Title

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

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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 complex 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 a complex behavioural trait: 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 can diverge rapidly through the action of direct and indirect selective effects (natural selection, sexual selection, reinforcing selection (M R 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 complex traits and identifying the signature of divergent selection acting on these underlying genomic regions remains 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 a complex behavioural trait: 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 preferred partner) have been evidenced in both sexes of both subspecies, though preference is stronger in *M. m. musculus* (Smadja and Ganem 2002; Smadja et al. 2004; Smadja and Ganem 2005; Ganem et al. 2008; Bimova et al. 2011; Latour et al. 2014).

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

To identify loci showing this expected pattern of divergence, we leveraged the high-quality reference genome available for this species (Waterston et al. 2002), produced whole-genome resequencing data, and finely characterised genomic divergence between Choosy and Non-Choosy populations within *M. m. musculus*, the subspecies where the contrast in behaviour is the strongest between these two types of populations (Smadja et al. 2004; Smadja and Ganem 2005; Ganem et al. 2008; Latour et al. 2014). To ensure the identification of genetic variation associated with the observed behavioural divergence, we combined multiple population comparisons (focal and control comparisons between groups of populations with or without contrasted behaviours), a replicated design within each group compared and robust outlier inference methods based on the recently implemented C_2 contrast statistic of the program BAYPASS which accounts for the possibly complex evolutionary history of the different populations (Gautier 2015; Olazcuaga et al. 2021) (Figure 1 and Methods).

We applied this combination of population comparisons for two types of outlier analyses at different genomic scales: SNP-based differentiation across the whole genome (based on the estimation of SNP-based C_2 contrast statistic), and gene-based differentiation across all annotated

genes. While most existing statistical methods for detecting outliers focus on analysing SNPs one-by-one, SNP-based analyses may have some limitations, among which a loss of power due to stringent correction applied to limit false positives in this large number of comparisons (millions of SNPs in a genome) and weak sensitivity to detecting multiple causal variants effects. Leveraging methods developed in the context of GWAS in humans (Neale and Sham 2004; Huang et al. 2011; Chung et al. 2019), we complemented SNP-based analyses with gene-based analyses to gain power in detecting outliers by aggregating disparate signals within a gene as well as to make more direct link of the outlier analyses with the phenotype of interest. To capture different potential underlying genetic architectures of adaptive divergence (single causal variant versus multiple weakly selected variants within a gene), we analysed two commonly used gene-based statistics representing the highest SNP-based differentiation value of a gene and the average differentiation value of a gene (Wang et al. 2017; Dickson et al. 2020), called respectively C_{2_max} and C_{2_mean} in our study.

Since functional expectations are also critical to further interpret genomic patterns of divergence and link phenotypic and genomic variation (Haasl and Payseur 2015; Ravinet et al. 2017; Wolf and Ellegren 2017), we additionally leveraged existing knowledge on the implication of olfaction in the expression of these assortative mate preferences in the house mouse to refine genomic differentiation analysis and interpretation. Previous behavioural assays and chemical analyses have shown that subspecies recognition and associated assortative mate preferences could be driven (at least partly) by olfactory cues present in the mouse urine (Smadja and Ganem 2007; Mucignat-Caretta et al. 2010; Hurst et al. 2017) and that urinary cues are sufficient to elicit mate choice between the two subspecies (Smadja and Ganem 2002; Smadja et al. 2004; Smadja and Ganem 2005; Ganem et al. 2008; Bimova et al. 2011). The olfactory system thus plays a major role in the expression of 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

148 this list of candidate genes to interrogate outlier regions and narrow down regions of interest.

149 Patterns of genomic differentiation between Choosy and Non-Choosy populations confirmed that
150 some regions of the genome show the patterns of divergence expected under selection. Among
151 those, a handful of olfactory and vomeronasal receptors showed genetic divergence associated
152 with the observed behavioural divergence between hybrid zone and allopatric populations. Our
153 study thus provides new insights into the role of olfactory and vomeronasal receptors in mammal
154 adaptation and speciation. It also offers a novel experimental framework to identify loci showing
155 the expected patterns of divergence under reinforcement, and confirms that combining
156 expectations on the genomic patterns of divergence with functional expectations is a promising
157 route to decipher the genetic architecture of behavioural trait variation.
158

Results

Sequence divergence between Choosy and Non-Choosy populations

Whole-genome resequencing of pools of individuals representing two Choosy and two Non-Choosy populations of *M. m. musculus* (see Figure 1a and Table S1 for a detailed sample description) identified a total of 28,500,265 SNPs in the dataset after stringent filtering. Genetic dissimilarity indexes estimated from the scaled genome-wide covariance matrix of the population allele frequencies (Ω matrix, produced by the program BAYPASS) indicated that the two Choosy populations do not cluster together (Figure 1b), a situation that makes the identification of outlier divergent loci in the Focal Test comparing Choosy and Non-Choosy populations (Figure 1c) conservative. A Control Test was performed to remove outlier loci from the Focal Test representing population-specific divergence and therefore not a consistent divergence between both Choosy versus both Non-Choosy populations (Figure 1c and Methods).

Outlier SNPs

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

Outlier genes

In the Focal Test, at the $P < 0.05$ threshold, we identified 999 C_{2_max} outlier genes (over 41,702 annotated genes included in this permutation test) (Table S4a) and 1756 C_{2_mean} outlier genes (over 39,598 annotated genes included in this analysis) (Table S4b). In the Control Test, comparable numbers of outlier genes were found: 1108 C_{2_max} outlier genes (Table S4c) and 1612 C_{2_mean} outlier genes (over 39,447 tested genes, among which 1472 genes with no permutation results due to insufficient SNP number or too large gene size) (Table S4d). However, the large majority of genes found significant in the Focal Test were not found significant in the Control Test: 77 genes had significant C_{2_max} in both the Focal and the Control Tests (considered as non-consistently

191 associated with the behavioural contrast); as a result, from the 999 C_{2_max} initial outlier genes in
192 the Focal Test, 922 were retained as the final C_{2_max} outlier genes; similarly, out of the 1756 initial
193 C_{2_mean} outlier genes, 1622 were retained as the final C_{2_mean} outlier genes after removing 134
194 outliers not consistently associated with the behavioural contrast. The union of these two cured
195 outlier gene sets, composing the final and complete list of outlier genes, included 2118 genes,
196 among which 496 were C_{2_max} specific outliers, 1196 were C_{2_mean} specific outliers and 426 were
197 C_{2_max} and C_{2_mean} outlier genes (Table S5).

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

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

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

210 Using the ENSEMBL Variant Effect Predictor (VEP), we found that the 4818 outlier SNPs identified as
211 significantly differentiated between Choosy and Non-Choosy populations include 1390 novel
212 variants and 3428 already known variants (71.1%). Those outlier SNPs overlap 977 annotated
213 genes, 2878 transcripts and 412 regulatory features (Table S6). Genic variants represent a majority
214 of those outlier SNPs (54.03%), with 50.76% of intronic variants and the rest corresponding to
215 exonic (1.31%) and UTRs (1.96%) variants (Table S6, Figure 2a). Coding consequences include 64%
216 of synonymous variants, 32% of missense variants and 3% of stop-gained variants (respectively
217 0.48%, 0.24% and 0.02% of the total, Figure 2a). Non-genic variants are slightly less represented
218 (45.97%) but include a large number of variants (32.91% of all outlier SNPs) potentially involved in
219 gene regulation: down- and upstream variants as well as already known regulatory region variants
220 (Table S6, Figure 2a). Only 13% of all outlier SNPs fall in intergenic regions. In comparison,
221 consequences predicted on all SNPs (and not only outlier SNPs) in the dataset show fewer variants
222 potentially involved in gene regulation (24%), fewer UTR variants (0.70%), fewer synonymous and
223 missense variants (0.37% and 0.19% respectively) and 10 times less stop-gained variants (0.0022%).

224 In contrast, they show more intergenic and intronic variants (16.15% and 57.62%, respectively).

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

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

244 A total of 127 Olfactory Receptor (*Olfr*) genes and 27 Vomeronasal Receptor (*Vmn*) genes were
245 identified as outlier genes (mostly on chromosomes 2, 7 and 9), and 55 *Olfr* and 13 *Vmn* are
246 among the highly significant ($p < 0.01$) outlier genes (Table 1 and Table S5). Consistent with DAVID
247 functional enrichment results, this corresponds to an over-representation of *Olfr* genes among the
248 complete list of outlier genes (hypergeometric test, $n_{\text{Olf outliers}} = 127$, $n_{\text{Olf all}} = 1356$; $n_{\text{genes outliers}} =$
249 2118; $n_{\text{genes all}} = 50942$, p-value = 1.90 e-17), and an over-representation of both *Olfr* genes (n_{Olf}
250 $_{\text{outliers}} = 55$, $n_{\text{Olf all}} = 1356$; $n_{\text{genes outliers}} = 479$; $n_{\text{genes all}} = 50942$, p-value = 1.37 e-19) and *Vmn* genes (n
251 $_{\text{Vmn outliers}} = 13$, $n_{\text{Vmn all}} = 543$; $n_{\text{genes outliers}} = 479$; $n_{\text{genes all}} = 50942$, p-value = 0.0021) among highly
252 significant outlier genes. Among Vomeronasal Receptor gene candidates, 10 belong to family 1 of
253 Vomeronasal Receptors and 17 to family 2 of Vomeronasal Receptors (Yang and Zhang 2007;
254 Zhang et al. 2007), the proportion of family 2 *Vmn* genes increasing when reducing the outlier
255 gene set to the most significant ones (number of family 1 *Vmn* genes = 1, number of family 2 *Vmn*
256 genes = 12) (Table 1 and Table S5). The majority of *Olfr* outlier genes are C_{2_mean} outliers while the

257 majority of *Vmn* outlier genes are C_{2_max} outliers (Table S5). Among the 'top' outlier genes defined
258 when combining information from the C_{2_mean} and C_{2_max} statistics, we found 23 *Olfr* and 4 *Vmn*
259 genes (Table 1 and Table S5).

260 Beyond the over-representation of Olfactory Receptor and Vomeronasal Receptor genes among
261 outlier gene sets, some of them appeared among the top most significantly differentiated outlier
262 genes: 10 *Olfr* and *Vmn* are among the 30 most differentiated C_{2_max} outlier genes (*Olfr304*,
263 *Olfr907*, *Olfr911-ps1*, *Olfr913*, *Olfr915*, *Olfr908*, *Olfr914*, *Olfr143*, *Olfr906* and *Vmn2r74*) and 8 *Olfr*
264 among the 30 most differentiated C_{2_mean} outlier genes (*Olfr906*, *Olfr908*, *Olfr914*, *Olfr907*, *Olfr143*,
265 *Olfr151*, *Olfr894* and *Olfr1265*) (Table S5). Five *Olfr* genes, all in a gene cluster on chromosome 9,
266 were common to these two lists and among the 10 most differentiated 'top' outlier genes: *Olfr906*,
267 *Olfr907*, *Olfr908*, *Olfr914* and *Olfr143,* with *Olfr906, Olfr907* and *Olfr908* among the 4 most
268 differentiated 'top' outliers (Table 1 and Table S5). Comparatively to *Olfr* genes, *Vmn* outlier genes
269 were less found in the very most significantly differentiated genes, but four of them were found in
270 the list of 'top' outlier genes: *Vmn2r74*, *Vmn2r76*, *Vmn2r70* and *Vmn2r12* (Table S5).

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

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

284 Genetic characteristics of regions associated with behavioural divergence

285 *Association with some other genomic features*

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

287 Genetic diversity *Π* did not differ significantly between outlier and non-outlier gene regions (Table
288 S8), indicating that there is no global reduction in genetic diversity in outlier gene regions. To

289 assess how recombination rate correlates with candidate outlier gene regions, we used
290 recombination rate estimates provided by Morgan et al. 2017. We found that recombination rates
291 did not differ significantly between outlier and non-outlier gene regions (Table S8). Of 2118,
292 196 outlier genes occurred in a recombination coldspot (Figure 3), which did not correspond to an
293 over-representation of outlier genes in coldspots of recombination (10,000 permutations, Z-score
294 = -0.379, p = 0.663, Figure S2).

295 *Genetic differentiation outliers do not show SFS-based signatures of selective sweeps*

296 To test whether differentiation-based outlier regions also show a signature of selective sweeps
297 estimated from SFS-based methods, we run the program POOL-HMM (Boitard et al. 2013) on the
298 two Choosy populations, the expected targets of reinforcing selection. We found 936 annotated
299 genes within which at least one significant selective sweep window was detected in the Choosy1-
300 Borum population, among which 28 C_2 outlier genes (including *Olfr291* on chromosome 7, and
301 *Olfr1328*, *Olfr1339*, *Olfr1340* and *Olfr1342* on chromosome 4) (Table S9a). In the Choosy2-Låsby
302 population, we found 1247 genes with at least one significant selective sweep window, among
303 which 31 C_2 outlier genes (including *Olfr32*, *Olfr1258* and *Olfr1270* on chr2, and *Olfr62*, *Olfr1339*,
304 *Olfr1340* and *Olfr1342* on chromosome 4) (Table S9b). Overall, we found 118 genes with
305 significant selective sweep windows in both Choosy populations (expected under a reinforcement
306 scenario), among which 3 C_2 outlier genes. Those three genes are *Olfr1339*, *Olfr1340* and *Olfr1342*.

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

309 We also tested whether outlier genes significantly co-localise with the 54 genomic regions
310 identified as candidates for hybrid sterility (postmating isolation candidates) between *M. m.*
311 *musculus* and *M. m. domesticus* (Turner and Harr 2014; Mukaj et al. 2020). We found that 143
312 outlier genes identified in our study overlap with candidate sterility regions (see Table S5 and
313 Figure 3), which is not more than expected by chance (10,000 permutations, Z-score = -2.686, p =
314 0.997; Figure S3a). However, we found that out of the nine highly supported candidate sterility
315 regions (i.e. showing concordant association with the two sterility phenotypes: relative testis
316 weight and testis expression, see (Turner and Harr 2014)), 8 contained at least one outlier gene.
317 The mean distance between outlier genes and sterility genomic regions was 23.70 Mb, which is
318 more distant than expected by chance (10,000 permutations, Z-score = 3.929, p = 0.0001, Figure
319 S3b). We note that PRDM9 gene, located on chromosome 17 and known as a major hybrid sterility
320 gene in the house mouse (Mukaj et al. 2020), is distant by ~ 5 Mb from the closest outlier gene
321 (*Vmn1r228*, see Table S5).

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

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

324 Data from 10-kb, 50-kb and 100-kb non-overlapping windows were examined, for each
325 chromosome separately, for autocorrelation in genetic differentiation associated with the
326 choosy/non-choosy trait, as measured by the BAYPASS C_2 contrast statistic. We found some
327 significant autocorrelation and partial autocorrelation between neighbouring windows (Figure S4),
328 with the autocorrelation parameter rho of a first-order autoregressive process estimated between
329 0.70 and 0.77 (e.g. 50-kb-windows, chr9: rho = 0.74, *p*-value = 0.039). These results indicated that
330 there is a long-range dependence in genetic differentiation measures (BAYPASS C_2 contrast statistic)
331 present over distances up to 100 kb. This dependence among C_2 measurements has a conservative
332 effect on the identification of outlier SNPs: using only FDR and not an adjusted α based on the
333 autocorrelation estimates, we corrected for an overinflated number of apparently independent
334 tests, making the *p*-value cut-off conservative. Given this autocorrelation affecting SNP-based C_2
335 measurements, we applied the local score approach (Fariello et al. 2017) to identify outlier
336 genomic segments cumulating signals from single SNP markers and characterize their distribution
337 along the genome. This analysis identified 557 significant local score genomic regions, distributed
338 on all chromosomes (Figure S5, Table S10 and Figure 3). Their mean size (+/- SE) is 99.139 kb (+/-
339 SE: 6.528 kb). Two regions stood out as the most significant ones: one 1-Mb long region on
340 chromosome 9 (peak value of the local score = 12769.03, coordinates: 9:37721717-38752579),
341 containing 52 outlier genes among which 40 outlier Olfactory Receptor genes (Table S5); one 0.8
342 Mb long region on chromosome 7 (peak value of the local score = 9260.49, coordinates:
343 7:85585560-86388933), containing 17 outlier genes, among which 10 Vomeronasal Receptor and
344 5 Olfactory Receptor outlier genes (Table S5).

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

347 Using the expected number of genes per 500 kb and assuming a random genome-wide
348 distribution, a permutation test showed that outlier genes were not homogeneously distributed
349 along the genome (p = 2e−05, 50,000 permutations) (Figure S6). We, therefore, analysed this
350 pattern of genomic clustering in more detail, by investigating whether some outlier gene sets were
351 more clustered in the genome than expected by chance. We identified 63 outlier gene sets found
352 significantly clustered in the genome (Table S11 and Figure 3). The most significantly clustered
353 outlier gene sets were found on chromosome 1 (cluster #3, p = 9.37E-14), chromosome 5 (cluster
354 #20, p = 7.31E-05), chromosome 9 (cluster #39, p = 7.31E-05; cluster #35 (*Olfr* cluster), p = 5.24E-

355 04), chromosome 11 (cluster #49, *p* = 5.24E-04), chromosome 12 (cluster #52, *p* = 5.24E-04) and
356 chromosome 7 (cluster #28 (*Vmn*+*Olfr* cluster), *p* = 2.97E-03). The mean size of those significant
357 outlier gene clusters was 919.88 kb +/- SE: 66.10 kb. The largest gene clusters were found on
358 chromosome 2 (cluster #6: 3.7 Mb), chromosome 1 (cluster #3: 2.45 Mb), chromosome 7 (cluster
359 #28 (*Vmn*+*Olfr* cluster): 2.3 Mb; cluster #30 (*Olfr* cluster): 1.95 Mb) and chromosome 9 (cluster
360 #35 (*Olfr* cluster): 1.8 Mb). These cluster sizes extend far beyond the estimated mean
361 autocorrelation effect on each chromosome, present over distances up to 100 kb. Figure 3
362 recapitulates the distribution of significant local score regions, outlier genes including *Olfr* and
363 *Vmn* ones, and significant outlier gene clusters along the genome.

364 By counting the total number of outlier and 'top' outlier genes and applying weights to this
365 content (see Table S11 for details), we could identify the gene clusters showing the strongest
366 degree of genetic differentiation between Choosy and Non-Choosy populations. Three gene
367 clusters stood out as the most differentiated ones: gene cluster #35 on chromosome 9, gene
368 cluster #28 on chromosome 7, and to some extent gene cluster #5 on chromosome 2 (Table S11).
369 Strikingly, these 3 gene clusters correspond to the three main olfactory/vomeronasal receptor
370 outlier gene clusters: cluster #35 contains 43 *Olfr* outlier genes; cluster #28 contains 14 *Vmn* and 6
371 *Olfr* outlier genes; cluster #5 contains 11 *Olfr* outlier genes. These three outlier gene clusters stand
372 out from genome-wide patterns of differentiation in Figure 3 and the two most important ones on
373 chromosomes 9 and 7 are detailed in Figure 4. Cluster #35 on chromosome 9 contains the five *Olfr*
374 outlier genes identified among the 10 most differentiated 'top' outlier genes (Table 1 and Table
375 S5).
376

377 ## Discussion

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

389 ### Population genomics and the genetics of complex trait divergence

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

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

409 differentiation, while confirming functional enrichment results obtained with gene-based analyses.
410 However, our study confirmed that genome-wide gene-based differentiation analyses are efficient
411 for gaining power and precision as well as for facilitating the link with the phenotype of interest
412 (Neale and Sham 2004; Huang et al. 2011; Chung et al. 2019). The identification of enriched
413 functional categories and the most promising individual genes was eased. Moreover, by capturing
414 different potential underlying genetic architectures of adaptive divergence (single causal variant
415 versus multiple weakly selected variants within a gene) (Wang et al. 2017), the two different gene-
416 based statistics (C_{2_max} and C_{2_mean}) provided two lists of outlier genes partially overlapping but also
417 complementary. For example, most Olfactory Receptor candidates are C_{2_mean} outlier genes, while
418 most Vomeronasal Receptor ones are C_{2_max} outlier genes.

419 The use of the C_2 contrast statistic of BAYPASS enabled us to assess genetic differentiation based on
420 contrasts of allele frequencies corrected for population structure (Olazcuaga et al. 2021). The
421 combination of biological replicates per behaviour group and multiple population comparisons
422 (Focal and Control tests) allowed us to identify the genomic regions consistently differentiated
423 between Choosy and Non-Choosy populations, beyond population-specific differentiation (Figure
424 1). These multi-way comparisons across populations had been advocated as a promising way
425 forward to separate processes acting similarly on all populations from processes that are specific
426 to a single population or population comparison (Wolf and Ellegren 2017) but are rarely
427 implemented (but see Roesti *et al.* 2014; Vijay *et al.* 2016). In our study, this procedure narrowed
428 down the list of genomic regions of interest, the control test removing respectively 26% and 7.65%
429 of the outlier SNPs and genes, with the final sets of outlier SNPs and genes eventually representing
430 0.017% and ~5 % of the total number of SNPs and annotated genes in the dataset. Yet, this
431 corresponds to a relatively large number of outlier SNPs and genes (4818 and 2118 respectively)
432 that show the expected patterns of genetic differentiation associated with behavioural divergence
433 and reinforcement.

434 Importantly, we do not expect all of these differentiated genomic regions between Choosy and
435 Non-Choosy populations to be involved in the observed behavioural divergence nor to be subject
436 to reinforcing selection. Because these sets of populations can differ in more than one trait and
437 selective regime, some differentiated regions may not affect the behaviour of interest. Moreover,
438 hitchhiking effects can induce genetic differentiation at variants in the vicinity of causal variants.
439 By selecting populations that are known to show distinct mating preferences despite being closely
440 related and inhabiting a similar environment (in Jutland Denmark, separated by no more than 50
441 km), we minimized the risk of confounding selective effects, but the list of outlier loci extends very
442 likely beyond the variants directly associated with the behavioural divergence. One key step of this

443 study was therefore to confront genome scan results with functional predictions, here available
444 from a series of previous studies that had characterised the behaviour of interest in detail (Smadja
445 and Ganem 2002; Smadja et al. 2004; Smadja and Ganem 2005; Ganem et al. 2008). Matching very
446 clear functional analyses' results (functional enrichment, nature of outlier genes), indicating a
447 prominent role of some olfactory and vomeronasal receptor genes, with information on the
448 involvement of olfactory-based processes in the expression of assortative mate preferences,
449 provides very strong support for considering these highly significantly differentiated olfactory and
450 vomeronasal receptor genes as the prime candidates potentially associated with behavioural
451 divergence and reinforcing selection (Table 1). Since our previous studies had shown that
452 individuals from Non-Choosy populations can discriminate between mating signals of the two
453 subspecies, but do not establish a directional mate choice (Smadja and Ganem 2005; Smadja and
454 Ganem 2008), the genomic regions identified in our study likely underlie the evolution of
455 choosiness rather than discrimination. Our study illustrates the importance of integrating detailed
456 phenotypic characterisation with genomic data to gain power and accuracy in population genomic
457 approaches that tackle the genetics of complex trait divergence.

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

459 Autocorrelation analyses indicated a long-range dependence in genetic differentiation measures
460 present over distances up to 100 kb, consistent with previous estimates of background linkage
461 disequilibrium in wild house mouse genomes (Laurie et al. 2007; Staubach et al. 2012) and that
462 could inflate the number of outlier loci (Hahn 2006). However, local score analyses (Fariello et al.
463 2017), which take into account this autocorrelation and background linkage disequilibrium,
464 consistently pointed to numerous outlier local score genomic segments spread across several
465 chromosomes (Figure 3). Whatever the criteria we applied (significance, consistency across
466 analyses and statistics) to stringently narrow down the list of candidate genes and genomic
467 regions, evidence supports a highly polygenic basis underlying the mate preference shift. From the
468 initial list of 2118 outlier genes containing 127 Olfactory receptor (*Olfr*) and 27 Vomeronasal
469 receptor (*Vmn*) genes, the most restricted set of 'top' outlier genes still include 95 genes spread
470 across several chromosomes and among which 23 are *Olfr* and 4 are *Vmn* genes. A highly
471 polygenic basis is expected for this complex olfactory-based behaviour and is in line with the few
472 studies addressing the genetics of variation in olfactory-mediated behaviours in insects (Swarup et
473 al. 2013; Arya et al. 2015) and mammals (Santos et al. 2018).

474 Given the highly polygenic nature of genetic architecture underlying the behavioural shift and the
475 apparent strong linkage disequilibrium among genes involved in this phenotypic divergence,

476 candidate loci are likely under polygenic selection, expected for such complex traits (Latta 1998;
477 Sella and Barton 2019). The fact that we did not find signatures of selective sweeps using SFS-
478 based methods in most candidate outlier regions may be another indication of polygenic
479 adaptation, known to be more detectable with pairwise tests for allelic differentiation (Stölting et
480 al. 2015; Schneider et al. 2021). This result may also suggest that those candidate regions are more
481 likely subject to soft sweeps than hard sweeps (Wang et al. unpublished data). Since outlier gene
482 regions are not associated with regions of reduced genetic diversity or reduced recombination
483 rate, a general effect of background selection inflating the levels of genetic differentiation in our
484 data seems unlikely. However, further analyses on individual data would be necessary to get fine-
485 scale estimates of recombination rate along these genomes. Interestingly, previous studies have
486 shown that polygenic adaptation can play a major role in the evolution of species-specific innate
487 behaviours through the modifications of pre-existing genetic networks (Anholt 2020) and in the
488 rapid evolution of chemosensory systems (Librado and Rozas 2016).

489 Another striking result is that outlier loci showed consistent signals clustered distributions in the
490 genome. While the signals of outlier SNPs via local score analyses accumulated in two main
491 genomic regions on chromosomes 9 and 7, clustering analyses of outlier genes consistently
492 identified numerous sets of genes significantly clustered in the genome, amongst which the two
493 most significant clusters pinpointing the same candidate regions on chromosomes 9 and 7. These
494 two clusters of outlier genes contain several candidate olfactory and/or vomeronasal receptor
495 genes and extend up to 1.8 Mb (Figure 4). These patterns suggest strong linkage disequilibrium in
496 these large candidate regions, potentially indicative of the strength of selection acting on its
497 targets or of complex correlations among loci belonging to the same gene regulatory network
498 (Fagny and Austerlitz 2021). Within these clusters, it is difficult to say which genes and nucleotides
499 are the direct targets of selection and pinpointing individual genes' contributions to the observed
500 behavioural shift in these genomic clusters will require additional experiments. Nevertheless,
501 some outlier genes are more significantly differentiated than others in those clusters and can be
502 considered prime candidates (*Vmn2r70*, *Vmn2r74*, *Vmn2r76*, *Olfr304* and *Olfr308* in the cluster on
503 chromosome 7; *Olfr143*, *Olfr906*, *Olfr907*, *Olfr908* and *Olfr914* in the cluster on chromosome 9).

504 The source of this genetic variation specific to the *M. m. musculus* Choosy populations is still
505 unknown. Considering our results, it may originate from standing variation present in allopatric
506 populations or differential introgression of *Mus musculus domesticus* alleles across the hybrid
507 zone. The large genomic clusters identified as outlier regions could fit the second scenario, which
508 echoes the recent evidence of adaptive introgression of OR and VR gene clusters across species
509 boundaries in the *Mus* genus (Baird et al. unpublished data; Ullrich et al. unpublished data; Banker

510 et al. 2022). This scenario would be compatible with the role of these genomic regions in
511 reproductive isolation with the nearby subspecies *M. m. domesticus*: the selective pressure of
512 reinforcement could have favoured the adaptive introgression of alleles conferring choosiness on
513 either side of the hybrid zone, the direction of the choice being expressed according to the
514 genomic background, including at the mating signal loci, of the individuals. It would correspond to
515 a case of one-allele mechanism (at the preference loci) and of a matching rule for assortative
516 mating, which is known to favour the evolution and maintenance of the barrier trait in the context
517 of gene flow (Kopp et al. 2018). Additional genomic data for *M. m. domesticus* populations from
518 the other side of the hybrid zone in Denmark would be needed to first test if variation comes from
519 introgression.

520 Overall, these results on the genetic architecture and selective regimes associated with the
521 divergence in mate preferences correspond to theoretical predictions for loci involved in
522 adaptation and reinforcement. Concentrated architectures of adaptive loci are expected in the
523 presence of gene flow (Yeaman and Whitlock 2011; Yeaman 2013), and fit with the few other
524 empirical studies testing for this pattern of clustering in natural systems (e.g. (Fustier et al. 2017;
525 Roda et al. 2017). Moreover, the selective regime of reinforcement corresponds to indirect
526 selection on prezygotic isolating traits in hybrid zone populations, usually from standing genetic
527 variation present in allopatry (Felsenstein 1981; Kirkpatrick and Servedio 1999) via linkage
528 disequilibrium between loci under direct selection (hybrid sterility genes) and assortative mating
529 loci. We did not find any significant co-localisation between known candidate hybrid sterility
530 genomic regions (Turner and Harr 2014) and outlier regions that could have facilitated the
531 maintenance of linkage disequilibrium among those co-adapted traits (mate preference and
532 hybrid sterility), but linkage disequilibrium can still evolve and be maintained between unlinked or
533 loosely linked and is not necessary under the scenario mentioned above of a case of one-allele
534 mechanism (Servedio 2009; Butlin and Smadja 2018; Hench et al. 2019). Our study, therefore,
535 provides an important addition to the rare studies that have investigated the genetics of
536 reinforcement (Ortiz-Barrientos et al. 2004; Hopkins and Rausher 2011; Ospina et al. 2021), by
537 testing predictions on expected genomic patterns of differentiation to characterize the genetic
538 basis of a complex premating isolating trait and the genomic signatures of reinforcing selection
539 acting on it.

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

541 Chemoreceptor genes are one of the most extensive and diverse multigene families in most
542 vertebrate and non-vertebrate genomes (Sánchez-Gracia et al. 2011; Ibarra-Soria, M. Levitin, et al.

543 2014; Benton 2015; Bear et al. 2016). Yet, their precise role in key evolutionary processes such as
544 adaptation and speciation remains largely unknown, although chemosensory processes have been
545 identified as key players at the phenotypic level (Smadja and Butlin 2009). Comparative analyses
546 have provided great insights into the role of OR or VR repertoire evolution in diversification and
547 ecological adaptations (e.g. dietary specialisation (Hayden et al. 2014; Yohe et al. 2021);
548 adaptation to different life-styles (Shi and Zhang 2007; Hayden et al. 2010; Khan et al. 2015; Jiao et
549 al. 2019). At microevolutionary scales, studies are scarce due to the difficulty of analysing these
550 gene families and linking their genetic variation to relevant adaptive phenotypes and selective
551 regimes (but see (Smadja et al. 2012; Li et al. 2015; Poelstra et al. 2018; Auer et al. 2020). Our
552 study provides one of the clearest and most detailed examples of the involvement of olfactory and
553 vomeronasal receptors in the evolution of an adaptive behavioural trait, assortative mate choice,
554 and how natural selection drives their genetic divergence.

555 Our results indicated the highly significant enrichment of this category of receptor genes among
556 outlier genomic regions (Figure 2B), in agreement with our functional prediction. Most Olfactory
557 and Vomeronasal receptor outlier genes belong to OR class II and VR type 2 gene superfamilies,
558 which can both be involved in odorant and pheromone recognition (Vargas-Barroso et al. 2017).
559 Some Olfactory Receptors, all on chromosome 9, are among the 10 most differentiated 'top'
560 outlier genes (*Olfr906*, *Olfr907*, *Olfr908*, *Olfr914* and *Olfr143*), with *Olfr906, Olfr907* and *Olfr908*
561 among the four most differentiated ones. Comparatively to Olfactory Receptors, Vomeronasal
562 Receptors are slightly less differentiated but we identified four of them among the 'top' outlier
563 genes: *Vmn2r74*, *Vmn2r76*, *Vmn2r70* and *Vmn2r12.* This indicates that divergence of these
564 categories of receptor genes concerns both differences in the number of receptors involved and
565 the degree of differentiation affecting some of them.

566 General variant effect predictor analyses pointed to the possible dual role of regulatory and
567 protein-coding changes in the evolution of behavioural divergence, with the presence of missense
568 and stop gained variants as well as variants in already known regulatory regions or putative
569 regulatory regions in downstream / upstream gene regions (Figure 2A). For candidate *Olfr* and
570 *Vmn* genes, many SNP variants fall within upstream or downstream gene regions, UTR regions or
571 intronic regions potentially affecting gene regulation, but we identified a few protein-coding
572 changes affecting *Olfr250*, *Olfr906*, *Olfr908*, *Olfr915*, *Olfr916* in the candidate cluster on
573 chromosome 9, and *Olfr304* and *Vmn2r76* in the cluster on chromosome 7 (Table 1). Consistent
574 with a putative role of regulatory changes are the results of two of our previous studies
575 investigating the expression and copy-number divergence between the same Choosy and Non-
576 Choosy populations. We found significant differential expression at some Vomeronasal Receptor

genes expressed in the vomeronasal organ (Loire et al. 2017), four out of five (*Vmn2r69*, *Vmn2r70*, *Vmn2r73*, *Vmn2r75*) being among the *Vmn* outlier genes identified in the present study and all belonging to the promising candidate gene cluster on chromosome 7. Similar investigations will be needed on the main olfactory epithelium to test for changes in expression affecting *Olfr* genes. We also identified deletions specific to the Choosy populations in upstream or intronic regions of some Olfactory Receptor genes (*Olfr907* in the candidate cluster on chromosome 9, *Olfr301*, in the candidate cluster on chromosome 7, *Olfr564* and *Olfr461* on chromosomes 7 and 6), that could affect their expression or the expression of nearby *Olfr* genes (North et al. 2020). The combination of nucleotide, structural and expression variation primarily at Olfactory and Vomeronasal Receptor genes could therefore shape the evolution of this complex behavioural trait under selection (Niepoth and Bendesky 2020; Fagny and Austerlitz 2021).

The Olfactory and Vomeronasal Receptor prime candidates are members of some specific phylogenetic clades of closely-related receptors within the respective gene superfamilies: prime candidate *Olfr* genes belong to clade 8 (chromosome 9), clade 4 (chromosome 2) and clades 6 and 14 (chromosome 7) of the OR class II superfamily, while prime candidate *Vmn* genes belong mostly to clade 3 (and one to clade 6) of the V2R gene superfamily (Chamero et al. 2012; Olender et al. 2020). Although the molecular, neurogenetic and physiological bases of olfaction are the focus of extensive research efforts in vertebrates, and in humans and mice in particular (Chamero et al. 2012; Hayden and Teeling 2014; Ibarra-Soria, M. Levitin, et al. 2014; Bear et al. 2016), very little is known about the functional characterisation of individual receptors. The mouse ORs identified in our study have not been functionally characterised (we just found that *Olfr913* is an ortholog to a musk receptor in humans (Ahmed et al. 2018) and is a eugenol-responsive receptor in mice (McClintock et al. 2014)). For VRs, most insights come from Isogai *et al.*'s study (Isogai et al. 2011). They showed that individual V2Rs are specifically tuned to detect the nature of the signal emitter (e.g., sex of conspecifics, heterospecifics, individual recognition), whereas individual V1Rs are activated by cues from multiple species. They also found that distinct mouse VR receptor subfamilies have evolved towards the specific recognition of certain animal groups (heterospecific signals from predators or sympatric closely related species/subspecies, male or female conspecific signals) or chemical structures (volatile steroids, non-volatile proteins). Interestingly, some members of V2R clades 3 and 6 (our main VR candidate clusters) were shown to be involved in the recognition of closely-related and sympatric species or conspecifics of the opposite sex, leading the authors to suggest that "through the activation of specialized receptors, *M. musculus* may readily discriminate scents emitted by closely related but reproductively incompatible species, a property that could be linked to the reproductive isolation of these species". Considering the

results of our study, and if we extrapolate Isogai's results to ORs, we can hypothesize a specialization of some VR and OR gene clusters towards the function of assortative mating in the hybrid zone. An interesting follow-up from our work would be to apply the methods developed by Isogai et al. to functionally characterize our candidate ORs and VRs in wild mice using stimuli from the two subspecies.

617 Material and Methods

618 **Samples**

619 We trapped *M. m. musculus* wild adult mice in Jutland, Denmark in several sites (indoor farms and
620 other human dwellings) and maintained them in the laboratory under controlled conditions before
621 being behaviourally tested and euthanized for dissection. Sampling sites represented two distinct
622 geographical areas in Jutland, which are characterised by populations with distinct mate
623 preference behaviours but sufficiently geographically close to avoid any geographical effect of
624 distant allopatry: (1) the border of the hybrid zone on the *M. m. musculus* side (50 km North to the
625 genetic centre of the hybrid zone (defined in Raufaste et al. 2005)) where strong assortative
626 mating was previously documented (Smadja et al. 2004; Smadja and Ganem 2005; Ganem et al.
627 2008) and confirmed on these newly sampled populations (Latour et al. 2014; Smadja et al. 2015)
628 ('Choosy' populations), (2) another area in Jutland but further north from the hybrid zone, where a
629 previous study did not find any significant directional mate preference in these sampled
630 populations ('Non-Choosy' populations) (Latour et al. 2014; Smadja et al. 2015).

631 Two populations per geographical area were included as biological replicates, each composed of
632 several trapping sites (four populations in total, hereafter called « Borum Choosy1 », « Låsby
633 Choosy2 », « Hobro Non-Choosy1 » and « Randers Non-Choosy2) (see Figure 1a and Table S1 for a
634 detailed sample description). For all individuals from each of the four populations n_{Borum} = 33, $n_{Låsby}$
635 = 27, n_{Hobro} = 29, $n_{Randers}$ = 29), spleen was extracted rapidly after death by cervical dislocation,
636 immersed in ethanol and stored at -80°C. We extracted genomic DNA from individual mouse
637 spleens, using the Macherey-Nagel kit standard protocol.

638 **Data generation and variant processing**

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

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

655 ***Mapping, SNP identification and filtering.*** We applied stringent and optimized mapping and SNP
656 filtering conditions to obtain accurate allele frequency estimations from pool-seq data (Gautier et
657 al. 2013; Schlötterer et al. 2014). The program Bowtie2 v2.2.3 (Langmead and Salzberg 2012) was
658 used to map the reads against the house mouse reference genome (http://www.ensembl.org/,
659 version GRCm38.p2), with the –very-sensitive-local option to get a stringent alignment and a
660 fragment size limit of 600 (-X parameter) to increase the number of concordant mapping count
661 and reduce the discordant mapping count. The Samtools suite v1.3.1 (Li et al. 2009) was used to
662 sort, index and filter for read duplicates the resulting BAM files. An overview of sequencing and
663 mapping statistics can be found in Table S2. BAM files were combined into mpileup files and *sync*
664 files (using samtools mpileup and mpileup2sync.pl script from Popoolation2 package (Kofler et al.
665 2011)) and first filtered based on base quality (min-qual 20). We filtered out *sync* files for indels
666 using Popoolation2, and developed a custom Python script to further filter bi-allelic SNP variants
667 based on minimum coverage (= 10X), maximum coverage (= 100X, to avoid repeated/duplicated
668 regions of the genome), and minimum count of minor allele (= 3). Minimum and maximum
669 coverage filters were applied per population, and a minimum allele count filter was applied across
670 all populations. From this set of filtered SNPs, we estimated mean population genetic diversity Π
671 (NPSTAT package v1, Ferretti et al. 2013) and pairwise-population F_{ST} (Pool-Seq estimator
672 implemented in the *poolfstat* R package, Hivert et al. 2018; Gautier et al. 2022) to describe the
673 populations under study (Figure 1a).

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

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

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

705 Custom scripts were used to format filtered *sync* files into BAYPASS input files. We run BAYPASS
706 separately for the autosomes and the X chromosome and specified the pool haploid sample sizes
707 to activate the Pool-Seq mode of BAYPASS. For the autosomes, the pool haploid sizes were simply
708 based on sample sizes ($n_{_aut_Borum\text{-}Choosy1}$ = 66; $n_{_aut_Låsby\text{-}Choosy2}$ = 54; $n_{_aut_Hobro\text{-}NonChoosy1}$ = 58; $n_{_aut_Randers\text{-}}$
709 $_{NonChoosy2}$ = 58). For the X chromosome, we adapted the haploid pool size information to the
710 effective sex ratio of each pool (Table S1; $n_{_chrX_Borum\text{-}Choosy1}$ = 48; $n_{_chrX_Låsby\text{-}Choosy2}$= 41; $n_{_chrX_Hobro\text{-}}$
711 $_{NonChoosy1}$ = 45; $n_{_chrX_Randers\text{-}NonChoosy2}$ = 43). We used the BAYPASS core model with default options for
712 the MCMC algorithm to obtain estimates of the scaled covariance matrix (Ω) and the C_2 contrast
713 statistic. Following the BAYPASS manual instructions, the autosomal dataset was divided into sub-
714 datasets of 110,905 SNPs (by taking one SNP every 250 SNPs along each chromosome) and three
715 independent runs (using option -seed) were performed to assess the reproducibility of the MCMC

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

723 ***Identification of outlier SNPs.*** For both the Focal and Control Tests, we computed SNP-based *p*-
724 values from the C_2 values, the C_2 statistic following a Chi-squared distribution with one degree of
725 freedom under the null hypothesis of no association (Olazcuaga et al. 2021). We identified outlier
726 SNPs in the Focal Test (Choosy versus Non-Choosy) after correcting for multiple testing (*FDR* <
727 0.05). Only outlier SNPs that were not significantly differentiated in the Control Test (*p*-values >
728 0.05 without correction for multiple testing) were retained for further analyses.

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

750 conditions: i) the same number of SNPs and ii) a length in base pairs not deviating from more than
751 10% of the observed one. For each fragment that satisfied these conditions (and thus mimicked
752 the genomic properties of the gene), we calculated C_{2_mean}. Repeating this process 10,000 for each
753 gene allowed us to estimate an empirical *p*-value for the observed gene-based C_{2_mean} statistic.
754 Significant genes (for C_{2_max} or C_{2_mean}) in each population comparison (Focal, Control) were
755 therefore those with *p-values* < 0.05 or 0.01 (see below).

756 Only outlier genes that were significantly differentiated in the Focal comparison (*p*-values < 0.05)
757 and not significantly differentiated in the Control comparison (*p*-values > 0.05) were retained. We
758 defined as the final and complete list of outlier genes the union of C_{2_max} and C_{2_mean} outlier genes
759 so that C_{2_max} specific and C_{2_mean} specific outlier genes are taken into account. All downstream
760 analyses were performed on this set of outlier genes (with two stringency categories: *p*-values <
761 0.05 or *p*-values < 0.01). However, we also defined a restricted list of outlier genes (hereafter
762 called 'top outlier genes'), identified as highly differentiated based on the combined information
763 from the two C_{2_max} and C_{2_mean} contrast statistics. To implement this classification, we first
764 assigned each outlier gene to a significance category for each gene-based statistic (1: most
765 significant; 5: least significant), assigned weights to these categories and calculated an overall
766 differentiation score as the sum of the weights obtained from C_{2_max} and C_{2_mean} information.
767 Genes were classified as 'top outlier genes' when showing a minimum overall differentiation score
768 of 10.

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

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

783 tests (*phyper* R function).

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

794 We tested for non-random co-localization of candidate outlier genes identified in this study with
795 (i) coldspots of recombination reported by Morgan et al. 2017 and (ii) candidate hybrid sterility
796 genomic regions between *M. m. musculus* and *M. m. domesticus* identified by Turner and Harr
797 2014. Candidate hybrid sterility regions correspond to the 53 GWAS genomic regions significantly
798 associated with relative weight testis variation (named RTW regions) and/or testis expression
799 variation (named PC regions) (Turner and Harr 2014). We added to this list of candidate genes the
800 gene *PRDM9* (chr17:15543079-15564354) known as a major hybrid sterility gene in the house
801 mouse. We counted the number of outlier genes overlapping these two genomic region sets
802 (coldspots of recombination and candidate sterility regions) and used permutations to assess
803 whether outlier genes overlap significantly more than by chance with these genomic elements
804 (outlier genes resampled 10,000 times among all annotated genes). To perform these permutation
805 tests, we used the bioconductor *RegioneR* (Gel et al. 2015) package in R version 3.6.2 (R Core
806 Team 2019). We also performed a Kruskall-Wallis test to investigate whether recombination rates
807 differ significantly between outlier and non-outlier gene regions.

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

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

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

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

840 **Data accessibility**

841 Raw mapping files (BAM files) and metadata have been uploaded to the Short Read Archive (SRA,
842 NCBI) under the bioproject ID PRJNA603262.
843

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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.

Competing interests

The authors have declared that no competing interests exist

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1233 Figure legends

1234 **Figure 1**: **Sampling and experimental design. A)** Populations of *Mus musculus musculus* sampled
1235 in localities in the *M. m. musculus - domesticus* hybrid zone (dark blue points) or further away
1236 from the centre of the hybrid zone (light blue points). Mean nucleotide diversity: Låsby (n=27, Π
1237 = 0.002 +/- 1.136e-05), Borum (n=33, Π = 0.003 +/- 1.151e-05), Randers (n=29, Π = 0.002 +/-
1238 1.027e-05), Hobro (n=29, Π = 0.002 +/- 1.159e-05); pairwise F_{ST} estimates between Choosy and
1239 Non-Choosy populations: $F_{ST\ \text{Borum-Choosy1}\ -\ \text{Randers-NonChoosy2}}$ = 0.057; $F_{ST\ \text{Låsby-Choosy2}\ -\ \text{Randers-NonChoosy2}}$ =
1240 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
1241 behavioural groups: $F_{ST\ \text{Borum-Choosy1}\ -\ \text{Låsby-Choosy2}}$ = 0.065; $F_{ST\ \text{Hobro-NonChoosy1}\ -\ \text{Randers-NonChoosy2}}$ = 0.076. **B)**
1242 Relatedness among populations based on the genome-wide covariance matrix (Ω) across
1243 population allele frequencies for autosomal chromosomes (similar results for the X chromosome).
1244 **C)** The two series of population comparisons were used to identify loci showing an association
1245 with behavioural divergence. The Focal Test compared replicated sets of Choosy and Non-Choosy
1246 populations to identify loci showing significant divergence (as measured by the contrast statistic C_2
1247 from the BAYPASS program) between populations with contrasting behaviour. The Control Test
1248 compared two other combinations of the same populations, this time not grouped by their
1249 behavioural profile. Only loci found divergent specifically in the Focal Test were retained as the
1250 final list of candidate loci.
1251

1252 **Figure 2**: **Functional analysis results. A)** Summary of Ensembl *VEP* analysis performed on all outlier
1253 SNPs. The pie chart shows the proportions of the different variant consequences found in this set
1254 of outlier SNPs. Consequences include information on the location and possible functional effect
1255 of each variant (e.g. intergenic, within 5k up-/down-stream of annotated genes, UTRs, intronic,
1256 exonic, synonymous, missense, stop_gained, as well as known regulatory regions). **B)** *DAVID*
1257 functional enrichment analyses. Up to top 10 functional annotation clusters (enrichment score >
1258 1.3) are shown for analyses performed on (i) genes found within 5 kb of outlier SNPs (ii) genes
1259 identified as outlier genes (C_{2_max} and/or C_{2_mean}) at 0.05 threshold, (iii) genes identified as outlier
1260 genes at 0.01 threshold.
1261

1262 **Figure 3**: **Genome-wide distribution of candidate regions showing significant genetic**
1263 **differentiation between Choosy and Non-Choosy populations.** Track 1 (top): chromosomes; Track
1264 2: local score values for significant genomic segments based on information on outlier SNPs;
1265 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
1266 genes shown respectively over 41,702 annotated genes); vomeronasal (*Vmn*) and olfactory (*Olfr*)

1267 outlier genes are shown in blue and purple respectively; Track 5: significant outlier gene clusters;
1268 Track 6: recombination cold spots observed in the Diversity Outbred hybrid mouse population by
1269 Morgan et al. 2017; Track 7 (bottom): candidate hybrid sterility genes identified by Turner & Harr
1270 2014. The three most significant outlier gene clusters are highlighted, on chr9, chr7 and to some
1271 extent chr2.
1272
1273 **Figure 4**: **The two main candidate gene clusters composed of Olfactory and Vomeronasal**
1274 **Receptor genes on chromosomes 9 and 7**. *Ensembl* protein-coding names and locations are
1275 shown within these two 1.8 Mb long genomic regions. Non-outlier genes are in grey and outlier
1276 genes are in colours (purple: Olfactory Receptor genes; blue: Vomeronasal Receptor genes; green:
1277 any other outlier genes). For outlier genes, different levels of significance are indicated as follows:
1278 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 $p < 0.01$;
1279 bold and underlined: top significant outlier genes, based on the combined information from C_{2_max}
1280 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

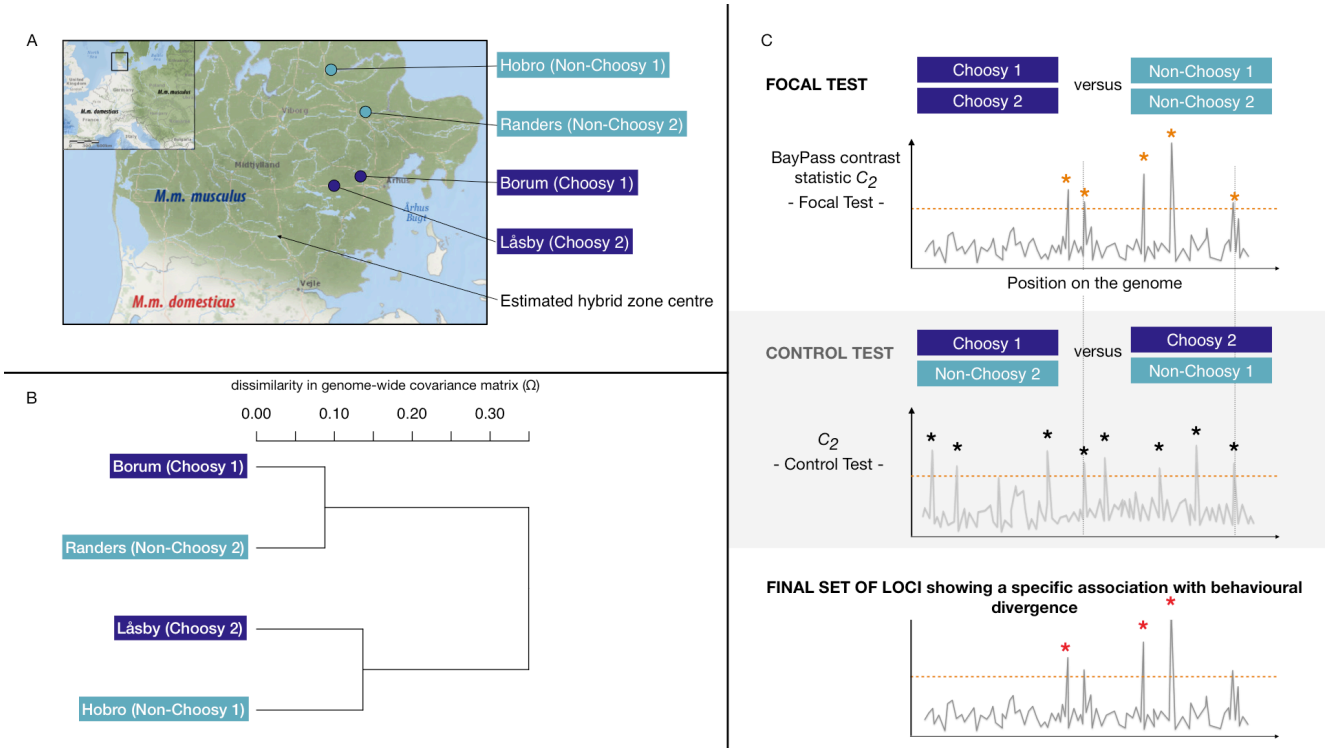


Figure 1: **Sampling and experimental design. A)** Populations of *Mus musculus musculus* sampled in localities in the *M. m. musculus - domesticus* hybrid zone (dark blue points) or further away from the centre of the hybrid zone (light blue points). Mean nucleotide diversity: Låsby (n=27, Π = 0.002 +/- 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 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. Only loci found divergent specifically in the Focal Test were retained as the final list of candidate loci.

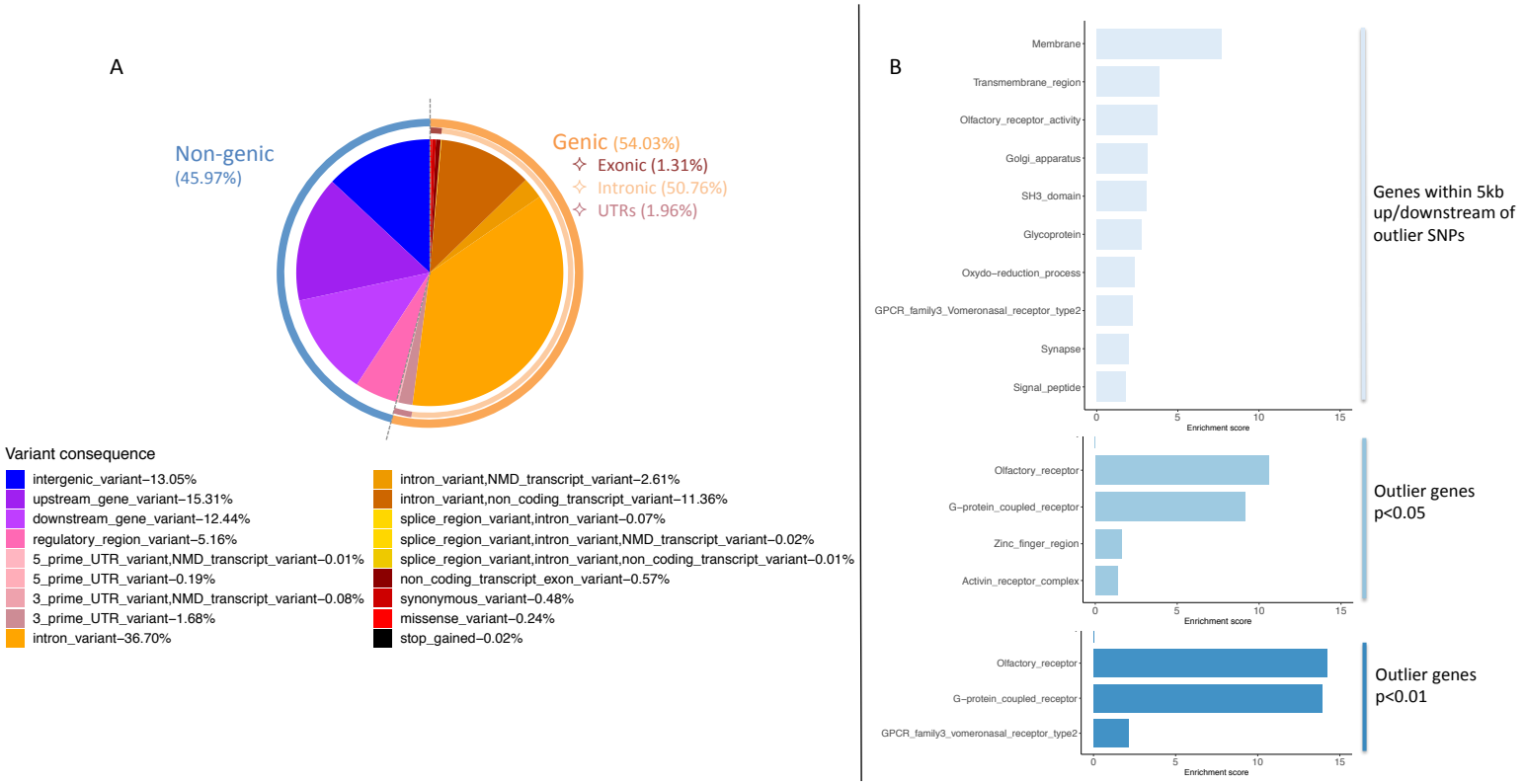


Figure 2: **Functional analysis results. A)** Summary of Ensembl *VEP* analysis performed on all outlier SNPs. The pie chart shows the proportions of the different variant consequences found in this set of outlier SNPs. Consequences include information on the location and possible functional effect of 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.

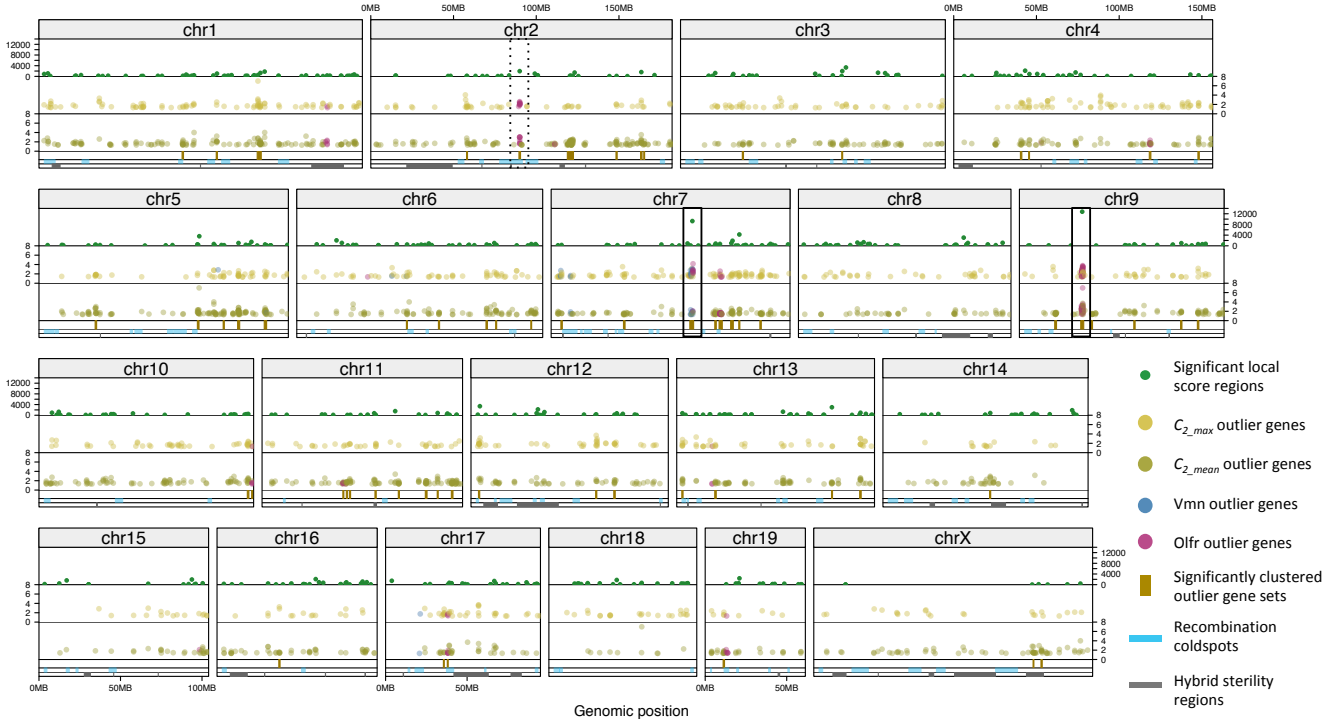
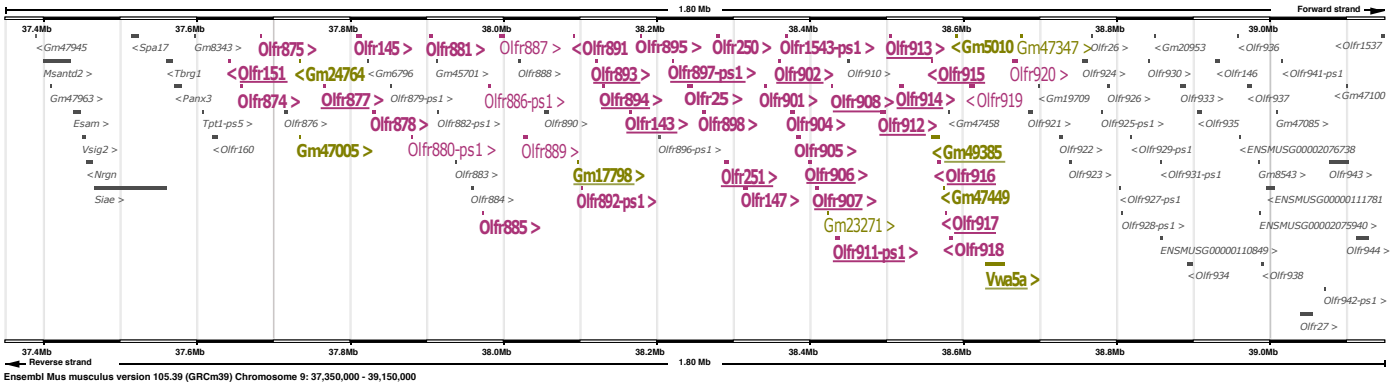


Figure 3: **Genome-wide distribution of candidate regions showing significant genetic differentiation between Choosy and Non-Choosy populations.** Track 1 (top): chromosomes; Track 2: local score values for significant genomic segments based on information on outlier SNPs; Tracks 3 & 4: C_{2_max} and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

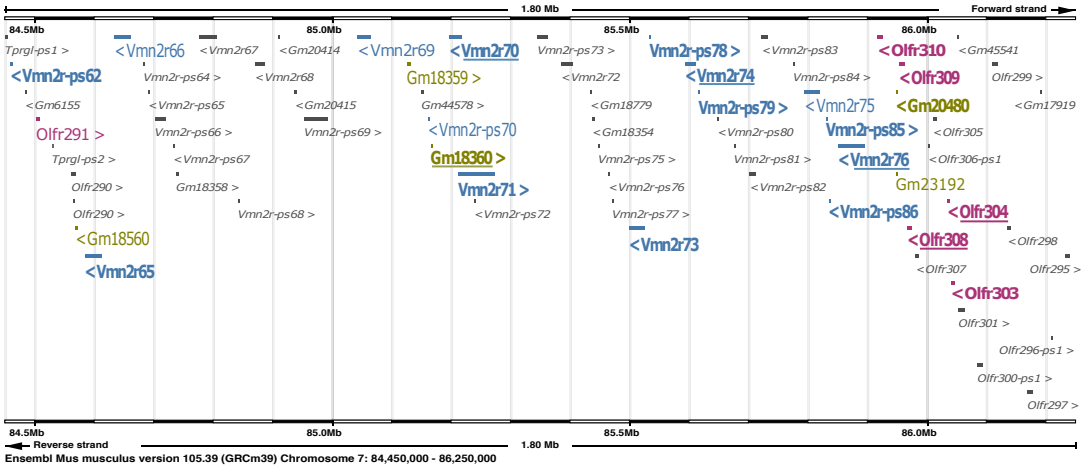


Figure 4: **The two main candidate gene clusters composed of Olfactory and Vomeronasal Receptor genes on chromosomes 9 and 7**. *Ensembl* protein-coding names and locations are shown within these two 1.8 Mb long genomic regions. Non-outlier genes are in grey and outlier genes are in colours (purple: Olfactory Receptor genes; blue: Vomeronasal Receptor genes; green: any other outlier genes). For outlier genes, 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.