g-h: 'top' outlier
257 genes).

258 A total of 127 Olfactory Receptor (*Olfr*) genes and 27 Vomeronasal Receptor (*Vmn*) genes were
259 identified as outlier genes (mostly on chromosomes 2, 7 and 9), and 55 *Olfr* and 13 *Vmn* are
260 among the highly significant ($p < 0.01$) outlier genes (Table 1 and Table S5). Consistent with DAVID
261 functional enrichment results, this corresponds to an over-representation of *Olfr* genes among the
262 complete list of outlier genes (hypergeometric test, $n_{\text{Olf outliers}} = 127$, $n_{\text{Olf all}} = 1356$; $n_{\text{genes outliers}} =$
263 2118; $n_{\text{genes all}} = 50942$, p-value = 1.90 e-17), and an over-representation of both *Olfr* genes (n_{Olf}
264 $_{\text{outliers}} = 55$, $n_{\text{Olf all}} = 1356$; $n_{\text{genes outliers}} = 479$; $n_{\text{genes all}} = 50942$, p-value = 1.37 e-19) and *Vmn* genes (n
265 $_{\text{Vmn outliers}} = 13$, $n_{\text{Vmn all}} = 543$; $n_{\text{genes outliers}} = 479$; $n_{\text{genes all}} = 50942$, p-value = 0.0021) among highly
266 significant outlier genes. Among Vomeronasal Receptor gene candidates, 10 belong to family 1 of
267 Vomeronasal Receptors and 17 to family 2 of Vomeronasal Receptors (Yang and Zhang 2007;

268 Zhang et al. 2007), the proportion of family 2 *Vmn* genes increasing when reducing the outlier
269 gene set to the most significant ones (number of family 1 *Vmn* genes = 1, number of family 2 *Vmn*
270 genes = 12) (Table 1 and Table S5). The majority of *Olfr* outlier genes are C_{2_mean} outliers while the
271 majority of *Vmn* outlier genes are C_{2_max} outliers (Table S5). Among the 'top' outlier genes defined
272 when combining information from the C_{2_mean} and C_{2_max} statistics, we found 23 *Olfr* and 4 *Vmn*
273 genes (Table 1 and Table S5).

274 Beyond the over-representation of Olfactory Receptor and Vomeronasal Receptor genes among
275 outlier gene sets, some of them appeared among the top most significantly differentiated outlier
276 genes: 10 *Olfr* and *Vmn* are among the 30 most differentiated C_{2_max} outlier genes (*Olfr304*,
277 *Olfr907*, *Olfr911-ps1*, *Olfr913*, *Olfr915*, *Olfr908*, *Olfr914*, *Olfr143*, *Olfr906* and *Vmn2r74*) and 8 *Olfr*
278 among the 30 most differentiated C_{2_mean} outlier genes (*Olfr906*, *Olfr908*, *Olfr914*, *Olfr907*, *Olfr143*,
279 *Olfr151*, *Olfr894* and *Olfr1265*) (Table S5). Five *Olfr* genes, all in a gene cluster on chromosome 9,
280 were common to these two lists and among the 10 most differentiated 'top' outlier genes: *Olfr906*,
281 *Olfr907*, *Olfr908*, *Olfr914* and *Olfr143,* with *Olfr906, Olfr907* and *Olfr908* among the 4 most
282 differentiated 'top' outliers (Table 1 and Table S5). Comparatively to *Olfr* genes, *Vmn* outlier genes
283 were proportionally less represented among the most differentiated genes, but four of them were
284 found in the list of 'top' outlier genes: *Vmn2r74*, *Vmn2r76*, *Vmn2r70* and *Vmn2r12* (Table S5).

285 Table 1 also reports, when available from VEP results (Table S6), the functional impact of outlier
286 SNPs found in candidate outlier *Vmn* and *Olfr* genes. The large majority of reported functional
287 consequences corresponded to up- and down-stream variants, UTR variants, intronic variants and
288 synonymous variants. However, five *Olfr* genes on chromosome 9 (*Olfr906*, *Olfr908*, *Olfr915*,
289 *Olfr916* and *Olfr250*) and two receptor genes on chromosome 7 (*Olfr304* and *Vmn2r76*)
290 additionally showed a missense mutation between Choosy and Non-Choosy populations, and
291 *Olfr891* and *Olfr895* on chromosome 9 showed a stop-gain variant.

292 Genes that are not Olfactory or Vomeronasal Receptor genes were also among outlier genes
293 (Table S5). Most of them do not have a well-characterized function, and when they do, their link
294 with the behavioural phenotype of interest is unclear. We nevertheless noted some genes
295 involved in gene regulation (*Khsrp*, among the four most differentiated 'top' outlier genes),
296 regulation of neural genes (*Barhl2*), acting as transcription factor (*Gtf2f1*), or expressed in testes
297 and potentially inducing male infertility (*Tex21*, *Dazl*).

298 **Genetic characteristics of regions associated with behavioural divergence**

299 ***Association with some other genomic features***

300 *No association with regions of reduced genetic diversity and coldspots of recombination*

301 Genetic diversity *Π* did not differ significantly between outlier and non-outlier gene regions (Table
302 S8), indicating that there is no global reduction in genetic diversity in outlier gene regions. To
303 assess how recombination rate correlates with candidate outlier gene regions, we used
304 recombination rate estimates provided by Morgan et al. 2017. We found that recombination rates
305 did not differ significantly between outlier and non-outlier gene regions (Table S8). Of 2118,
306 196 outlier genes occurred in a recombination coldspot (Figure 3), which did not correspond to an
307 over-representation of outlier genes in coldspots of recombination (10,000 permutations, Z-score
308 = -0.379, p = 0.663, Figure S2).

309 *SFS-based signatures of selective sweeps in genetic differentiation outliers*

310 To test whether differentiation-based outlier regions also show a signature of selective sweeps
311 estimated from SFS-based methods, we run the program POOL-HMM (Boitard et al. 2013) on the
312 two Choosy populations, the expected targets of reinforcing selection. We found 936 annotated
313 genes within which at least one significant selective sweep window was detected in the Choosy1-
314 Borum population, among which 28 C_2 outlier genes (including *Olfr291* on chromosome 7, and
315 *Olfr1328*, *Olfr1339*, *Olfr1340* and *Olfr1342* on chromosome 4) (Table S9a). In the Choosy2-Låsby
316 population, we found 1247 genes with at least one significant selective sweep window, among
317 which 31 C_2 outlier genes (including *Olfr32*, *Olfr1258* and *Olfr1270* on chr2, and *Olfr62*, *Olfr1339*,
318 *Olfr1340* and *Olfr1342* on chromosome 4) (Table S9b). However, only 3 C_2 outlier genes were
319 found with significant selective sweep windows in both Choosy populations (expected under a
320 reinforcement scenario). Those three genes are *Olfr1339*, *Olfr1340* and *Olfr1342*.

321 *No significant overlap between outlier genes and candidate hybrid sterility regions but some*
322 *punctual associations exist*

323 We also tested whether outlier genes significantly co-localise with the 54 genomic regions
324 identified as candidates for hybrid sterility (postmating isolation candidates) between *M. m.*
325 *musculus* and *M. m. domesticus* (Turner and Harr 2014; Mukaj et al. 2020). We found that 143
326 outlier genes identified in our study overlap with candidate sterility regions (see Table S5 and
327 Figure 3), which is not more than expected by chance (10,000 permutations, Z-score = -2.686, p =
328 0.997; Figure S3a). However, we found that out of the nine highly supported candidate sterility
329 regions (i.e. showing concordant association with the two sterility phenotypes: relative testis
330 weight and testis expression, see (Turner and Harr 2014)), 8 contained at least one outlier gene.
331 The mean distance between outlier genes and sterility genomic regions was 23.70 Mb, which is

332 more distant than expected by chance (10,000 permutations, Z-score = 3.929, p = 0.0001, Figure
333 S3b). We note that PRDM9 gene, located on chromosome 17 and known as a major hybrid sterility
334 gene in the house mouse (Mukaj et al. 2020), is distant by ~ 5 Mb (less than expected by chance,
335 Figure S3b) from the closest outlier gene (*Vmn1r228*, see Table S5).

336 ***Clustered distribution of outlier loci in the genome***

337 *Signals of outlier SNPs accumulate in two main genomic regions on chromosomes 9 and 7*

338 Data from 10-kb, 50-kb and 100-kb non-overlapping windows were examined, for each
339 chromosome separately, for autocorrelation in genetic differentiation associated with the
340 choosy/non-choosy trait, as measured by the BAYPASS C_2 contrast statistic. We found some
341 significant autocorrelation and partial autocorrelation between neighbouring windows (Figure S4),
342 with the autocorrelation parameter rho of a first-order autoregressive process estimated between
343 0.70 and 0.77 (e.g. 50-kb-windows, chr9: rho = 0.74, p-value = 0.039). These results indicated that
344 there is a long-range dependence in genetic differentiation measures (BAYPASS C_2 contrast statistic)
345 present over distances up to 100 kb. This dependence among C_2 measurements has a conservative
346 effect on the identification of outlier SNPs: using only FDR and not an adjusted α based on the
347 autocorrelation estimates, we corrected for an overinflated number of apparently independent
348 tests, making the p-value cut-off conservative. Given this autocorrelation affecting SNP-based C_2
349 measurements, we applied the local score approach (Fariello et al. 2017) to identify outlier
350 genomic segments cumulating signals from single SNP markers and characterize their distribution
351 along the genome. This analysis identified 557 significant local score genomic regions, distributed
352 on all chromosomes (Figure S5, Table S10 and Figure 3). Their mean size (+/- SE) is 99.139 kb (+/-
353 SE: 6.528 kb). Two regions stood out as the most significant ones: one 1-Mb long region on
354 chromosome 9 (peak value of the local score = 12769.03, coordinates: 9:37721717-38752579),
355 containing 52 outlier genes among which 40 outlier Olfactory Receptor genes (Table S5); one 0.8
356 Mb long region on chromosome 7 (peak value of the local score = 9260.49, coordinates:
357 7:85585560-86388933), containing 17 outlier genes, among which 10 Vomeronasal Receptor and
358 5 Olfactory Receptor outlier genes (Table S5).

359 *Two ~ 2 Mb long Olfactory and Vomeronasal receptor gene clusters on chromosomes 9 and 7*
360 *represent the main candidate genomic regions underlying behavioural divergence*

361 Using the expected number of genes per 500 kb and assuming a random genome-wide
362 distribution, a permutation test showed that outlier genes were not homogeneously distributed
363 along the genome (p = 2e−05, 50,000 permutations) (Figure S6). We, therefore, analysed this
364 pattern of genomic clustering in more detail, by investigating whether some outlier gene sets were

365 more clustered in the genome than expected by chance. We identified 63 outlier gene sets found
366 significantly clustered in the genome (Table S11 and Figure 3). The most significantly clustered
367 outlier gene sets were found on chromosome 1 (cluster #3, p = 9.37E-14), chromosome 5 (cluster
368 #20, p = 7.31E-05), chromosome 9 (cluster #39, p = 7.31E-05; cluster #35 (*Olfr* cluster), p = 5.24E-
369 04), chromosome 11 (cluster #49, p = 5.24E-04), chromosome 12 (cluster #52, p = 5.24E-04) and
370 chromosome 7 (cluster #28 (*Vmn*+*Olfr* cluster), p = 2.97E-03). The mean size of those significant
371 outlier gene clusters was 919.88 kb +/- SE: 66.10 kb. The largest gene clusters were found on
372 chromosome 2 (cluster #6: 3.7 Mb), chromosome 1 (cluster #3: 2.45 Mb), chromosome 7 (cluster
373 #28 (*Vmn*+*Olfr* cluster): 2.3 Mb; cluster #30 (*Olfr* cluster): 1.95 Mb) and chromosome 9 (cluster
374 #35 (*Olfr* cluster): 1.8 Mb). These cluster sizes extend far beyond the estimated mean
375 autocorrelation effect on each chromosome, present over distances up to 100 kb. Figure 3
376 recapitulates the distribution of significant local score regions, outlier genes including *Olfr* and
377 *Vmn* ones, and significant outlier gene clusters along the genome.

378 By counting the total number of outlier and 'top' outlier genes and applying weights to this
379 content (see Table S11 for details), we could identify the gene clusters showing the strongest
380 degree of genetic differentiation between Choosy and Non-Choosy populations. Three gene
381 clusters stood out as the most differentiated ones: gene cluster #35 on chromosome 9, gene
382 cluster #28 on chromosome 7, and to some extent gene cluster #5 on chromosome 2 (Table S11).
383 Strikingly, these 3 gene clusters correspond to the three main olfactory/vomeronasal receptor
384 outlier gene clusters: cluster #35 contains 43 *Olfr* outlier genes; cluster #28 contains 14 *Vmn* and 6
385 *Olfr* outlier genes; cluster #5 contains 11 *Olfr* outlier genes. These three outlier gene clusters stand
386 out from genome-wide patterns of differentiation (Figure 3, Figure S7) and the two most
387 important ones on chromosomes 9 and 7 are detailed in Figure 4. Cluster #35 on chromosome 9
388 contains the five *Olfr* outlier genes identified among the 10 most differentiated 'top' outlier genes
389 (Table 1 and Table S5).
390

391 # Discussion

392 In this study, we addressed the genetic basis of assortative mate preferences in the house mouse,
393 expected to be evolving under the action of reinforcing selection, using a population genomics
394 approach coupled with functional predictions. We identified some regions of the genome showing
395 significant and consistent divergence between populations with contrasted behaviours (Choosy
396 populations from the hybrid zone and Non-Choosy populations from allopatric areas). A series of
397 Olfactory and Vomeronasal Receptor (OR and VR) genes, among the most differentiated regions
398 and in line with functional predictions, stand out as the prime candidates potentially underlying
399 this olfactory-based behavioural divergence. Those outlier genes form significantly clustered sets
400 of genes in the genome, with two main candidate gene clusters on chromosomes 9 and 7,
401 extending up to 1.8 Mb and containing several highly significant Olfactory and Vomeronasal
402 receptor outlier genes.

403 **Population genomics and the genetics of complex trait divergence**

404 Population differentiation scans, between two or more populations that differ in a behaviour trait
405 of interest, have rarely been applied to address the genetic basis of polygenic behaviours (but see
406 (Toews et al. 2019)). However, they can be powerful for mapping genetic variation at a high
407 resolution, can be applied to natural populations, and can additionally provide evidence of
408 selection (Niepoth and Bendesky 2020). Potential drawbacks to these approaches include their
409 agnosticism to phenotypic traits and the challenges to interpret signals of genetic differentiation
410 (e.g. populations often differ in more than one trait, other factors than divergent selection can
411 drive genetic differentiation - Cruickshank & Hahn 2014; Ravinet *et al.* 2017; Wolf & Ellegren 2017).
412 In this study, we took advantage of this powerful population genomics approach but combined it
413 with specific experimental and data analysis strategies as well as clear functional predictions to
414 overcome its potential limitations and to refine interpretations of the results in terms of genetic
415 variation associated with the observed behavioural divergence.

416 Our strategy to investigate population differentiation, with whole-genome resequencing data, at
417 different genomic scales (SNPs, genes, different gene-based statistics) enabled us to identify highly
418 supported candidate regions found consistently divergent regardless of the scales and the
419 statistics used, while also characterising more comprehensively the catalogue of genomic regions
420 and architectures potentially underlying this behavioural shift. Genome-wide SNP-based
421 differentiation analyses, despite their limitations (Huang et al. 2011), informed about the
422 respective role of genic versus non-genic regions (mostly regulatory regions) in population

423 differentiation, while confirming functional enrichment results obtained with gene-based analyses.
424 However, our study confirmed that genome-wide gene-based differentiation analyses are efficient
425 for gaining power and precision as well as for facilitating the link with the phenotype of interest
426 (Neale and Sham 2004; Huang et al. 2011; Chung et al. 2019). The identification of enriched
427 functional categories and the most promising individual genes was eased. Moreover, by capturing
428 different potential underlying genetic architectures of divergence (single versus multiple small-
429 effect causal variants within a gene) (Wang et al. 2017), the two different gene-based statistics
430 (C_{2_max} and C_{2_mean}) provided two lists of outlier genes partially overlapping but also
431 complementary. For example, most Olfactory Receptor candidates are C_{2_mean} outlier genes, while
432 most Vomeronasal Receptor ones are C_{2_max} outlier genes.

433 The use of the C_2 contrast statistic of BAYPASS enabled us to assess genetic differentiation based on
434 contrasts of allele frequencies corrected for population structure (Olazcuaga et al. 2021). The
435 combination of biological replicates per behaviour group and multiple population comparisons
436 (Focal and Control tests) allowed us to identify the genomic regions consistently differentiated
437 between Choosy and Non-Choosy populations, beyond population-specific differentiation (Figure
438 1). These multi-way comparisons across populations had been advocated as a promising way
439 forward to separate processes acting similarly on all populations from processes that are specific
440 to a single population or population comparison (Wolf and Ellegren 2017) but are rarely
441 implemented (but see Roesti *et al.* 2014; Vijay *et al.* 2016). In our study, this procedure narrowed
442 down the list of genomic regions of interest, the control test removing respectively 26% and 7.65%
443 of the outlier SNPs and genes, with the final sets of outlier SNPs and genes eventually representing
444 0.017% and ~5 % of the total number of SNPs and annotated genes in the dataset. Yet, this
445 corresponds to a relatively large number of outlier SNPs and genes (4818 and 2118 respectively)
446 that show the expected patterns of genetic differentiation associated with behavioural divergence
447 and reinforcement.

448 Importantly, we do not expect all of these differentiated genomic regions between Choosy and
449 Non-Choosy populations to be involved in the observed behavioural divergence nor to be subject
450 to reinforcing selection. Because these sets of populations can differ in more than one trait and
451 selective regime, some differentiated regions may not affect the behaviour of interest. Moreover,
452 neutral clines across the *M. m. domesticus*/*M. m. musculus* hybrid zone can produce genetic
453 structure at specific loci between Choosy populations and Non-Choosy populations and hitchhiking
454 effects can induce genetic differentiation at variants in the vicinity of causal variants. By selecting
455 populations that are known to show distinct mating preferences despite being closely related and
456 inhabiting a similar environment (in Jutland Denmark, separated by no more than 50 km), we

457 minimized the risk of confounding selective effects, but the list of outlier loci extends very likely
458 beyond the variants directly associated with the behavioural divergence. One key step of this
459 study was therefore to confront genome scan results with functional predictions, here available
460 from a series of previous studies that had characterised the behaviour of interest in detail (Smadja
461 and Ganem 2002; Smadja et al. 2004; Smadja and Ganem 2005; Ganem et al. 2008). Matching very
462 clear functional analyses' results (functional enrichment, nature of outlier genes), indicating a
463 prominent role of some olfactory and vomeronasal receptor genes, with information on the
464 involvement of olfactory-based processes in the expression of assortative mate preferences,
465 provides very strong support for considering these highly significantly differentiated olfactory and
466 vomeronasal receptor genes as the prime candidates potentially associated with behavioural
467 divergence and reinforcing selection (Table 1). Since our previous studies had shown that
468 individuals from Non-Choosy populations can discriminate between mating signals of the two
469 subspecies, but do not establish a directional mate choice (Smadja and Ganem 2005; Smadja and
470 Ganem 2008), the genomic regions identified in our study likely underlie the evolution of
471 choosiness rather than discrimination. Our study illustrates the importance of integrating detailed
472 phenotypic characterisation with genomic data to gain power and accuracy in population genomic
473 approaches that tackle the genetics of complex trait divergence.

474 **Genetic architecture of candidate regions and selective regimes**

475 Autocorrelation analyses indicated a long-range dependence in genetic differentiation measures
476 present over distances up to 100 kb, consistent with previous estimates of background linkage
477 disequilibrium in wild house mouse genomes (Laurie et al. 2007; Staubach et al. 2012) and that
478 could inflate the number of outlier loci (Hahn 2006). However, local score analyses (Fariello et al.
479 2017), which take into account this autocorrelation and background linkage disequilibrium,
480 consistently pointed to numerous outlier local score genomic segments spread across several
481 chromosomes (Figure 3). Whatever the criteria we applied (significance, consistency across
482 analyses and statistics) to stringently narrow down the list of candidate genes and genomic
483 regions, evidence supports a highly polygenic basis underlying the mate preference shift. From the
484 initial list of 2118 outlier genes containing 127 Olfactory receptor (*Olfr*) and 27 Vomeronasal
485 receptor (*Vmn*) genes, the most restricted set of 'top' outlier genes still include 95 genes spread
486 across several chromosomes and among which 23 are *Olfr* and 4 are *Vmn* genes. A highly
487 polygenic basis for assortative mate preferences fits predictions from a previous phenotypic cline
488 analysis in the house mouse suggesting the involvement of a polygenic autosomal system with
489 dominance and epistasis (Ganem et al. 2008). This result is also in line with the few studies

490 addressing the genetics of variation in olfactory-mediated behaviours in insects (Swarup et al.
491 2013; Arya et al. 2015) and mammals (Santos et al. 2018).

492 Given the highly polygenic nature of genetic architecture underlying the behavioural shift and the
493 apparent strong linkage disequilibrium among genes involved in this phenotypic divergence,
494 candidate loci are likely under polygenic selection, expected for such complex traits (Latta 1998;
495 Sella and Barton 2019). Interestingly, previous studies have shown that polygenic adaptation can
496 play a major role in the evolution of species-specific innate behaviours through the modifications
497 of pre-existing genetic networks (Anholt 2020) and in the rapid evolution of chemosensory
498 systems (Librado and Rozas 2016). The fact that we did not find signatures of selective sweeps
499 using SFS-based methods in most candidate outlier regions may be another indication of polygenic
500 adaptation, known to be more detectable with pairwise tests for allelic differentiation (Stölting et
501 al. 2015; Schneider et al. 2021). This result, together with the absence of a significant reduction of
502 genetic diversity in outlier regions, may also suggest that those candidate regions are more likely
503 subject to soft sweeps than hard sweeps (Pritchard et al. 2010; Wang et al. 2022). The two types
504 of outlier genes (C_{2_max} and C_{2_mean}) may here not reflect different selection regimes but just
505 different variant architectures within outlier genes. Since outlier gene regions are not associated
506 with regions of reduced genetic diversity or reduced recombination rate, a general effect of
507 background selection inflating the levels of genetic differentiation in our data seems unlikely.
508 However, future analyses on individual data and a more extensive sampling of Choosy populations
509 would be an interesting perspective to get fine-scale estimates of recombination rate along these
510 genomes and more directly test for signatures of polygenic selection/soft sweeps (Kern and
511 Schrider 2018).

512 Another striking result is that outlier loci showed consistent signals clustered distributions in the
513 genome. While the signals of outlier SNPs via local score analyses accumulated in two main
514 genomic regions on chromosomes 9 and 7, clustering analyses of outlier genes consistently
515 identified numerous sets of genes significantly clustered in the genome, amongst which the two
516 most significant clusters pinpointing the same candidate regions on chromosomes 9 and 7. These
517 two clusters of outlier genes contain several candidate olfactory and/or vomeronasal receptor
518 genes and extend up to 1.8 Mb (Figure 4). These patterns suggest strong linkage disequilibrium in
519 these large candidate regions, potentially indicative of the strength of selection acting on its
520 targets or of complex correlations among loci belonging to the same gene regulatory network
521 (Fagny and Austerlitz 2021). Within these clusters, it is difficult to say which genes and nucleotides
522 are the direct targets of selection and pinpointing individual genes' contributions to the observed
523 behavioural shift in these genomic clusters will require additional experiments. Nevertheless,

524 some outlier genes are more significantly differentiated than others in those clusters and can be
525 considered prime candidates (*Vmn2r70*, *Vmn2r74*, *Vmn2r76*, *Olfr304* and *Olfr308* in the cluster on
526 chromosome 7; *Olfr143*, *Olfr906*, *Olfr907*, *Olfr908* and *Olfr914* in the cluster on chromosome 9).

527 The source of this genetic variation specific to the *M. m. musculus* Choosy populations is still
528 unknown. Considering our results, it may originate from standing variation present in allopatric
529 populations or differential introgression of *Mus musculus domesticus* alleles across the hybrid
530 zone. The large genomic clusters identified as outlier regions could fit the second scenario, which
531 echoes the recent evidence of adaptive introgression of OR and VR gene clusters across species
532 boundaries in the *Mus* genus (Ullrich et al. 2017; Baird et al. 2022; Banker et al. 2022). This
533 scenario would be compatible with the role of these genomic regions in reproductive isolation
534 with the nearby subspecies *M. m. domesticus*: the selective pressure of reinforcement could have
535 favoured the adaptive introgression of alleles conferring choosiness on either side of the hybrid
536 zone (a case of one-allele mechanism), the direction of the choice being possibly expressed via a
537 matching rule, i.e. towards a mating partner matching the chooser's genomic background (or
538 mating signal type). These mechanisms are theoretically predicted to favour the evolution and
539 maintenance of the barrier trait in the context of gene flow (Kopp et al. 2018). Additional genomic
540 data for *M. m. domesticus* populations from the other side of the hybrid zone in Denmark would
541 be needed to first test if variation comes from introgression.

542 Overall, these results on the genetic architecture and selective regimes associated with the
543 divergence in mate preferences correspond to theoretical predictions for loci involved in
544 adaptation and reinforcement. Concentrated architectures of adaptive loci are expected in the
545 presence of gene flow (Yeaman and Whitlock 2011; Yeaman 2013), and fit with the few other
546 empirical studies testing for this pattern of clustering in natural systems (e.g. (Fustier et al. 2017;
547 Roda et al. 2017). Moreover, the selective regime of reinforcement corresponds to indirect
548 selection on prezygotic isolating traits in hybrid zone populations, usually from standing genetic
549 variation present in allopatry (Felsenstein 1981; Kirkpatrick and Servedio 1999) via linkage
550 disequilibrium between loci under direct selection (hybrid sterility genes) and assortative mating
551 loci. We were expecting a co-localisation between candidate hybrid sterility genomic regions
552 (Turner and Harr 2014) and outlier regions that could facilitate the maintenance of linkage
553 disequilibrium among those co-adapted traits (mate preference and hybrid sterility). With the
554 exception of a few co-localising regions, we did not find any significant physical overlap between
555 these two sets of genes. However, this test may miss some unidentified hybrid sterility genes and
556 is quite stringent (testing overlap and not physical linkage). For example, the outlier gene cluster
557 on chromosome 9 lies in physical proximity with a candidate hybrid sterility region (Figure 3).

558 Moreover, linkage disequilibrium can still evolve and be maintained between unlinked or loosely
559 linked and is not necessary under the scenario mentioned above of a case of one-allele
560 mechanism (Servedio 2009; Butlin and Smadja 2018; Hench et al. 2019). Our study provides an
561 important addition to the rare studies that have investigated the genetics of reinforcement (Ortiz-
562 Barrientos et al. 2004; Hopkins and Rausher 2011; Ospina et al. 2021), by testing predictions on
563 expected genomic patterns of differentiation to characterize the genetic basis of a complex
564 premating isolating trait and the genomic signatures of reinforcing selection acting on it.

565 **The role of Olfactory and Vomeronasal Receptor genes in the evolution of mate choice divergence**

566 Chemoreceptor genes are one of the most extensive and diverse multigene families in most
567 vertebrate and non-vertebrate genomes (Sánchez-Gracia et al. 2011; Ibarra-Soria, M. Levitin, et al.
568 2014; Benton 2015; Bear et al. 2016). Yet, their precise role in key evolutionary processes such as
569 adaptation and speciation remains largely unknown, although chemosensory processes have been
570 identified as key players at the phenotypic level (Smadja and Butlin 2009). Comparative analyses
571 have provided great insights into the role of OR or VR repertoire evolution in diversification and
572 ecological adaptations (e.g. dietary specialisation (Hayden et al. 2014; Yohe et al. 2021) or
573 adaptation to different life-styles (Shi and Zhang 2007; Hayden et al. 2010; Khan et al. 2015; Jiao et
574 al. 2019). At microevolutionary scales, studies are scarce due to the difficulty of analysing these
575 gene families and linking their genetic variation to relevant adaptive phenotypes and selective
576 regimes (but see (Smadja et al. 2012; Li et al. 2015; Poelstra et al. 2018; Auer et al. 2020). Our
577 study provides one of the clearest and most detailed examples of the involvement of olfactory and
578 vomeronasal receptors in the evolution of an adaptive behavioural trait, assortative mate choice,
579 and how natural selection drives their genetic divergence.

580 Our results indicated the highly significant enrichment of this category of receptor genes among
581 outlier genomic regions (Figure 2B), in agreement with our functional prediction.

582 General variant effect predictor analyses pointed to the possible dual role of regulatory and
583 protein-coding changes in the evolution of behavioural divergence, with the presence of missense
584 and stop gained variants as well as variants in already known regulatory regions or putative
585 regulatory regions in downstream / upstream gene regions (Figure 2A). For candidate *Olfr* and
586 *Vmn* genes, many SNP variants fall within upstream or downstream gene regions, UTR regions or
587 intronic regions potentially affecting gene regulation, but we identified a few protein-coding
588 changes affecting *Olfr250*, *Olfr906*, *Olfr908*, *Olfr915*, *Olfr916* in the candidate cluster on
589 chromosome 9, and *Olfr304* and *Vmn2r76* in the cluster on chromosome 7 (Table 1). Consistent
590 with a putative role of regulatory changes are the results of two of our previous studies

591 investigating the expression and copy-number divergence between the same Choosy and Non-
592 Choosy populations. We found significant differential expression at some Vomeronasal Receptor
593 genes expressed in the vomeronasal organ (Loire et al. 2017), four out of five (*Vmn2r69*, *Vmn2r70*,
594 *Vmn2r73*, *Vmn2r75*) being among the *Vmn* outlier genes identified in the present study and all
595 belonging to the promising candidate gene cluster on chromosome 7. We also identified deletions
596 specific to the Choosy populations in upstream or intronic regions of some Olfactory Receptor
597 genes (*Olfr907* in the candidate cluster on chromosome 9, *Olfr301*, in the candidate cluster on
598 chromosome 7, *Olfr564* and *Olfr461* on chromosomes 7 and 6), that could affect their expression
599 or the expression of nearby *Olfr* genes (North et al. 2020). The combination of nucleotide,
600 structural and expression variation primarily at Olfactory and Vomeronasal Receptor genes could
601 therefore shape the evolution of this complex behavioural trait under selection (Niepoth and
602 Bendesky 2020; Fagny and Austerlitz 2021).

603 The Olfactory and Vomeronasal Receptor prime candidates are members of some specific
604 phylogenetic clades of closely-related receptors within each gene superfamilies. This result echoes
605 previous insights obtained by Isogai *et al.* on the functional characterisation of Vomeronasal
606 Receptors in the house mouse (Isogai et al. 2011). They showed that distinct mouse VR receptor
607 subfamilies have evolved towards the specific recognition of certain animal groups (heterospecific
608 signals from predators or sympatric closely related species/subspecies, male or female conspecific
609 signals) or chemical structures (volatile steroids, non-volatile proteins). Interestingly, some
610 members of V2R clades 3 and 6 (our main VR candidate clusters) were shown to be involved in the
611 recognition of closely-related and sympatric species or conspecifics of the opposite sex, leading
612 the authors to suggest that "through the activation of specialized receptors, *M. musculus* may
613 readily discriminate scents emitted by closely related but reproductively incompatible species, a
614 property that could be linked to the reproductive isolation of these species". Considering the
615 results of our study, and if we extrapolate Isogai's results to ORs, we can hypothesize a
616 specialization of some VR and OR gene clusters towards the function of assortative mating in the
617 hybrid zone.

618 In the future, several interesting follow-up from our work will help to better understand the role
619 of these candidate receptors in the evolution of assortative mating. Comparative transcriptomics
620 on the main olfactory epithelium will complement the study carried out on VNO (Loire et al. 2017)
621 to assess whether changes in expression affect *Olfr* genes between Choosy and Non-Choosy
622 populations. Haplotagging (Meier et al. 2021) and long-range sequencing will help to further test
623 for polygenic selection and structural changes in these candidate genomic regions. Finally,
624 functional characterisation of the candidate OR and VR gene clusters could be tackled by

leveraging the molecular tools developed by Isogai et al. (i.e. activity-dependent immunolabelling of sensory neurons coupled with *in situ* hybridisation) and applying them to wild mice from hybrid zone. With this approach, we could test whether those receptors are activated by subspecific cues (i.e. urinary stimuli), as expected if involved in the expression of assortative mate preferences.

631 Material and Methods

632 **Samples**

633 We trapped *M. m. musculus* wild adult mice in Jutland, Denmark in several sites (indoor farms and
634 other human dwellings) and maintained them in the laboratory under controlled conditions before
635 being behaviourally tested and euthanized for dissection. Sampling sites represented two distinct
636 geographical areas in Jutland, which are characterised by populations with distinct mate
637 preference behaviours but sufficiently geographically close to avoid any geographical effect of
638 distant allopatry: (1) the border of the hybrid zone on the *M. m. musculus* side (50 km North to the
639 genetic centre of the hybrid zone (defined in Raufaste et al. 2005)) where strong assortative
640 mating was previously documented (Smadja et al. 2004; Smadja and Ganem 2005; Ganem et al.
641 2008) and confirmed on these newly sampled populations (Latour et al. 2014; Smadja et al. 2015)
642 ('Choosy' populations), (2) another area in Jutland but further north from the hybrid zone, where a
643 previous study did not find any significant directional mate preference in these sampled
644 populations ('Non-Choosy' populations) (Latour et al. 2014; Smadja et al. 2015).

645 Two populations per geographical area were included as biological replicates, each composed of
646 several trapping sites (four populations in total, hereafter called « Borum Choosy1 », « Låsby
647 Choosy2 », « Hobro Non-Choosy1 » and « Randers Non-Choosy2) (see Figure 1a and Table S1 for a
648 detailed sample description). For all individuals from each of the four populations n_{Borum} = 33, $n_{\text{Låsby}}$
649 = 27, n_{Hobro} = 29, n_{Randers} = 29), spleen was extracted rapidly after death by cervical dislocation,
650 immersed in ethanol and stored at -80°C. We extracted genomic DNA from individual mouse
651 spleens, using the Macherey-Nagel kit standard protocol.

652 **Data generation and variant processing**

653 ***Whole-genome re-sequencing.*** To assess genomic divergence in two independent comparisons of
654 Choosy and Non-Choosy populations, at the level of the whole genome (genome size: 2.7 Gb), we
655 carried out a pool-sequencing approach. Pooled samples representing each population were
656 obtained by mixing individual DNA extracts from each population in equimolar proportions.
657 Library preparation and sequencing were carried out at the MGX-Montpellier GenomiX platform
658 (Institut de Génomique Fonctionnelle - Institut de Génétique Humaine, Montpellier, France).
659 Sequencing libraries (average insert size: 478 bp (Borum Choosy1), 497 bp (Låsby Choosy2), 478 bp
660 (Hobro Non-Choosy1) and 445 bp (Randers Non-Choosy2)) were prepared using two methods to
661 increase sequence diversity: Illumina TruSeq DNA Sample Prep Kit v2 (three-quarters of samples),
662 and Illumina Nextera DNA sample preparation kit. Overall, ¾ of all sequence data was obtained

using the TruSeq protocol. All libraries were sequenced on an Illumina HiSeq2000 machine using a paired-end 100-bp read-length protocol, targeting an average raw coverage per pool of 30X, chosen as a cost-effective optimum given the large house mouse genome and pool size/pool coverage balance recommendation for pool-seq sequencing (Futschik and Schlotterer 2010; Gautier et al. 2013; Schlötterer et al. 2014). Sequencing reads were trimmed and cleaned of adaptor sequences using Trimmomatic (Bolger et al. 2014).

Mapping, SNP identification and filtering. We applied stringent and optimized mapping and SNP filtering conditions to obtain accurate allele frequency estimations from pool-seq data (Gautier et al. 2013; Schlötterer et al. 2014). The program Bowtie2 v2.2.3 (Langmead and Salzberg 2012) was used to map the reads against the house mouse reference genome (http://www.ensembl.org/, version GRCm38.p2), with the –very-sensitive-local option to get a stringent alignment and a fragment size limit of 600 (-X parameter) to increase the number of concordant mapping count and reduce the discordant mapping count. The Samtools suite v1.3.1 (Li et al. 2009) was used to sort, index and filter for read duplicates the resulting BAM files. An overview of sequencing and mapping statistics can be found in Table S2. BAM files were combined into mpileup files and *sync* files (using samtools mpileup and mpileup2sync.pl script from Popoolation2 package (Kofler et al. 2011)) and first filtered based on base quality (min-qual 20). We filtered out *sync* files for indels using Popoolation2, and developed a custom Python script to further filter bi-allelic SNP variants based on minimum coverage (= 10X), maximum coverage (= 100X, to avoid repeated/duplicated regions of the genome), and minimum count of minor allele (= 3). Minimum and maximum coverage filters were applied per population, and a minimum allele count filter was applied across all populations. From this set of filtered SNPs, we estimated mean population genetic diversity Π (NPSTAT package v1, Ferretti et al. 2013) and pairwise-population F_{ST} (Pool-Seq estimator implemented in the *poolfstat* R package, Hivert et al. 2018; Gautier et al. 2022) to describe the populations under study (Figure 1a).

Identification of SNPs and genes associated with behavioural divergence and under divergent selection

Population contrasts and BAYPASS procedure. We used the program BAYPASS v2.2 (Gautier 2015) to identify Single-Nucleotide-Polymorphisms (SNPs) significantly and repeatedly divergent between Choosy and Non-Choosy populations, i.e. associated with the observed behavioural divergence between these populations. BAYPASS implements a method accounting for the neutral correlation of allele frequencies across populations to detect loci under selection or associated with covariables. We used BAYPASS to estimate the scaled covariance matrices (Ω) of the population allele frequencies for autosomal and X-linked SNPs (Figure 1b). This matrix captures

696 information about the shared demographic history of the sampled populations which is expected
697 to differ between autosomes and the X chromosome (Clemente et al. 2018). Using our repeated
698 experimental design (two biological replicates per behavioural group), we relied on the recently
699 implemented C_2 statistic of BAYPASS (Olazcuaga et al. 2021) to contrast SNP allele frequencies
700 corrected for the global effect of the shared population history captured by the matrix Ω on their
701 covariance (i.e. standardized allele frequencies) between the two groups of populations specified
702 by the behavioural trait. This approach allows correcting for population structure to identify SNP
703 associated with binary co-variables characterizing the populations under study which here
704 consisted of two distinct comparisons (Figure 1c). First, the Focal Test consisted in estimating the
705 contrast statistic between the two groups of populations with contrasted mating behaviours:
706 Choosy1+Choosy2 versus Non-Choosy1+Non-Choosy2 populations. This test aimed at identifying
707 SNPs showing significant association with the behavioural contrast between Choosy and Non-
708 Choosy populations. To further remove outlier loci from the Focal Test that would not show a
709 consistent divergence between both Choosy and both Non-Choosy populations, we carried out a
710 Control Test. This control test estimates the contrast statistic between two other combinations of
711 the same populations, this time not grouped by their behavioural profile (one Choosy and one
712 Non-Choosy population per group) (Figure 1c). We used the inference of genetic relationships
713 among populations based on the Ω matrix estimated by BAYPASS (see Figure 1b) to maximize the
714 distance between the control groups (i.e. comparing Choosy1+Non-Choosy2 versus Choosy2+Non-
715 Choosy1) to perform the most stringent control contrast. SNPs and genes found to be significantly
716 divergent in the Focal Test but also found to be significantly divergent in the Control Test
717 (potentially reflecting population-specific divergence in the Focal Test) were removed from the
718 final list of candidate loci.

719 Custom scripts were used to format filtered *sync* files into BAYPASS input files. We run BAYPASS
720 separately for the autosomes and the X chromosome and specified the pool haploid sample sizes
721 to activate the Pool-Seq mode of BAYPASS. For the autosomes, the pool haploid sizes were simply
722 based on sample sizes ($n_{_aut_Borum\text{-}Choosy1}$ = 66; $n_{_aut_Låsby\text{-}Choosy2}$ = 54; $n_{_aut_Hobro\text{-}NonChoosy1}$ = 58; $n_{_aut_Randers\text{-}}$
723 $_{NonChoosy2}$ = 58). For the X chromosome, we adapted the haploid pool size information to the
724 effective sex ratio of each pool (Table S1; $n_{_chrX_Borum\text{-}Choosy1}$ = 48; $n_{_chrX_Låsby\text{-}Choosy2}$= 41; $n_{_chrX_Hobro\text{-}}$
725 $_{NonChoosy1}$ = 45; $n_{_chrX_Randers\text{-}NonChoosy2}$ = 43). We used the BAYPASS core model with default options for
726 the MCMC algorithm to obtain estimates of the scaled covariance matrix (Ω) and the C_2 contrast
727 statistic. Following the BAYPASS manual instructions, the autosomal dataset was divided into sub-
728 datasets of 110,905 SNPs (by taking one SNP every 250 SNPs along each chromosome) and three
729 independent runs (using option -seed) were performed to assess the reproducibility of the MCMC

730 estimates. We found a fairly high correlation across the different independent runs for the
731 different estimators (Pearson's $r > 0.95$ for the C_2 estimator) and thus only presented results from
732 the first run. Similarly, a near-perfect correlation of the posterior means of the estimated Ω matrix
733 elements was observed across independent runs as well as within each run across SNP subsamples,
734 with the corresponding FMD distances (Gautier 2015) being always <0.4. We thus only reported
735 BAYPASS results that were obtained from a single randomly chosen sub-dataset analysed in the first
736 run.

737 ***Identification of outlier SNPs.*** For both the Focal and Control Tests, we computed SNP-based *p*-
738 values from the C_2 values, the C_2 statistic following a Chi-squared distribution with one degree of
739 freedom under the null hypothesis of no association (Olazcuaga et al. 2021). We identified outlier
740 SNPs in the Focal Test (Choosy versus Non-Choosy) after correcting for multiple testing (*FDR* <
741 0.05). Only outlier SNPs that were not significantly differentiated in the Control Test (*p*-values >
742 0.05 without correction for multiple testing) were retained for further analyses.

743 ***Identification of outlier genes.*** Gene annotations were obtained from ENSEMBL v90
744 (Mus_musculus.GRCm38.90.geneset). Using SNP-based C_2 estimates and the BEDTOOLS suite
745 v2.16.1 (Quinlan and Hall 2010) (*bedtools map*), we computed for each annotated gene in the
746 house mouse genome two gene-based differentiation statistics: C_{2_max} and C_{2_mean}, corresponding
747 to the highest SNP-based C_2 value of a gene and the average C_2 value of a gene, respectively.
748 These two gene-based statistics correspond to two groups of gene-based methods: C_{2_max} has the
749 advantage of detecting genes with a single main causal variant while reducing the total number of
750 tests, while C_{2_mean} appropriately captures the combined effect of multiple small-effect SNP
751 variants within a gene (Wang et al. 2017). Since we had no *a priori* knowledge of the genetic
752 architecture of our phenotype of interest (mate preference), nor on which one of the test statistics
753 was optimal for our analysis, we used those two different statistics and reported as outlier genes
754 those showing significant C_{2_max} or C_{2_mean} statistics. The significance of each gene-based
755 differentiation statistic was assessed using custom permutation procedures. C_{2_max} estimates are
756 influenced by the nucleotide diversity of each gene: the larger the number of SNPs it contains, the
757 more likely are extreme C_{2_max} values. Thus, our permutation procedure consisted in sampling SNP-
758 based C_2 values among gene sequences (including introns) in the genome equal to the number of
759 SNPs in the focal gene. We then recorded the maximum C_2 value of the sample and generated a
760 random distribution specific to the tested gene by repeating the resampling 10,000 times. C_{2_mean}
761 estimates are further influenced by gene length, a proxy for the degree of linkage disequilibrium
762 among SNPs occurring in a given gene. To control for polymorphism and gene length in this
763 permutation procedure, we thus sampled, for each gene, genomic fragments which satisfied two

764 conditions: i) the same number of SNPs and ii) a length in base pairs not deviating from more than
765 10% of the observed one. For each fragment that satisfied these conditions (and thus mimicked
766 the genomic properties of the gene), we calculated C_{2_mean}. Repeating this process 10,000 for each
767 gene allowed us to estimate an empirical *p*-value for the observed gene-based C_{2_mean} statistic.
768 Significant genes (for C_{2_max} or C_{2_mean}) in each population comparison (Focal, Control) were
769 therefore those with *p-values* < 0.05 or 0.01 (see below).

770 Only outlier genes that were significantly differentiated in the Focal comparison (*p*-values < 0.05)
771 and not significantly differentiated in the Control comparison (*p*-values > 0.05) were retained. We
772 defined as the final and complete list of outlier genes the union of C_{2_max} and C_{2_mean} outlier genes
773 so that C_{2_max} specific and C_{2_mean} specific outlier genes are taken into account. All downstream
774 analyses were performed on this set of outlier genes (with two stringency categories: *p*-values <
775 0.05 or *p*-values < 0.01). However, we also defined a restricted list of outlier genes (hereafter
776 called 'top outlier genes'), identified as highly differentiated based on the combined information
777 from the two C_{2_max} and C_{2_mean} contrast statistics. To implement this classification, we first
778 assigned each outlier gene to a significance category for each gene-based statistic (1: most
779 significant; 5: least significant), assigned weights to these categories and calculated an overall
780 differentiation score as the sum of the weights obtained from C_{2_max} and C_{2_mean} information.
781 Genes were classified as 'top outlier genes' when showing a minimum overall differentiation score
782 of 10.

783 **Functional and genomic characteristics of candidate genomic regions**

784 ***Functional analyses of candidate genomic regions***. The final set of outlier SNPs inferred from the
785 combination of the Focal and Control BayPass Tests was analysed using the ENSEMBL Variant Effect
786 Predictor (VEP) (McLaren et al. 2016) to annotate outlier SNPs in coding and non-coding genomic
787 regions as well as to determine their impact on the protein sequence or gene regulation. We used
788 the program DAVID v6.8 (Huang et al. 2009) to carry out functional annotation analyses on both
789 outlier SNP and gene sets. Enriched annotation terms were identified using a significance
790 threshold of 0.01 (EASE score) after Benjamini & Hochberg's correction. To reduce the functional
791 redundancy among annotation terms, we also carried out Functional Annotation Clustering
792 analyses provided by DAVID, which report functional groups with similar annotation terms
793 (significant enrichment score (i.e. the geometric mean of all the enrichment *p*-values (EASE scores)
794 for each annotation term associated with the gene members in the group) > 1.3). Over-
795 representation of some candidate gene families such as Olfactory Receptor (*Olfr*) and
796 Vomerosanal Receptor (*Vmn*) genes among outlier gene sets was tested using hypergeometric

797 tests (*phyper* R function).

798 ***Genomic features associated with outlier gene regions.*** We used the NPSTAT package v1 (Ferretti
799 et al. 2013) to estimate nucleotide diversity Π (Tajima 1983), measured as the average pairwise
800 nucleotide diversity for a given position across all reads and summing it over all positions, and
801 Watterson's theta θ (Watterson 1975) statistics in 5 kb and 50 kb non-overlapping windows across
802 all 20 chromosomes for each of the four studied populations. Using the BEDTOOLS (bedtools flank
803 and map) and custom scripts, we computed nucleotide diversity Π for all annotated gene regions
804 (gene sequence +/- 5 kb) and tested for differences between outlier and non-outlier gene regions
805 using Kruskall-Wallis tests. POOL-HMM v1.4.3 program (Boitard et al. 2013) was used to detect
806 selective sweep windows across the genome in each of the two Choosy populations, using
807 estimates of Watterson's θ provided by NPSTAT.

808 We tested for non-random co-localization of candidate outlier genes identified in this study with
809 (i) coldspots of recombination reported by Morgan et al. 2017 and (ii) candidate hybrid sterility
810 genomic regions between *M. m. musculus* and *M. m. domesticus* identified by Turner and Harr
811 2014. Candidate hybrid sterility regions correspond to the 53 GWAS genomic regions significantly
812 associated with relative weight testis variation (named RTW regions) and/or testis expression
813 variation (named PC regions) (Turner and Harr 2014). We added to this list of candidate genes the
814 gene *PRDM9* (chr17:15543079-15564354) known as a major hybrid sterility gene in the house
815 mouse. We counted the number of outlier genes overlapping these two genomic region sets
816 (coldspots of recombination and candidate sterility regions) and used permutations to assess
817 whether outlier genes overlap significantly more than by chance with these genomic elements
818 (outlier genes resampled 10,000 times among all annotated genes). To perform these permutation
819 tests, we used the bioconductor *RegioneR* (Gel et al. 2015) package in R version 3.6.2 (R Core
820 Team 2019). We also performed a Kruskall-Wallis test to investigate whether recombination rates
821 differ significantly between outlier and non-outlier gene regions.

822 ***Genomic distribution of outlier SNPs and genes***

823 *Outlier SNPs.* We first estimated the autocorrelation between linked loci in the level of genetic
824 differentiation (BAYPASS SNP-based contrast statistics C_2) using a first-order autoregressive model
825 (Hahn 2006). For each chromosome, 10 kb, 50 kb and 100 kb non-overlapping windows were
826 analysed for autocorrelation and partial autocorrelation in C_2 (using the *acf* and *pacf* R functions
827 respectively). We then applied the local score approach (Fariello et al. 2017), which accounts for
828 linkage disequilibrium using autocorrelation information, to identify outlier genomic segments
829 cumulating signals from single SNP markers and characterize their distribution along the genome.

830 We looked at the distribution of $-\log_{10}(p\text{-value})$ to determine the tuning parameter ξ, which must
831 be comprised between mean($-\log_{10}(p\text{-value})$) (here equal to 0.425) and max($-\log_{10}(p\text{-value})$) (here
832 equal to 10.365). We chose a value of $\xi = 1$ to run the excursions of the Lindley process so that we
833 could expect cumulating a larger number of intermediate signals and the identification of wider
834 outlier regions, putting more emphasis on recent soft or incomplete sweeps. Although this
835 method does not provide absolute information about the number and the size of candidate
836 genomic regions since they depend on the tuning parameter ξ, it is useful to identify regions with
837 outstanding genetic differentiation between populations and the genomic range at which
838 cumulating effects at multiple differentiated SNPs spread.

839 *Outlier genes.* We first assessed whether outlier genes were distributed heterogeneously in the
840 genome (permutation procedure testing whether the observed number of outlier genes per 500
841 kb was larger or smaller than expected by chance). We then performed a cluster analysis
842 (developed by (Haque et al. 2016)) to identify the number, location and size of outlier gene
843 clusters in the genome. For the clustering approach, the window size used was 500 kb (basal
844 autocorrelation being non-significant at distance > 100 kb) and this 500-kb window was shifted 50
845 kb at a time in an overlapped fashion. For each sliding window, the number of outlier genes was
846 counted. The sliding window was used from the start of the chromosome until the end for each
847 chromosome separately. Once the number of outlier genes had been calculated for each window a
848 z -test was used to calculate the z -score for each window from which the p-value was calculated.
849 Any window with a p-value < 0.05 was taken as statistically significant in the over-representation
850 of outlier genes. Windows falling within 50,000 bases (consecutive windows) of each other were
851 merged to create final clusters. We graphically represented the distribution of significant local
852 score segments, outlier genes and outlier gene clusters along the genome using the Gtrellis v1.4.2
853 package (Gu et al. 2016).

854 **Data and scripts availability**

855 Raw mapping files (BAM files) and metadata have been uploaded to the Short Read Archive (SRA,
856 NCBI) under the bioproject ID PRJNA603262. Custom scripts produced for data analysis are
857 deposited in Zenodo (10.5281/zenodo.7225771).

858 **Supplementary information**

859 Supplementary material is deposited in Zenodo (10.5281/zenodo.6900875). The list of
860 supplementary tables and figures is as follows:

861 Table S1: sample description

862 Table S2: sequencing and mapping statistics

863 Table S3: list of BayPass outlier SNPs

864 Table S4: gene-based results

865 Table S5: complete list of outlier genes

866 Table S6: Variant Effect Predictor results

867 Table S7: DAVID functional enrichment results

868 Table S8: Comparison of genetic diversity and recombination rate between outlier and non-outlier
869 gene regions

870 Table S9 : list of annotated genes with significant selective sweep windows (pool-hmm)

871 Table S10 : significant local score regions

872 Table S11 : list of outlier gene sets significantly clustered in the genome (called gene clusters)

873 Figure S1: Distributions of SNP-based C_2 values in the Focal and Control tests

874 Figure S2: Results of permutation tests assessing co-location of outlier genes with coldspots of
875 recombination

876 Figure S3: Results of permutation tests assessing co-location of outlier genes with candidate
877 hybrid sterility regions

878 Figure S4: Autocorrelation and partial autocorrelation estimates in genetic differentiation

879 Figure S5: Local score distribution along the autosomes and the X chromosome

880 Figure S6: Results of a parameter-free test for homogeneous distribution of outlier genes in the
881 genome

882 Figure S7: Details of Figure 3 for the three main gene clusters on chromosomes 2, 7 and 9

883

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Authors' Contributions

CMS: conceptualization, funding acquisition, data analyses, study and data analyses supervision, drafting the article; EL: bioinformatic developments and data analyses; PC: sampling and DNA sample preparation; DS: library preparation and sequencing; MG: BayPass analyses; GG: participation to research formulation, funding acquisition, sampling. All authors contributed to revising and editing the manuscript.

Conflict of interest disclosure

The authors declare they have no conflict of interest relating to the content of this article. C. Smadja and M. Gautier are recommenders for PCI.

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1282 Figure legends

1283 **Figure 1**: **Sampling and experimental design. A)** Populations of *Mus musculus musculus* sampled
1284 in localities in the *M. m. musculus* - *domesticus* hybrid zone (dark blue points) or further away
1285 from the centre of the hybrid zone (light blue points). Mean nucleotide diversity: Låsby (n=27, Π
1286 = 0.002 +/- 1.136e-05), Borum (n=33, Π = 0.003 +/- 1.151e-05), Randers (n=29, Π = 0.002 +/-
1287 1.027e-05), Hobro (n=29, Π = 0.002 +/- 1.159e-05); pairwise F_{ST} estimates between Choosy and
1288 Non-Choosy populations: $F_{ST\ \text{Borum-Choosy1 - Randers-NonChoosy2}}$ = 0.057; $F_{ST\ \text{Låsby-Choosy2 - Randers-NonChoosy2}}$ =
1289 0.087; $F_{ST\ \text{Hobro-NonChoosy1 - Borum-Choosy1}}$ = 0.089; $F_{ST\ \text{Hobro-NonChoosy1 - Låsby-Choosy2}}$ = 0.114); F_{ST} within
1290 behavioural groups: $F_{ST\ \text{Borum-Choosy1 - Låsby-Choosy2}}$ = 0.065; $F_{ST\ \text{Hobro-NonChoosy1 - Randers-NonChoosy2}}$ = 0.076. **B)**
1291 Relatedness among populations based on the genome-wide covariance matrix (Ω) across
1292 population allele frequencies for autosomal chromosomes (similar results for the X chromosome).
1293 **C)** The two series of population comparisons were used to identify loci showing an association
1294 with behavioural divergence. The Focal Test compared replicated sets of Choosy and Non-Choosy
1295 populations (Choosy1+Choosy2 versus Non-Choosy1+Non-Choosy2) to identify loci showing
1296 significant divergence (as measured by the contrast statistic C_2 from the BAYPASS program)
1297 between populations with contrasting behaviour. The Control Test compared two other
1298 combinations of the same populations, this time not grouped by their behavioural profile
1299 (Choosy1+Non-Choosy2 versus Choosy2+Non-Choosy1). Only loci found divergent specifically in
1300 the Focal Test were retained as the final list of candidate loci.
1301

1302 **Figure 2**: **Functional analysis results. A)** Summary of Ensembl *VEP* analysis performed on all outlier
1303 SNPs. The pie chart shows the proportions of the different variant consequences found in this set
1304 of outlier SNPs. Consequences include information on the location and possible functional effect
1305 of each variant (e.g. intergenic, within 5k up-/down-stream of annotated genes, UTRs, intronic,
1306 exonic, synonymous, missense, stop_gained, as well as known regulatory regions). **B)** *DAVID*
1307 functional enrichment analyses. Up to top 10 functional annotation clusters (enrichment score >
1308 1.3) are shown for analyses performed on (i) genes found within 5 kb of outlier SNPs (ii) genes
1309 identified as outlier genes (C_{2_max} and/or C_{2_mean}) at 0.05 threshold, (iii) genes identified as outlier
1310 genes at 0.01 threshold.
1311

1312 **Figure 3**: **Genome-wide distribution of candidate regions showing significant genetic**
1313 **differentiation between Choosy and Non-Choosy populations.** Track 1 (top): chromosomes; Track
1314 2: local score values for significant genomic segments based on information on outlier SNPs;
1315 Tracks 3 & 4: C_{2_max} and C_{2_mean} values of outlier genes (922 and 1622 C_{2_max} and C_{2_mean} outlier

1316 genes shown respectively over 41,702 annotated genes); vomeronasal (*Vmn*) and olfactory (*Olfr*)
1317 outlier genes are shown in blue and purple respectively; Track 5: significant outlier gene clusters;
1318 Track 6: recombination cold spots observed in the Diversity Outbred hybrid mouse population by
1319 Morgan et al. 2017; Track 7 (bottom): candidate hybrid sterility genes identified by Turner & Harr
1320 2014. The three most significant outlier gene clusters are highlighted, on chr9, chr7 and to some
1321 extent chr2.
1322
1323 **Figure 4**: **The two main candidate gene clusters composed of Olfactory and Vomeronasal**
1324 **Receptor genes on chromosomes 9 and 7**. *Ensembl* protein-coding names and locations are
1325 shown within these two 1.8 Mb long genomic regions. The ">" or "<" symbols indicate the
1326 orientation of the gene on the chromosome. Non-outlier genes are in grey and outlier genes are in
1327 colours (purple: Olfactory Receptor genes; blue: Vomeronasal Receptor genes; green: any other
1328 outlier genes). For outlier genes in colours, different levels of significance are indicated as follows:
1329 regular text: C_{2_max} or C_{2_mean} outlier genes at $p < 0.05$; bold text: C_{2_max} or C_{2_mean} outliers at $p < 0.01$;
1330 bold and underlined: top significant outlier genes, based on the combined information from C_{2_max}
1331 and C_{2_mean} statistics.

Tables

Table 1: list of significantly differentiated Olfactory (Olfr) and Vomeronasal (Vmn) Receptor genes between Choosy and Non-Choosy populations

Chr	Gene_names*	Types of SNP variant consequences identified by VEP (see Table S6 for details)
1	**Olfr1406**, **Olfr258-ps1**, **Olfr421-ps1**, Olfr420, Olfr419, Olfr417	
2	Olfr1235-ps1, Olfr1258, **Olfr1259, Olfr1260, Olfr1261, Olfr1262**, Olfr1263, **Olfr1264, Olfr1265**, **Olfr140**, Olfr32, **Olfr1270**, Olfr1275, Olfr1276	Olfr: up-/down-stream; intron; synonymous; 3'_UTR;
4	Olfr273, Olfr62, Olfr1343-ps1, **Olfr1342,** Olfr1340, Olfr1339, Olfr1328	Not in Table S6
5	**Vmn2r12**, Vmn2r-ps24	Not in Table S6
6	Olfr435, Vmn1r22, Vmn1r-ps24	Not in Table S6
7	Vmn1r62, Vmn1r63, **Vmn1r64**, Vmn1r74, Vmn1r-ps55, Vmn1r-ps65, **Vmn2r-ps62**, Olfr291, **Vmn2r65**, Vmn2r66, Vmn2r69, Vmn2r-ps70, **Vmn2r70**, **Vmn2r71**, **Vmn2r73**, **Vmn2r-ps78**, **Vmn2r74**, **Vmn2r-ps79**, Vmn2r75, **Vmn2r-ps85**, **Vmn2r-ps86**, **Vmn2r76**, **Olfr310**, **Olfr309**, **Olfr308**, **Olfr305**, **Olfr304**, **Olfr303**, Olfr520, Olfr521, Olfr556, Olfr557, Olfr558, Olfr33, Olfr561, Olfr562-ps1, Olfr563-ps1, Olfr566, Olfr567-ps1, **Olfr568**, Olfr570, Olfr571, Olfr595-ps1, Olfr597, Olfr598, Olfr599, Olfr601, Olfr611, Olfr618, Olfr621-ps1, Olfr624, Olfr628, Olfr66, Olfr639	Vmn: up-/down-stream; intron; synonymous; 3'_UTR; Vmn2r76: missense Olfr: up-/down-stream; intron; synonymous; 3'_UTR; 5'_UTR; Olfr304: missense;
9	**Olfr151, Olfr874, Olfr875, Olfr876, Olfr877**, **Olfr145**, **Olfr878**, Olfr880-ps1, **Olfr881**, **Olfr885**, Olfr886-ps1, Olfr887, Olfr889, **Olfr891, Olfr892-ps1, Olfr893, Olfr894**, **Olfr143**, **Olfr895, Olfr897-ps1, Olfr25, Olfr898**, **Olfr250, Olfr251, Olfr147, Olfr901, Olfr902, Olfr1543-ps1, Olfr904, Olfr905, Olfr906, Olfr907, Olfr908, Olfr911-ps1, Olfr912, Olfr913, Olfr914, Olfr915, Olfr916, Olfr917, Olfr918**, Olfr919, Olfr920	Olfr: up-/down-stream; intron; synonymous; 3'_UTR; 5'_UTR; Olfr891: stop_gained; Olfr895: stop_gained; Olfr250: missense x2; Olfr906: missense; Olfr908: missense; Olfr915: missense; Olfr916: missense
10	Olfr803, Olfr806, Olfr814, Olfr818, Olfr819	Not in Table S6
11	Olfr10, Olfr1387, Olfr1385, Olfr1384, Olfr1383, Olfr1378	Not in Table S6
13	Olfr1367, Olfr1360	Not in Table S6
15	**Olfr282**	Not in Table S6
17	Vmn1r228, Vmn1r-ps149, Olfr761, Olfr132, Olfr133, Olfr135, Olfr137, Olfr136	Olfr136: down-stream, 3'_UTR
19	Olfr1444, **Olfr1446**, Olfr1460-ps1, Olfr1492-ps1, Olfr1495, Olfr1497	Not in Table S6

* Regular: C_{2_max} or C_{2_mean} outliers at p<0.05; **Bold**: C_{2_max} or C_{2_mean} outliers at p<0.01; **bold underlined**: top outliers genes based on the combined information from C2_max and C2_mean statistics; **red**: among the 10 most differentiated 'top' outlier genes (see Methods and Table S5 for details)

Figures

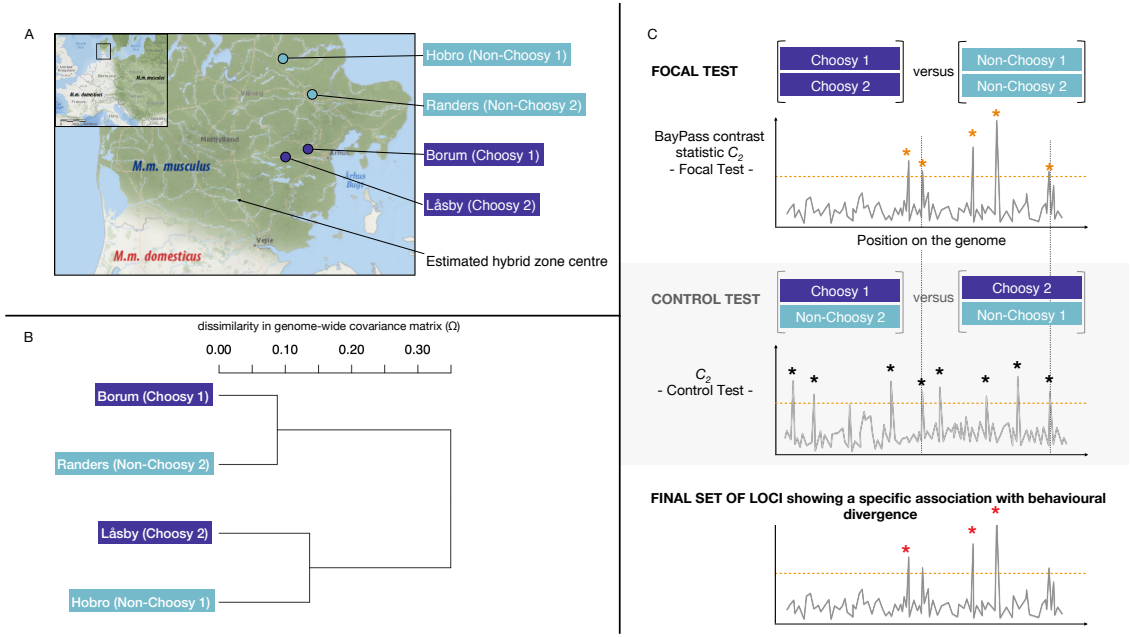


Figure 1: **Sampling and experimental design. A)** Populations of *Mus musculus musculus* sampled in localities in the *M. m. musculus - domesticus* hybrid zone (dark blue points) or further away from the centre of the hybrid zone (light blue points). Mean nucleotide diversity: Låsby (n=27, Π = 0.002 +/- 1.136e-05), Borum (n=33, Π = 0.003 +/- 1.151e-05), Randers (n=29, Π = 0.002 +/- 1.027e-05), Hobro (n=29, Π = 0.002 +/- 1.159e-05); pairwise F_{ST} estimates between Choosy and Non-Choosy populations: $F_{ST\ \text{Borum-Choosy1}\ -\ \text{Randers-NonChoosy2}}$ = 0.057; $F_{ST\ \text{Låsby-Choosy2}\ -\ \text{Randers-NonChoosy2}}$ = 0.087; $F_{ST\ \text{Hobro-NonChoosy1}\ -\ \text{Borum-Choosy1}}$ = 0.089; $F_{ST\ \text{Hobro-NonChoosy1}\ -\ \text{Låsby-Choosy2}}$ = 0.114); F_{ST} within behavioural groups: $F_{ST\ \text{Borum-Choosy1}\ -\ \text{Låsby-Choosy2}}$ = 0.065; $F_{ST\ \text{Hobro-NonChoosy1}\ -\ \text{Randers-NonChoosy2}}$ = 0.076. **B)** Relatedness among populations based on the genome-wide covariance matrix (Ω) across population allele frequencies for autosomal chromosomes (similar results for the X chromosome). **C)** The two series of population comparisons were used to identify loci showing an association with behavioural divergence. The Focal Test compared replicated sets of Choosy and Non-Choosy populations (Choosy1+Choosy2 versus Non-Choosy1+Non-Choosy2) to identify loci showing significant divergence (as measured by the contrast statistic C_2 from the BAYPASS program) between populations with contrasted behaviour. The Control Test compared two other combinations of the same populations, this time not grouped by their behavioural profile (Choosy1+Non-Choosy2 versus Choosy2+Non-Choosy1). Only loci found divergent specifically in the Focal Test were retained as the final list of candidate loci.

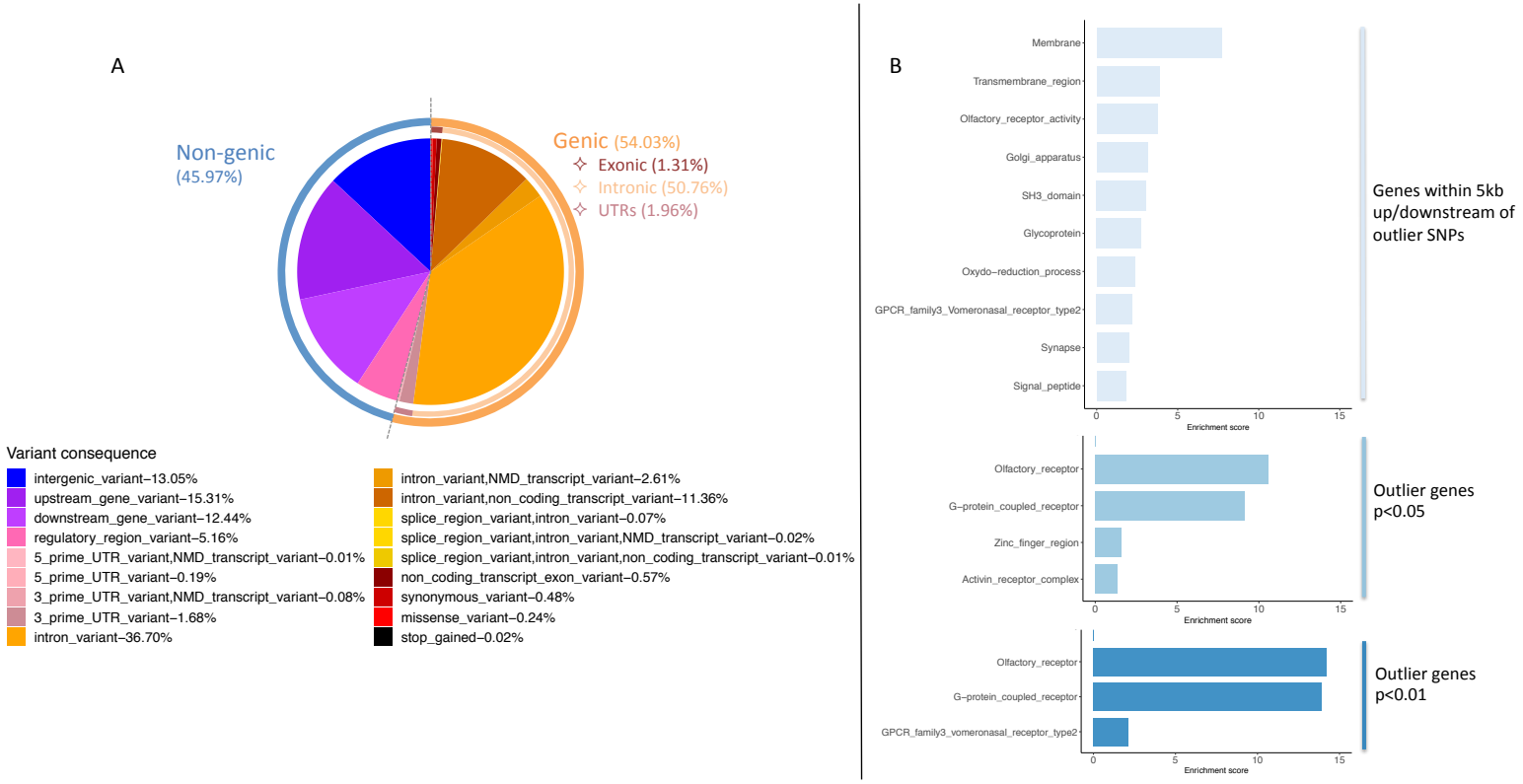


Figure 2: **Functional analysis results. A)** Summary of Ensembl *VEP* analysis performed on all outlier SNPs. The pie chart shows the proportions of the different variant consequences found in this set of outlier SNPs. Consequences include information on the location and possible functional effect of each variant (e.g. intergenic, within 5k up-/down-stream of an annotated genes, UTRs, intronic, exonic, synonymous, missense, stop_gained, as well as known regulatory regions). **B)** *DAVID* functional enrichment analyses. Up to top 10 functional annotation clusters (enrichment score > 1.3) are shown for analyses performed on (i) genes found within 5 kb of outlier SNPs (ii) genes identified as outlier genes (C_{2_max} and/or C_{2_mean}) at 0.05 threshold, (iii) genes identified as outlier genes at 0.01 threshold.

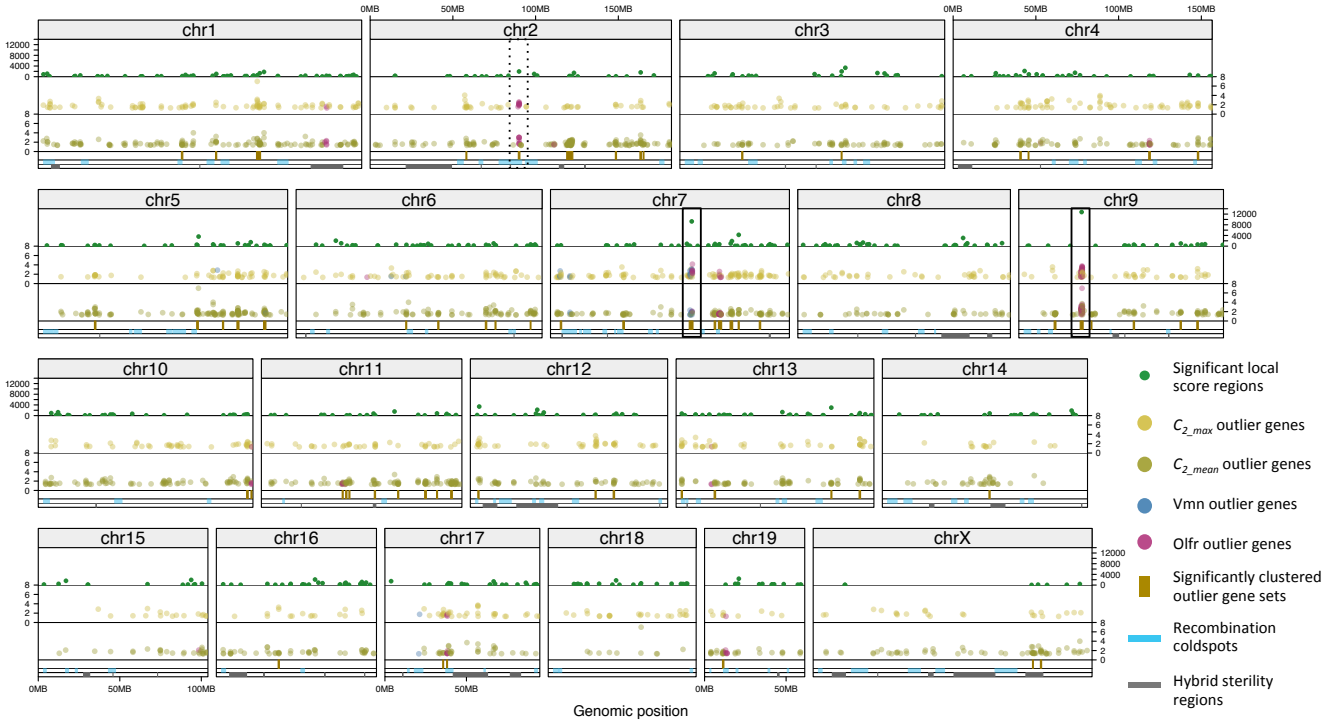
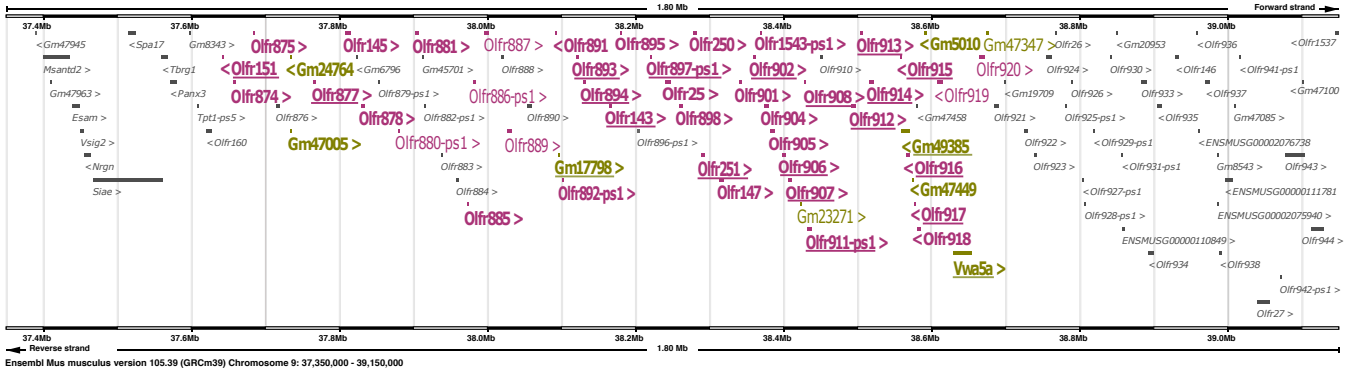


Figure 3: **Genome-wide distribution of candidate regions showing significant genetic differentiation between Choosy and Non-Choosy populations.** Track 1 (top): chromosomes; Track 2: local score values for significant genomic segments based on information on outlier SNPs; Tracks 3 & 4: C_{2_max} and C_{2_mean} values of outlier genes (922 and 1622 C_{2_max} and C_{2_mean} outlier genes shown respectively, over 41,702 annotated genes); vomeronasal (*Vmn*) and olfactory (*Olfr*) outlier genes are shown in blue and purple respectively; Track 5: significant outlier gene clusters; Track 6: recombination cold spots observed in the Diversity Outbred hybrid mouse population by Morgan et al. 2017; Track 7 (bottom): candidate hybrid sterility genes identified by Turner & Harr 2014. The three most significant outlier gene clusters are highlighted, on chr9, chr7 and to some extent chr2.

Chromosome 9, Olfactory Receptor (*Olfr*) outlier gene cluster



Chromosome 7, Vomeronasal Receptor (*Vmn*) and Olfactory Receptor (*Olfr*) outlier gene cluster

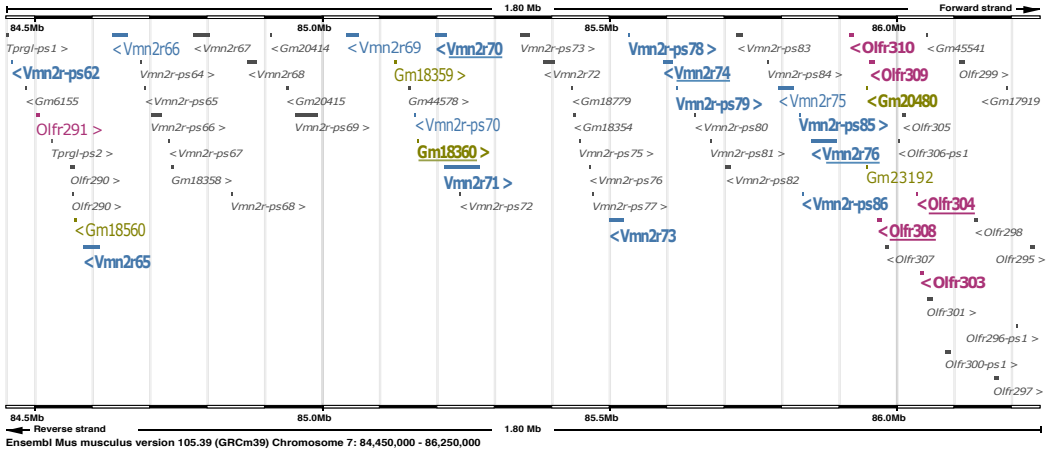
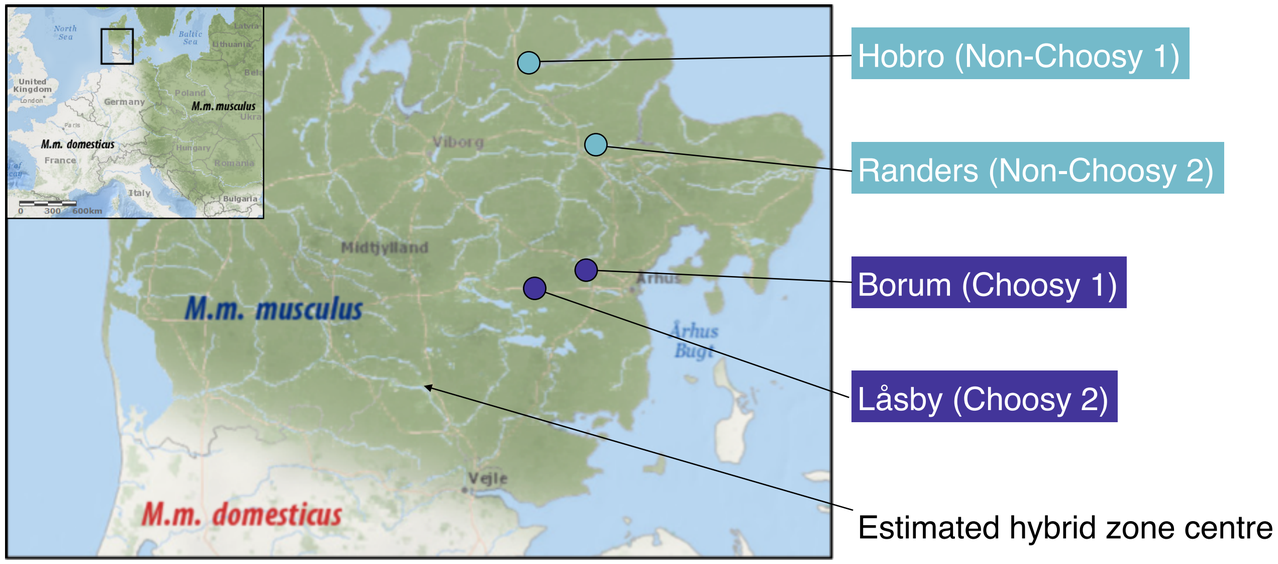
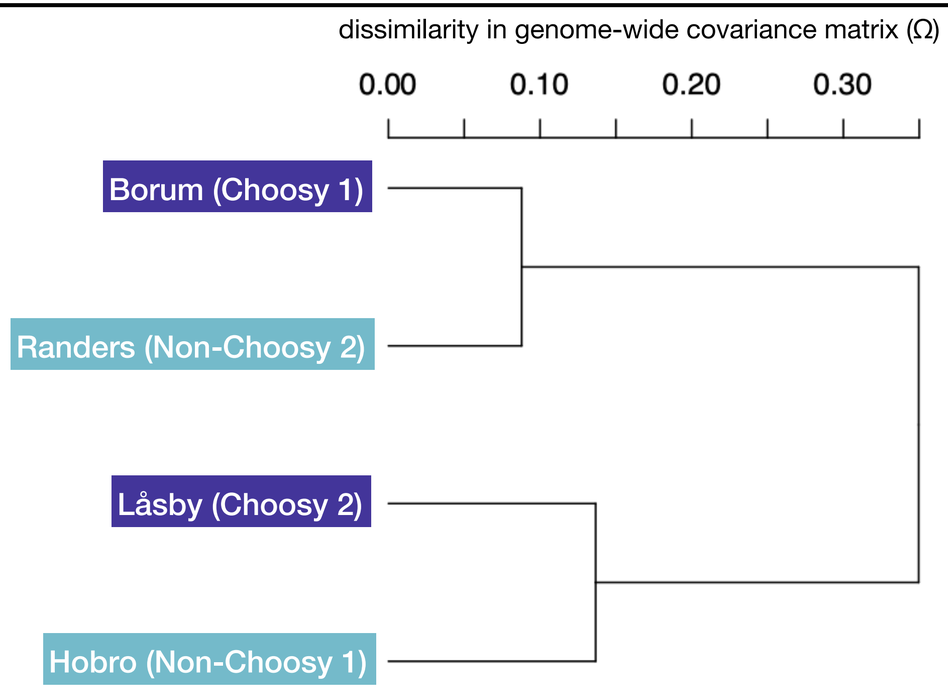


Figure 4: **The two main candidate gene clusters composed of Olfactory and Vomeronasal Receptor genes on chromosomes 9 and 7**. *Ensembl* protein-coding names and locations are shown within these two 1.8 Mb long genomic regions. The ">" or "<" symbols indicate the orientation of the gene on the chromosome. Non-outlier genes are in grey and outlier genes are in colours (purple: Olfactory Receptor genes; blue: Vomeronasal Receptor genes; green: any other outlier genes). For outlier genes in colours, different levels of significance are indicated as follows: regular text: C_{2_max} or C_{2_mean} outlier genes at $p < 0.05$; bold text: C_{2_max} or C_{2_mean} outliers at $p < 0.01$; bold and underlined: top significant outlier genes, based on the combined information from C_{2_max} and C_{2_mean} statistics.

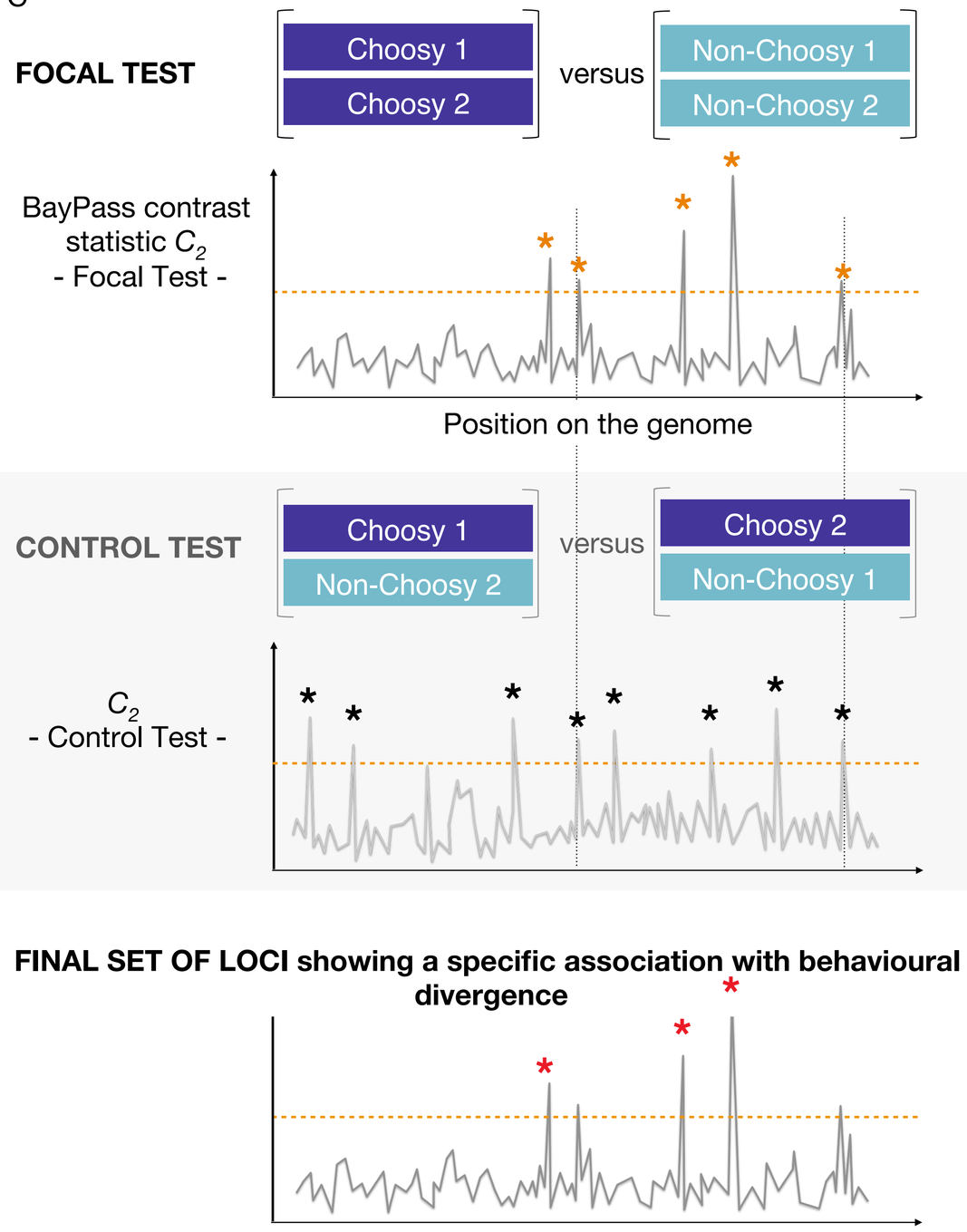
A


Hobro (Non-Choosy 1)
Randers (Non-Choosy 2)
Borum (Choosy 1)
Låsby (Choosy 2)
Estimated hybrid zone centre
M.m. musculus
M.m. domesticus
Viborg
Midtjylland
Århus
Århus Bugt
Vejle

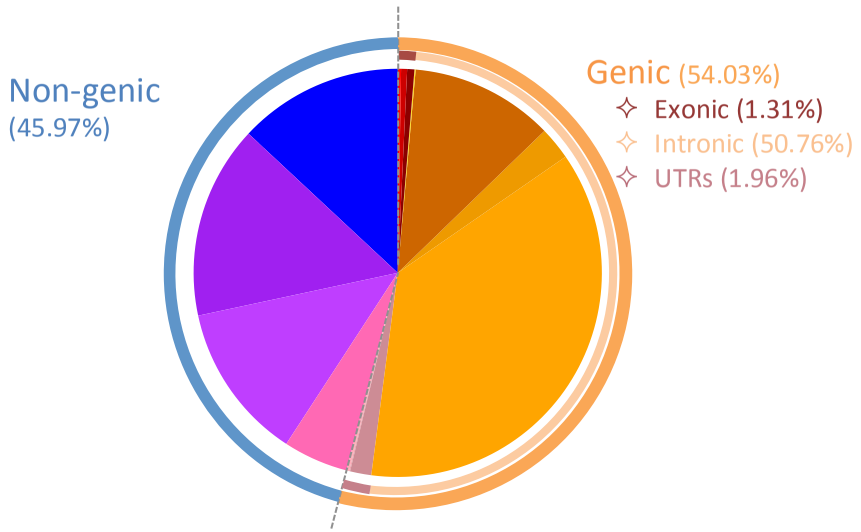
B


dissimilarity in genome-wide covariance matrix (Ω)
0.00
0.10
0.20
0.30
Borum (Choosy 1)
Randers (Non-Choosy 2)
Låsby (Choosy 2)
Hobro (Non-Choosy 1)

C


FOCAL TEST
Choosy 1
Choosy 2
versus
Non-Choosy 1
Non-Choosy 2
BayPass contrast statistic C_2
- Focal Test -
Position on the genome
CONTROL TEST
Choosy 1
Non-Choosy 2
versus
Choosy 2
Non-Choosy 1
C_2
- Control Test -
FINAL SET OF LOCI showing a specific association with behavioural divergence

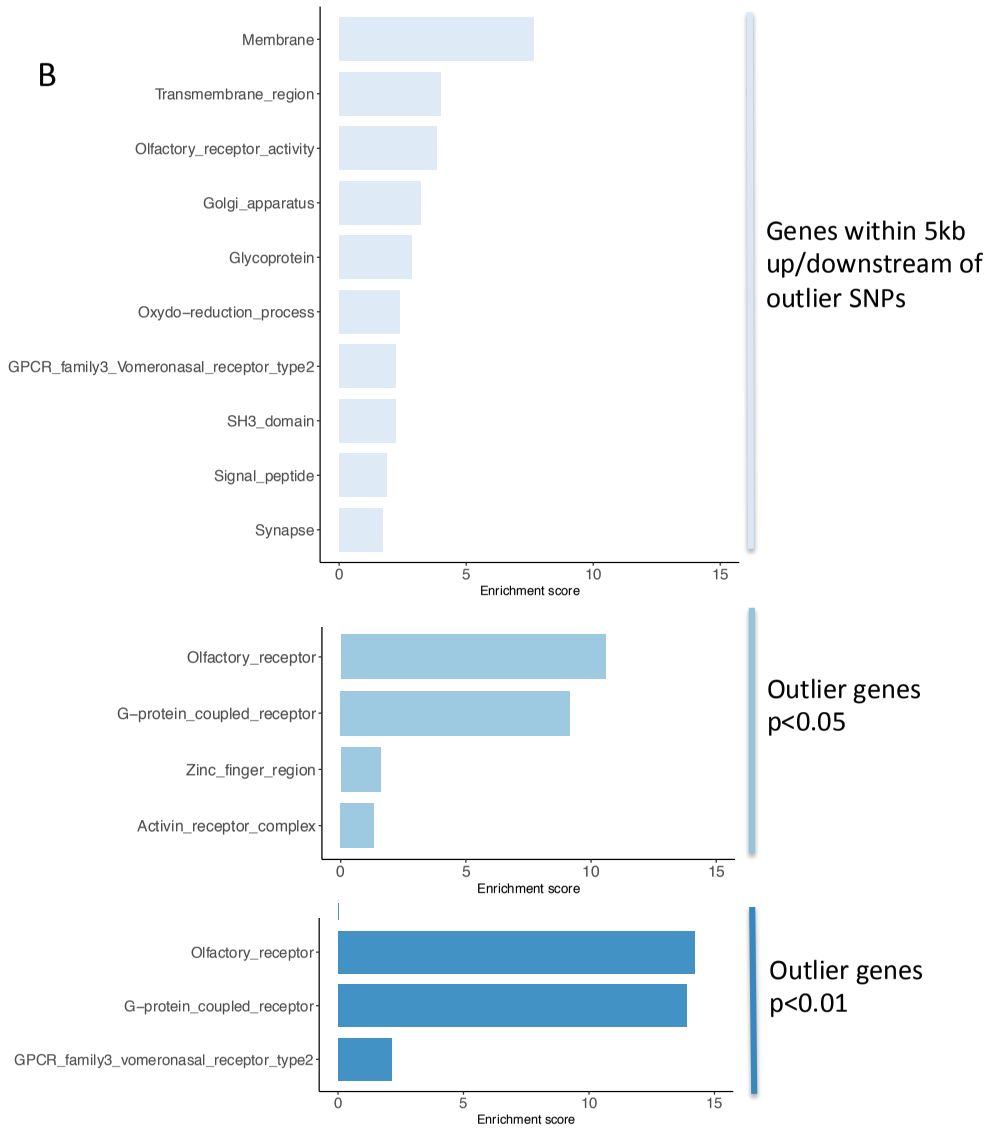
A

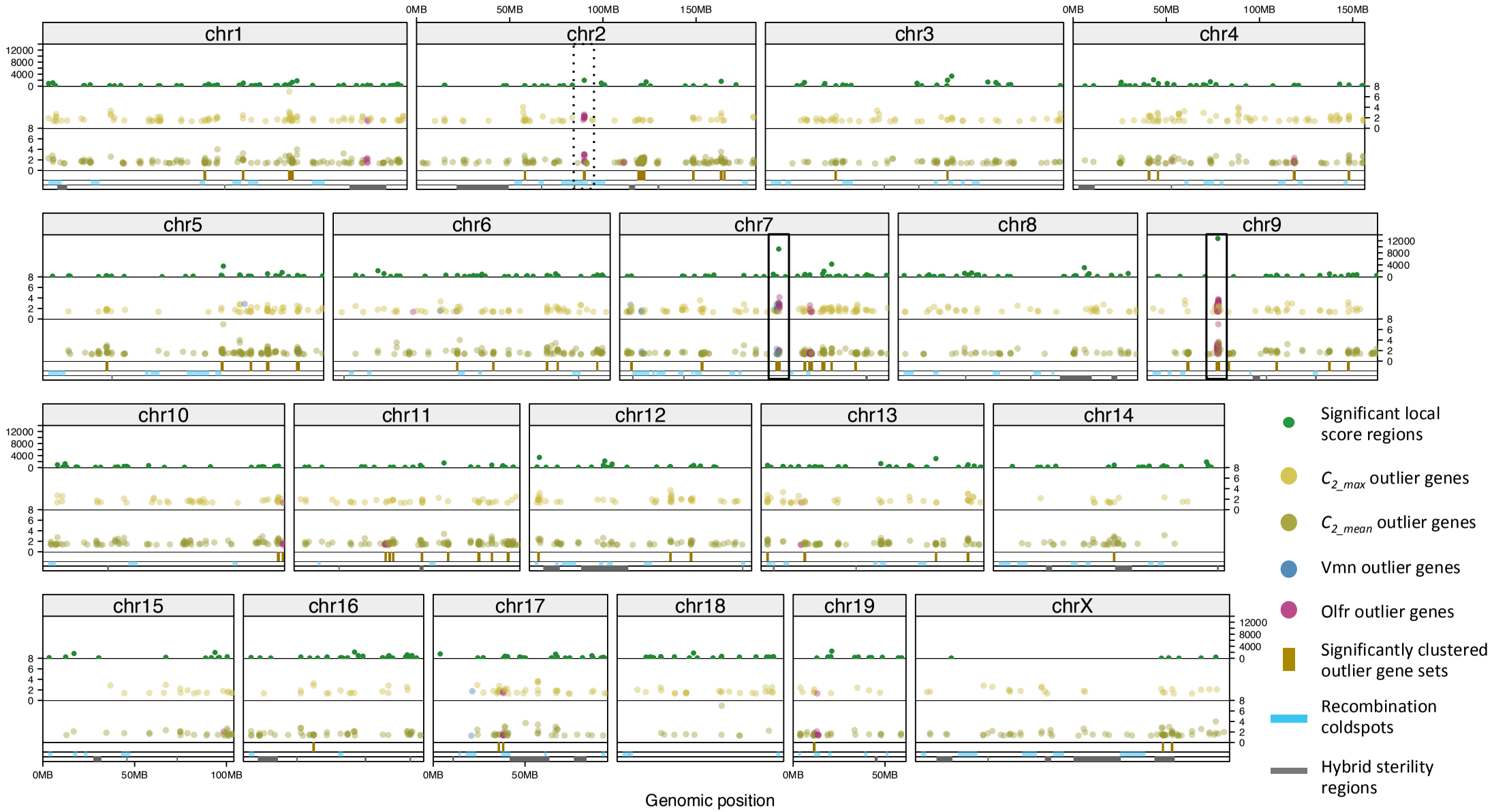


Variant consequence

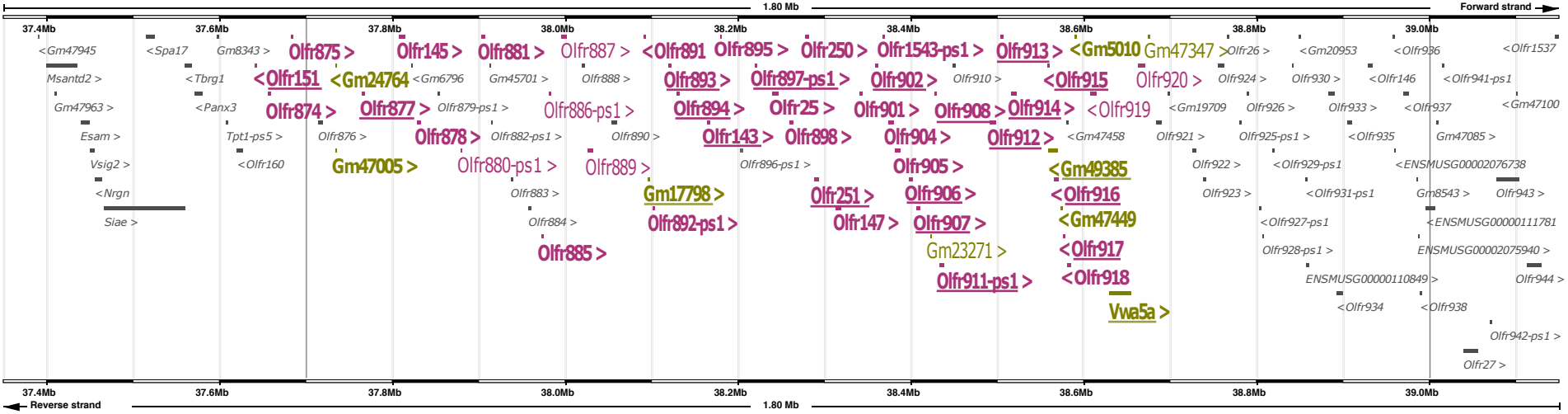
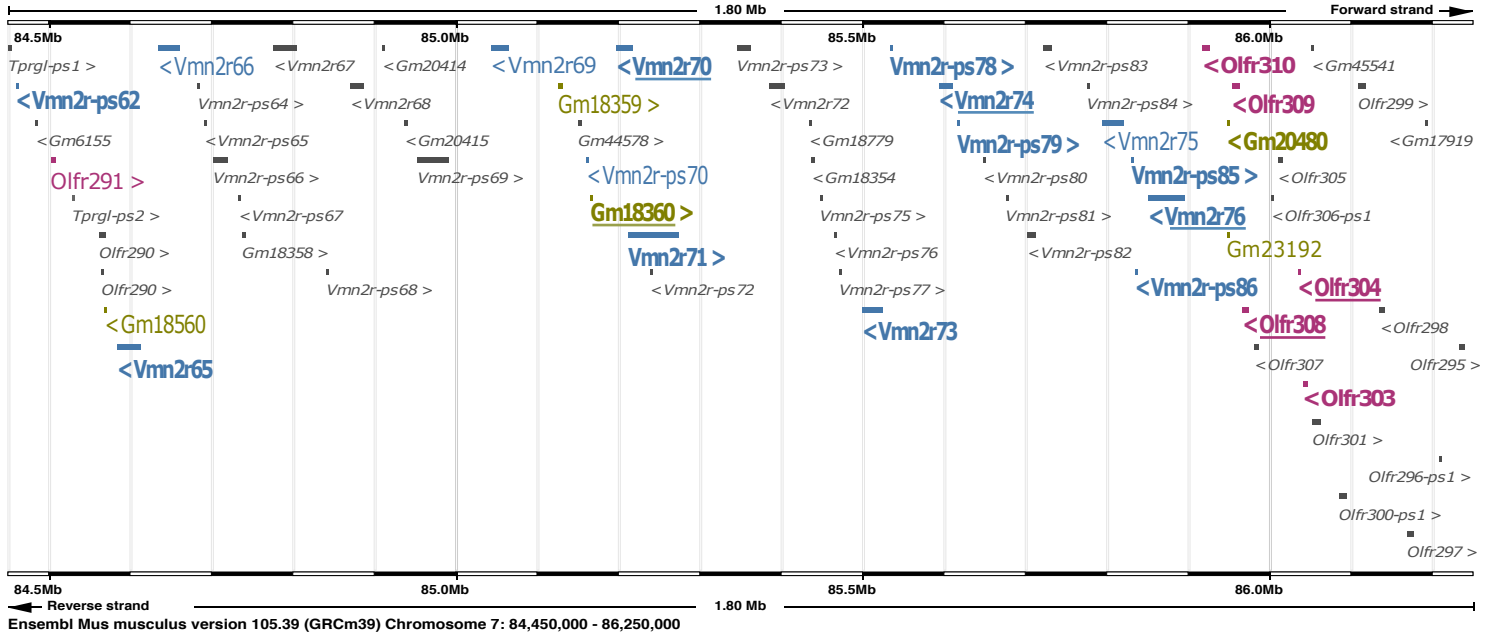
- intergenic_variant–13.05%
- upstream_gene_variant–15.31%
- downstream_gene_variant–12.44%
- regulatory_region_variant–5.16%
- 5_prime_UTR_variant,NMD_transcript_variant–0.01%
- 5_prime_UTR_variant–0.19%
- 3_prime_UTR_variant,NMD_transcript_variant–0.08%
- 3_prime_UTR_variant–1.68%
- intron_variant–36.70%
- intron_variant,NMD_transcript_variant–2.61%
- intron_variant,non_coding_transcript_variant–11.36%
- splice_region_variant,intron_variant–0.07%
- splice_region_variant,intron_variant,NMD_transcript_variant
- splice_region_variant,intron_variant,non_coding_transcript_
- non_coding_transcript_exon_variant–0.57%
- synonymous_variant–0.48%
- missense_variant–0.24%
- stop_gained–0.02%

B




chr1
chr2
chr3
chr4
chr5
chr6
chr7
chr8
chr9
chr10
chr11
chr12
chr13
chr14
chr15
chr16
chr17
chr18
chr19
chrX
0MB
50MB
100MB
150MB
Genomic position
Significant local score regions
C_{2_max} outlier genes
C_{2_mean} outlier genes
Vmn outlier genes
Olfr outlier genes
Significantly clustered outlier gene sets
Recombination coldspots
Hybrid sterility regions

Chromosome 9, Olfactory Receptor (*Olfr*) outlier gene cluster



Chromosome 7, Vomeronasal Receptor (*Vmn*) and Olfactory Receptor (*Olfr*) outlier gene cluster