Do changes in gene expression contribute to sexual isolation and reinforcement in the house mouse?

Etienne Loire, Sergio Tusso, Pierre Caminade, Dany Severac, Pierre Boursot, Guila Ganem, Carole Smadja

Title

Do changes in gene expression contribute to sexual isolation and reinforcement in the house mouse?

Etienne Loire [§,1,2], Sergio Tusso [§,1,4], Pierre Caminade [1], Dany Severac [3], Pierre Boursot [1], Guila Ganem [1] and Carole M. Smadja [1]

[§] These authors contributed equally

[1] Institut des Sciences de l'Evolution UMR 5554 (Centre National de la Recherche Scientifique CNRS, Institut pour la Recherche et le Développement IRD, EPHE, Université de Montpellier), cc065 Université de Montpellier, 34095 Montpellier, France

[2] Current address: CIRAD, UMR15 CMAEE, Campus international de Baillarguet, 34398 Montpellier Cedex 5, France

[3] MGX-Montpellier GenomiX; c/o Institut de Génomique Fonctionnelle; 141 rue de la cardonille; 34094 Montpellier Cedex 5; France

[4] Current address: Evolutionsbiologiskt Centrum, Norbyvägen 18D, University of Uppsala, 75236 Uppsala, Sweden

Corresponding author: Carole M. Smadja: carole.smadja@univ-montp2.fr

Running title: candidate genes for sexual isolation in mice

Keywords: assortative mate preference, speciation, hybrid zone, RNA-seq, olfaction, vomeronasal receptors

Abstract

Expression divergence, rather than sequence divergence, has been shown to be important in speciation, particularly in the early stages of divergence of traits involved in reproductive isolation. In the two European subspecies of house mice, *Mus musculus musculus* and *M. m. domesticus*, earlier studies have demonstrated olfactory-based assortative mate preference in populations close to their hybrid zone. It has been suggested that this behaviour evolved following the recent secondary contact between the two taxa (~3,000 years ago) in response to selection against hybridisation. To test for a role of changes in gene expression in the observed behavioural shift, we conducted a RNA-sequencing experiment on mouse vomeronasal organs. Key candidate genes for pheromone-based subspecies recognition, the vomeronasal receptors, are expressed in these organs. Overall patterns of gene expression varied significantly between samples from the two subspecies, with a large number of differentially expressed genes between the two taxa. In contrast, only ~200 genes were found repeatedly differentially expressed between populations within *M. m. musculus* that did, or did not displayassortative mate preferences (close to, or more distant from the hybrid zone, respectively), with an overrepresentation of genes belonging to vomeronasal receptor family 2. These receptors are known to play a key role in recognition of chemical cues that handle information about genetic identity. Interestingly, four out of five of these differentially expressed receptors belong to the same phylogenetic cluster, suggesting specialisation of a group of closely-related receptors in the recognition of odorant signals that may allow subspecies recognition and assortative mating.

Introduction

Understanding the genetic basis of speciation remains a key challenge in evolutionary biology (Seehausen *et al.* 2014), in particular determining the genetic architecture of differentiation and reproductive isolation (Nosil & Feder 2012), the types of genes involved (Nosil & Schluter, 2011), and the types of genetic changes responsible for the evolution of reproductive isolation between diverging taxa (Hoekstra & Coyne 2007). Although evidence is rapidly accumulating for the role of protein coding and structural changes in adaptation and reproductive isolation (Stapley *et al.* 2010), it has also long been argued that gene expression differences may be of prime importance in species differentiation (King & Wilson 1975; Carroll *et al.* 2005), allowing rapid divergence (Wolf *et al.* 2010). Moreover, measures of gene expression provide an important bridge between genotype and phenotype (Huestis & Marshall 2009). In recent years, the study of gene expression has been greatly facilitated by high-throughput sequencing-based methods such as RNA-seq (Mortazavi *et al.* 2008; Wang *et al.* 2009), contributing to understand the importance of expression variation in both local adaptation and speciation.

Gene expression changes and the evolution of reproductive isolation

There are many examples of differential gene expression causing adaptive phenotypic changes (e.g., Chan *et al.* 2010a; McBride *et al.* 2014), and large-scale transcriptomics studies have shown heritable expression differences between diverging populations (e.g., Wolf *et al*. 2010; Uebbing *et al*. 2016; Davidson & Balakrishnan, 2016). How these components of gene expression can be associated with reproductive isolation is still a largely unexplored question. The major advances on this point come from studies investigating the role of gene regulation in the evolution of postzygotic isolation and, in particular, how misexpression in hybrids can reflect Dobzhansky-Muller incompatibilities (e.g. Haerty & Singh, 2006; Ortiz-Barrientos, Counterman, & Noor, 2007; Dion-Cote, Renaut, Normandeau,

& Bernatchez, 2014; Davidson & Balakrishnan, 2016; Mack *et al*. 2016). A recent study also identified significant changes in expression associated with a postmating-prezygotic reproductive barrier between two *Solanum* plant species, that involve effector molecules possibly responsible for pollen tube arrest (Pease *et al.* 2016). As far as premating isolation is concerned, evidence is mostly found in phytophagous insect species that are reproductively isolated due to host plant preference and specialisation. For example, recent studies have shown expression differences between populations specialised on different plants, involving genes related to host selection (odorant receptors, salivary proteins, detoxification genes) (e.g. cactus-specialised populations of *Drosophila mettleri*: Hoang, Matzkin, & Bono, 2015; *Rhagoletis* host races: Ragland *et al*. 2015; pea aphid host races: Eyres *et al.* 2016). These differences in expression have therefore been proposed to play a key role for adaptive genetic divergence in traits causing reproductive isolation during ecological speciation (Pavey, Collin, Nosil, & Rogers, 2010; Etges, 2014). Despite growing evidence for the role of gene regulation in mate choice and sexual selection (Wilkinson *et al.* 2015), support for expression changes contributing to sexual isolation is relatively scarce, and again concerns insect species. Examples include sex-specific gene expression changes underlying shifts in sex-pheromone preferences between populations of the mosquito *Anopheles gambiae* (Cassone *et al.* 2008), the silkmoth *Bombyx mori* (Fujii *et al.* 2011) and *D. melanogaster* (Bailey *et al.* 2011), and a change in expression of a fatty acid synthase responsible for cuticular hydrocarbon production inducing sexual isolation between *D. serrata* and *D. birchii* (Chung *et al.* 2014).

Gene expression and the evolution of assortative mate preference in a house mouse hybrid zone

In this study, we investigate the role of expression divergence in the evolution of olfactory-based assortative mate preference between two subspecies of the house mouse (*Mus*

musculus) in order to gain insights into the genetic basis of sexual isolation in this mammalian system. The two European subspecies of the house mouse, *M. m. musculus* and *M. m. domesticus* have diverged in allopatry for 0.5 million years in the Indian sub-continent (Boursot *et al.* 1996), before meeting secondarily in Europe around 5,000 years ago (and around 3,000 years ago in Denmark) (Cucchi *et al.* 2012). They now form a hybrid zone stretching from Norway to the Black Sea. Assortative mate preference between the two subspecies has been demonstrated in populations at the edges of the hybrid zone in Denmark in both subspecies, and in both males and females, but appears particularly strong in *M. m. musculus* populations (Smadja & Ganem 2002, 2005; Smadja *et al.* 2004a; Ganem *et al.* 2008). These results suggest a degree of sexual isolation between the two taxa. In contrast, populations of both subspecies occurring in distant allopatry or slightly further away from the Danish hybrid zone do not display any directional mate preference (Smadja & Ganem 2005; Smadja *et al.* 2015). This pattern of reproductive character displacement has been confirmed in the Czech part of the hybrid zone (Bimova *et al.* 2011). Since selection against hybrids is acting in the mouse hybrid zone (reduced hybrid fertility: Britton-Davidian *et al*. 2005; Albrechtova *et al*. 2012; Turner *et al*. 2012; Bhattacharyya *et al*. 2013; Turner & Harr 2014; sexual selection against hybrids: Latour *et al.* 2014; hybrid microbiome dysfunction: Wang *et al.* 2015) a reinforcement process has been hypothesised in this system, by which premating barriers could have evolved as a response to selection against hybrids (Bimova *et al*. 2011; Smadja & Ganem, 2005; Smadja *et al*. 2015).

A current challenge is to determine the genetic basis of assortative mate preferences between the two subspecies and of the behavioural shift within each subspecies between populations distant from or close to the hybrid zone. Previous studies have shown that chemical signals present in urine are sufficient to elicit discrimination and assortative mate preferences between the two subspecies (Smadja & Ganem 2002, 2005; Smadja *et al.*

2004a; Ganem *et al.* 2008; Bimova *et al.* 2011), suggesting a role of the olfactory system in this behavioural shift. In mammals, two organs are responsible for odorant signal recognition: the main olfactory epithelium (MOE), primarily involved in recognition of airborne chemicals present in the environment (odorants and pheromones), and the accessory olfactory organ or vomeronasal organ (VNO), known to play a key role in both volatile and non-volatile kairomone and pheromone recognition (Isogai *et al*. 2011; Chamero, Leinders-Zufall, & Zufall, 2012; Liberles, 2014; Bear *et al.* 2016). Recognition in these organs involves chemoreceptors (olfactory receptors (ORs) in the MOE and vomeronasal receptors (VRs) in the VNO) that are organised in very large multigene families in the mouse genome (more than 1,200 ORs and 530 VRs, Ibarra-Soria, Levitin, & Logan, 2013; Ibarra-Soria, Levitin, Saraiva, & Logan, 2014; Zhang & Firestein, 2002). Although duplications, mutations, and adaptive evolution certainly play an important role in the evolution of chemoreceptors and their associated behaviours (Horth, 2007; Karn *et al.* 2010; Smadja *et al*. 2015), variation in expression at these genes can also strongly influence mouse behaviour (Keller *et al.* 2009; Zhang *et al.* 2010; Bear *et al.* 2016). A recent study has demonstrated that changes in expression patterns of vomeronasal receptors can induce changes in patterns of mate preference between laboratory strains of the house mouse (Broad & Keverne 2012), confirming the key role of the vomeronasal organ and its receptors in the recognition and transduction of olfactory signals used in mate choice, as well as the possible link between variation in VR expression and variation in mate preference in mice.

Predictions and experimental design

We hypothesized that the observed behavioural divergence between populations close to the hybrid zone and displaying strong assortative mate preferences (hereafter 'Choosy' populations) and populations further away from the hybrid zone and not displaying any directional mate preferences (hereafter 'Non-Choosy' populations) could originate from

153 divergence in expression of some vomeronasal receptors. To test this hypothesis, we
154 conducted deep RNA-sequencing of whole vomeronasal organs, an approach that has
155 already been validated for detection of expression variation at VRs (Ibarra-Soria *et al.* 2014).
156 We compared adult female and male mice originating from different 'Non-Choosy' and
157 'Choosy' populations of *M. m. musculus* and *M. m. domesticus*. Estimates of differential
158 expression allowed us to identify specific sets of vomeronasal receptors and quantify
159 differences in receptor expression potentially related to the shift toward assortative mate
160 preference in the hybrid zone.
161

MATERIAL AND METHODS

Samples

Mice used in the study

Patterns of gene expression were studied in adult mice that were born in the wild, trapped in Jutland, Denmark in October 2010 in several sites (indoor farms and other human dwellings) and then maintained in the laboratory under controlled conditions before being behaviourally tested and euthanised for dissection. As it is known that postnatal odour exposure can influence patterns of mate choice and VNO gene expression of adult mice (Broad & Keverne 2012; Chamero *et al.* 2012; Cadiou *et al.* 2014), this experimental strategy allowed us to study behavioural and expression patterns characterising wild animals, while controlling for some factors (odorant environment, age range, diet) just before dissection that could inflate variance in expression among samples.

Mice were trapped in three distinct sampling areas, characterised by populations with distinct mate preference behaviours (Figure S1): (1) the border of the hybrid zone on the *M. m. musculus* side (50 km North to the genetic centre of hybrid zone (defined in Raufaste *et al.* 2005)) where strong assortative mating has been documented ('*musculus* Choosy' samples) (Smadja *et al.* 2004b, 2015; Smadja & Ganem 2005; Ganem *et al.* 2008), (2) another area in Denmark but further away from the hybrid zone, where mate preference was not assortative ('*musculus* Non-Choosy' samples) (Smadja *et al.* 2015); and (3) the border of the hybrid zone on the *M. m. domesticus* side (50 km South to the genetic centre of the hybrid zone), where weak assortative mating was documented ('*domesticus*' Choosy samples) (Smadja & Ganem 2005; Ganem *et al.* 2008; Latour *et al.* 2014). The *M. m. domesticus* samples were used as a control for the mapping of *M. m. musculus* reads against the house mouse reference genome, which is primarily of *M. m. domesticus* origin, and for

188 interspecific comparisons. We decided to focus on the *M. m. musculus* side for comparisons
189 between 'Choosy' and 'Non-Choosy' populations, because the strongest behavioural
190 contrasts were observed there (Smadja *et al.* 2004b; Smadja & Ganem 2005; Ganem *et al.*
191 2008). Two populations per geographical area were included as biological replicates, each
192 composed of several trapping sites (six populations in total).
193
194 Mice were brought back to the laboratory in Montpellier (France) and maintained in
195 quarantine under controlled and homogenized conditions for two months (same facility
196 room, odorant environment, 12/12 photoperiod, and diet - food ad libitum). Only mice
197 trapped as young adults were selected for this study, so that the ages of individuals
198 participating to the behavioural and expression analyses were likely to have been within the
199 age range at which VR expression in the VNO is optimal (between 90 and 120 days old,
200 Zhang *et al.* 2010). During the period in the laboratory, mice were tested for sex odour
201 preferences to confirm assortative preferences in the '*musculus* Choosy' group and an
202 absence of directional preference in the '*musculus* Non Choosy' group (protocol and results
203 reported in Smadja *et al*. 2015). We also confirmed weak assortative mate preferences in
204 the '*domesticus* Choosy' group (Latour *et al.* 2014). We then selected eight males and eight
205 females per population, resulting in a total of 96 adult male and female mice taking part to
206 the RNA-seq experiment. The average mate preference of RNA-seq samples did not deviate
207 from the average mate preference assessed in larger population samples. Geographical,
208 genetic and behavioural characteristics of the mice used in this study are summarised in
209 Table S1 (the method used to estimate hybridisation index of each mouse is described in
210 detail in Latour *et al*. 2014 and Smadja *et al*. 2015 and summarised in Table S1).
211
212 *Vomeronasal organ dissection and RNA extraction*

For each of the 96 female and male mice, the VNO was dissected rapidly after death by cervical dislocation. The VNO was immediately immersed in RNAlater and stored at -80C. Total RNA was extracted using Macherey-Nagel XS kits or the phenol-chloroform based TRIzol procedure. RNA extracts were subsequently quantified and checked for quality with Nanodrop to assess sample concentration and purity and with Agilent Bioanalyzer to assess sample integrity. To constitute samples representative of each of the six populations and sexes, and to obtain enough material for library preparation, equimolar mixes from the eight males or eight females per population were pooled and again checked for quantity and quality using the Agilent Bioanalyzer and Nanodrop, to obtain 3 to 5 µg per pool (200ng/µL).

Trapping and transport of mice were performed under permission from the French Ministry of Agriculture (authorization n°C34-265). Housing took place in the facilities of the University of Montpellier and followed the ethical and animal welfare guidelines of the University of Montpellier and the regional ethical commitee.

Transcriptome sequencing and raw data analysis

Library preparation and sequencing

RNA-seq libraries were prepared using the TruSeq RNA sample preparation kit (Illumina) and quantified using Agilent DNA 1000 Labchip kits (Agilent Technologies, Palo Alto, CA). The libraries were then sequenced on an Illumina HiSeq 2000 platform using a paired-end 100bp read-length protocol (3 libraries per lane, 4 lanes in total). Library preparation and sequencing were carried out at the MGX -Montpellier Genomix platform (Institut de Génomique Fonctionnelle- Institut de Génétique Humaine, Montpellier, France).

Filtering and mapping

Raw reads were filtered in order to discard reads with low quality sequence. The remaining

reads were trimmed to subtract primer sequences and low quality 3' ends using *cutadapt* (Martin 2011). The package *RSEM* (Li & Dewey 2011), with default mapping parameters, was used to map the reads against the house mouse reference genome and its known transcript annotation database (http://www.ensembl.org/, version GRCm38.p2) and to estimate gene expression abundance. Since the reference genome used for mapping corresponds to an inbred strain of the laboratory mouse (C57BL/6J) (primarily of *M. m. domesticus* origin), we compared the distribution of expression estimations between wild *M. m. domesticus* and *M. m. musculus* samples to detect any potential mapping biases among samples.

Identification of Vomeronasal Receptor and Olfactory Receptor transcripts

Coding sequences belonging to family 1 (V1R) and family 2 (V2R) of vomeronasal receptors (Yang & Zhang 2007; Zhang *et al.* 2007) as well as coding sequences of ORs were extracted with the BioMart tool from Ensembl (http://www.ensembl.org/). Information on chromosome location, genomic coordinates and strand for each gene was according to the NCBI-Build-37 mouse assembly (mm9).

Expression analyses

To compare the amount of gene expression among different classes of genes (e.g. VR genes versus non-VR genes), we normalised coverage values using the Bioconductor package *CQN* (conditional quantile normalization) (version 1.2.0) (Hansen *et al.* 2012), which accounts for possible bias due to library size, gene length and GC content. For comparisons of gene expression patterns among samples and differential expression analyses, read counts were normalised using the Bioconductor package *edgeR* (version 2.13) (McCarthy et al. 2012) implemented for the software *R* v.3.0.1 (http://www.r-project.org/) and the method of trimmed mean of M values (TMM) (Robinson *et al.* 2010), which normalises for the library size and for the total number of expressed transcripts in each sample. A gene was

265 considered as expressed in one sample if the number of reads was higher than 1 count per
266 million (CPM).
267
268 Multidimensional scaling (MDS) plots were generated from the 500 genes maximizing the
269 distances between points, to visualise similarity of global gene expression between samples.
270 Coverage at the gene level was obtained by summing up transcript coverage values
271 associated with a given gene. To assess whether global patterns of gene expression vary
272 between subspecies, mate preference profile groups ('*musculus* Choosy' versus '*musculus*
273 Non-Choosy') and sexes, we first verified equal dispersions among samples and then
274 performed permutation-based MANOVA analyses (Anderson 2001) using the PERMDISP and
275 PERMANOVA programs of the Vegan package *Adonis* (Dixon 2003) with 1000 permutations.
276 Two models were used: the effects of the explanatory variables "Subspecies" and "Sex"
277 were tested on the whole dataset (counts ~ Subspecies + Sex + Subspecies*Sex) and the
278 effects of the explanatory variables "Choosiness" (two levels: Choosy and Non-Choosy) and
279 "Sex" were tested on *M. m. musculus* samples only (counts ~ Choosiness + Sex +
280 Choosiness*Sex). All explanatory variables were defined as fixed factors in our models.
281
282 Differential expression analyses were conducted using *edgeR* (version 2.13) (McCarthy *et al*.
283 2012). The analyses compared the two subspecies ('d*omesticus* Choosy' versus '*musculus*
284 Choosy' samples) or the two groups of *M. m. musculus* mice showing distinct behavioural
285 profiles ('*musculus* Choosy' versus '*musculus* Non-Choosy'). Since males and females display
286 similar patterns of mate preference within each population (e.g., Smadja *et al.* 2004b, 2015;
287 Smadja & Ganem 2005), we considered four biological replicates per categorial group (two
288 sexes x two populations). Only genes expressed in all samples but one of the same
289 categorical group were included, leading to a different number of genes considered for each
290 comparison. Because simulation analyses have shown the tendency of *edgeR* to identify a

high number of false differentially expressed genes (Soneson & Delorenzi 2013), the significance level was reduced to 0.01. Additionally, a false discovery rate (FDR) method was used to correct the threshold of *P*-values for multiple testing (Benjamini & Yekutieli 2001). The outcome of this analysis was a list of up- and down-regulated differentially expressed (DE) genes.

Enrichment analyses

Functional annotation of the significantly differentially expressed genes was performed using the functional classification program *DAVID* (Version 6.8) (Huang *et al.* 2009). Enrichment analyses were performed for DE genes between the two subspecies and for DE genes between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus*. Functional categories were considered significantly enriched when FDR **<** 0.05 and we analysed functional annotation clusters, which group similar functional categories together, to identify enriched biological functions. In the case of VR genes, we ran an independent hyper-geometric test, as some of the genes are not annotated as VR genes in the database. In all cases, the reference background genome included only expressed genes for each comparison, instead of using the whole reference mouse genome.

Phylogenetic and clustering analyses

To map DE genes onto the phylogenies of vomeronasal receptor gene family 1 (V1R) and 2 (V2R), we first reconstructed these gene phylogenies using available coding sequences of V1R and V2R (191 and 110 sequences respectively). Sequences were aligned using *MACSE* (Multiple Alignment of Coding Sequences; Ranwez *et al.* 2011), with manual adjustments. *jModelTest* v0.1.1 (Posada 2008) identified GTR+G+I (General Time Reversible + Gamma + Invariable sites) as the model of sequence evolution that best described the observed pattern of sequence variation for both V1R and V2R, and this model was used to perform

317 maximum likelihood phylogenetic analyses using *RAxML* version 8 (Stamatakis 2014). We
318 then reported positions of DE genes identified previously between the two subspecies and
319 between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus* on the phylogenetic
320 trees of the V1R and V2R families.
321
322 Since the phylogenetic analyses indicated a close relationship among five V2R genes that
323 were found to be differentially expressed between the 'Choosy' and 'Non-Choosy' samples
324 within *M. m. musculus*, we assessed the probability of phylogenetic clustering using a
325 randomisation procedure. We randomly sampled five receptor genes in the phylogenetic
326 tree of vomeronasal receptor family 2 and computed the total genetic distance among them
327 using the *ape* R package (Paradis *et al.* 2004). We performed this random sampling 500,000
328 times to obtain an expected distribution of genetic distance. The observed value of genetic
329 distance among the five DE V2R genes was then compared to this distribution.
330
331 Since all VR genes belong to only two different gene families, they share a significant amount
332 of genetic identity. Since mapping was based on sequence identity, we were concerned that
333 reads mapping to one truly differentially expressed gene may also map to other VR paralogs
334 with close sequence identity, leading to false positive results in the clustering analysis.
335 Therefore, for each VR gene found to be differentially expressed in the '*musculus* Choosy'
336 versus '*musculus* Non-Choosy' comparison, we retrieved the 10 closest paralogs with the
337 program *cd-hit-est* (http://weizhong-lab.ucsd.edu/cd-hit/). Sequences were aligned using
338 the program *MACSE* (Ranwez *et al.* 2011) and positions that were specific to the
339 differentially expressed genes were recorded. Then, using mapping information, we
340 retrieved the coverage at each position for each of the DE genes in the relevant samples,
341 and checked that positions specific to individual genes were covered at the same level as
342 shared positions.

RESULTS

Variation in gene expression among samples

All samples were sequenced successfully, except one from the '*Musculus* Non-Choosy' group ('*Musculus* Non-Choosy population2 Females', Table S1) due to failure in library construction. For the eleven sequenced samples, the percentage of mapped reads per sample was between 79% and 85%, and the total number of transcripts per sample varied from 33457 to 39770 (Table S2). Table S3 reports the raw and normalised counts for all genes and all samples.

The MDS plots, representing variation in overall gene coverage among samples, indicated a clear separation along dimension 1 between samples of the two subspecies (Figure 1A) and some degree of separation between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus* (Figure 1B). Multivariate analysis of variance showed that the overall gene expression levels differed significantly between *M. m. musculus* and *M. m. domesticus* samples but not between sexes (perMANOVA Adonis test: $F_{subspecies} = 6.423$; $P = 0.004$; $F_{sex} = 0.804$; $P = 0.547$; $F_{subspecies \times sex} = 0.322$; $P = 0.916$). The trend for a separation between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus* was not statistically significant (perMANOVA Adonis test: $F_{choosiness} = 2.424$; $P = 0.089$; $F_{sex} = 0.782$; $P = 0.537$; $F_{choosiness \times sex} = 0.506$; $P = 0.835$).

The number of expressed VR genes varied slightly among samples (Table S4a) but remained comparable (range between 197 to 252), with no significant difference between samples from the most divergent groups, i.e. the two subspecies (Wilcoxon rank sum test, $P = 0.886$). We also detected expression of Olfactory Receptors (OR) in the vomeronasal organ samples

(Tables S3 and S4). The mean levels of expression of VR and OR genes were lower than the mean coverage for the rest of the genes (mean CPM $_{VR}$ = 3.414; mean CPM $_{OR}$ = 0.804; mean CPM $_{other\_genes}$ = 27.198), with ORs particularly lowly expressed (Table S4b and Figure S2). In contrast, the mean of median CPM values across all samples was higher for VRs than for the other genes (mean median $_{VR}$ = 0.57; mean median $_{other\_genes}$ = 0.29) (Figure S2).

Finally, we report expression of some mouse lipocalins in our VNO samples (Table S3). Seven Odorant Binding Proteins (OBP) were expressed in all samples (including *Obp3-p* annotated as a pseudogene in the mouse reference genome Stopková *et al.* 2016). Among the 12 Major Urinary Proteins (MUP) annotated as functional genes and pseudogenes in the reference genome and that belong to Group A (i.e. the ancestral group of MUPs for which mapping information is reliable, Logan *et al.* 2008), we detected expression of three MUPs in all samples (*Mup4*, *Mup5* and to a lesser extent *Mup6*), and four other MUPs were lowly expressed, mostly in *M. m. musculus* samples (*Mup3*, *Mup20* (also known as Darcin), *Mup-ps19* and *Mup-ps20*).

Differential expression among samples

Differential expression analyses were performed on the comparison between the two subspecies and the comparison between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus*. DE genes showed log fold changes from 1 to 10 and average coverage between 1 and 12 logCPM (counts per million) (Figure 2). We found a large number of genes (2624) differentially expressed between *M. m. domesticus* and *M. m. musculus* samples (Figure 2A, Table S5a). In contrast, we only found 236 genes differentially expressed between the 'Choosy' and 'Non-Choosy' *M. m. musculus* samples (Figure 2B, Table S5b). For this comparison, there were more differentially expressed genes up-regulated in the 'Choosy' samples (156 genes) than the 'Non-Choosy' samples (90 genes) (Table S5b), and in general

the values of log fold change were also higher in the 'Choosy' samples (Figure 2B).

We identified 90 VR genes and one OR gene (*Olfr460*) differentially expressed between the two subspecies (Table S5a). We also identified three lipocalins that were differentially expressed between the two taxa (Table S5a): two MUP genes from Group A (*Mup6* and *Mup20*) expressed mostly in *M. m. musculus* samples, and one OBP gene (*Obp6*) only expressed in *M. m. domesticus* samples. More generally, functional analyses revealed an enrichment in 35 functional categories (GO terms) in the set of genes differentially expressed between the two subspecies, with the most enriched clusters of annotation terms corresponding to signal-related functions, vomeronasal receptors of type 2 (V2Rs) and other G-protein-coupled receptors (Table S6a). For the comparison between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus,* we identified 34 enriched functional categories, with the most enriched clusters of annotation terms corresponding to immune-related functions, signal-related functions and sensory perception of smell (Table S6b). Among the genes found to be differentially expressed between 'Choosy' and 'Non-Choosy' samples, we found five V2R genes (*V2r17*, *V2r69*, *V2r70*, *V2r73* and *V2r75*), which corresponds to a significant enrichment of this category of genes (hypergeometric test: $P < 0.01$ with 5 out of 140 in a sample of 236 DE genes). Among the five DE V2Rs, four were up-regulated in the 'Choosy' samples and one in the 'Non-Choosy' samples (Figure 3). Differentially expressed genes between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus* also included two OR genes (*Olfr15* and *Olfr449*) but no members from family 1 of the VR genes (V1R) (Table S5b).

Phylogenetic relationships among differentially expressed vomeronasal receptor genes

When plotting the differentially expressed genes on the vomeronasal receptor phylogenies, we observed that genes differentially expressed between the two subspecies mapped

almost everywhere on the V1R and V2R phylogenies (Figure 4A and 4B). In contrast, genes differentially expressed between 'Choosy' and 'Non-Choosy' samples only mapped to family 2 of vomeronasal receptors (Figure 4B). One DE VR gene, *V2r17*, belongs to clade 6 of the V2R family. Interestingly, the other four DE VR genes belong to the same phylogenetic clade, clade 3 (genes *V2r69*, *V2r70*, *V2r73* and *V2r75*). The phylogenetic distance between the five DE VR genes was found to be significantly smaller than expected by chance (random sampling test, observed phylogenetic distance = 3.69; $p < 0.004$ and Figure S3).

The fact that closely related VR genes were found to be differentially expressed between the 'Choosy' and 'Non-Choosy' samples within *M. m. musculus* could reflect a bias in mapping due to sequence similarity among closely-related paralogs. Indeed, these four VR belong to the same genomic cluster on chromosome 7, and are probably tandem-repeat paralogs with relatively high sequence similarity. However, we found no evidence for such a bias. Although the coverage along the gene was variable, there was no difference between the coverage of conserved and variable regions in any gene, and there were numerous variable sites with high coverage (Figure S4). This was true even when DE genes were compared to their most closely-related paralogs (same phylogenetic cluster). For example, analysis of coverage information along *V2r70* (differentially expressed) and its closest relative *V2r74* (ENSMUSG00000090774) (not differentially expressed) showed prominent differences in coverage between these two genes for both variable and conserved regions, which indicates that the mapping was able to differentiate reads from different but closely related VR genes.

DISCUSSION

Changes in expression are expected to play an important role in the evolution and divergence of traits involved in reproductive isolation. Here we have used RNA-seq on whole vomeronasal organs collected from wild mice sampled in Denmark to test for the role of changes in expression of vomeronasal receptors in the behavioural shift toward olfactory-based assortative mating observed in the hybrid zone between the two European subspecies of the house mouse. Overall expression levels did not vary significantly between males and females in our dataset, a result in agreement with previous comparisons of male and female mouse VNO transcriptomes (Ibarra-Soria *et al.* 2014). Since assortative mate preferences are displayed by both males and females in the studied mouse populations (Smadja *et al.* 2004b; Smadja & Ganem 2005), we did not analyse differential expression between males and females further. Overall expression levels differed between the two subspecies, and we found a very large number of genes differentially expressed between the two taxa (more than 2,000 in total, and 90 VRs), consistent with the degree of divergence between the two taxa (Phifer-Rixey *et al.* 2014). In contrast, only 236 genes were found repeatedly differentially expressed between the *M. m. musculus* populations showing contrasting patterns of mate preference. Among these DE genes between 'Choosy' and 'Non-Choosy' samples, we found an overrepresentation of genes belonging to family 2 of vomeronasal receptors, known to play a key role in pheromone recognition in mice (Chamero *et al.* 2012). Interestingly, four out of five of these differentially expressed VRs belong to the same phylogenetic cluster. In the following paragraphs, we focus on discussing these findings related to expression divergence between populations of *M. m. musculus* showing contrasting mate preference behaviours.

How confident can we be that differences in expression are associated with differences in behaviour?

Comparative transcriptomics has previously been used to reveal candidate genes for behavioural differences or to reconstruct preference-gene networks (reviewed in Wilkinson *et al*. 2015). In our study, how confident can we be that these differences in gene expression are, at least in part, associated with the observed behavioural shift toward assortative mating in the populations nearer to the hybrid zone? First, the use of several biological replicates for each of the two types of natural populations known to differ in their mate preference patterns minimizes the risk of observing population-specific differences that could reflect different regimes of selection or demographic events among populations. Second, we sampled *M. m. musculus* populations with contrasting mate preference behaviours but are geographically close in Denmark. The different populations thus share a very recent common ancestor, have similar low rates of introgression from *M. m. domesticus* and live in similar environments. Our aim was to minimize any other possible natural sources of divergence between these populations and therefore maximize the chance that genetic/expression differences reflect, at least in part, the known behavioural difference between the groups. Since VR expression at the adult stage is known to be influenced by postnatal (from birth to 19 days old) exposure to odours (environment, parental imprinting) at least at the kin recognition level (Broad & Keverne 2012), we decided to study wild-caught mice to ensure assessment of patterns of gene expression as close as possible to natural populations, i.e. potentially influenced by a combination of innate and imprinted mechanisms. However, to homogenise replicates further and avoid any possible variation in gene expression due to differing physiological conditions, we maintained wild-caught mice in the laboratory before dissection to control for the main factors known to influence expression in general and VR expression in particular (diet, temperature, photoperiod, the olfactory environment, age range). Although we could not strictly control

494 for the age of these wild-caught individuals, we selected only mice trapped as young adults
495 expected to fall in the age range corresponding to the peaked plateau of VR expression in
496 adult mice (Zhang *et al.* 2010). This ensured that age-related variation in VR expression
497 would not strongly influence our results.
498 Considering that the experiment was performed using wild animals from natural
499 populations, we cannot completely exclude other sources of variation in the observed
500 profiles of gene expression. However, we consider that this series of experimental settings,
501 combined with a targeted approach to candidate genes likely to be involved in the behaviour
502 of interest, offers optimal conditions to detect association between assortative mate
503 preferences and differential gene expression.
504
505 *Identity and functions of differentially expressed genes*
506 Our main objective was to assess patterns of expression of vomeronasal receptors.
507 However, we identified several other classes of genes showing repeated patterns of
508 differential expression between 'Choosy' and 'Non-Choosy' samples. Several of these genes
509 have physiological functions related to signal transportation, perception and transduction.
510 Enrichment analyses showed an overrepresentation of genes involved in regulation of
511 odorant selectivity in the extracellular space and olfactory lumen. This includes genes coding
512 for androgen-binding proteins (ABP), previously suggested to act as subspecific signals in
513 mice (Laukaitis *et al.* 1997; Bimova *et al.* 2011), and some organic anion transporters (OAT)
514 expressed in the mouse olfactory mucosa. The latter are potentially involved in odour
515 signalling by binding and transporting organic anions present in urine (Kaler *et al.* 2006).
516 Other enriched categories include genes involved in regulation of signal transduction (cyclic-
517 nucleotide-gated channel (CNG) gene family; Francia, Pifferi, Menini, & Tirindelli, 2014). We
518 also identified two olfactory receptors differentially expressed between the two behavioural
519 groups. Although ORs are primarily expressed in the MOE, our results are congruent with a

recent study reporting expression of ORs in mouse VNOs (Ibarra-Soria *et al.* 2014). We did not detect differential expression of MUPs (from Group A) or OBPs between 'Choosy' and 'Non-Choosy' samples, but could confirm previous findings evidencing their expression in VNO tissues (Utsumi *et al.* 1999; Ibarra-Soria *et al.* 2014; Stopková *et al.* 2016) and expression divergence at some MUP and OBP genes between the two subspecies (although not affecting the same genes as in Stopková *et al.* 2016). These lipocalins expressed in the VNO may play a role in sequestering volatile pheromones and possibly transporting them to vomeronasal receptors (Sharrow *et al.* 2002; Stopková *et al.* 2016). Interestingly, the diversity of genes and functions identified as differentially expressed between 'Choosy' and 'Non-Choosy' samples suggests that the evolution of assortative mating involves a complex network of VNO genes that will need further investigation. Additionnally, genes expressed in other olfactory tissues (MOE) or involved in the integration of the signal in the brain, which we did not study here, may also play an important role in the expression of assortative mate preference.

As far as VR are concerned, we identified five receptor genes (*V2r17*, *V2r69*, *V2r70*, *V2r73* and *V2r75*) that showed repeated differential expression between the two behavioural groups. Since in most cases only one functional receptor is expressed per neuron in the vomeronasal organ (Dalton & Lomvardas 2015; Ishii & Mombaerts 2011), the observed expression divergence at individual VRs could result from differences in transcript level per neuron (Young *et al.* 2003), but also from differences in the numbers of neurons expressing a given receptor, a parameter that shapes the olfactory map in the accessory olfactory bulb and provides to the brain the signal for a behavioural response (Mombaerts 2004; Bear *et al.* 2016). Since we used RNA-seq on whole vomeronasal organs, our experiment does not allow us to distinguish these two levels of expression, but is informative about the overall pattern of variation and divergence at individual VRs that likely influence odour perception.

Four of the differentially expressed VR were found to be up-regulated in the 'Choosy' samples and one was up-regulated in the 'Non-Choosy' samples. A majority of VR genes upregulated in the 'Choosy' samples is consistent with the idea that some receptors are recruited in the zone of contact with the other subspecies to allow the expression of assortative mate preferences.

In contrast to differentially expressed VRs between the two subspecies belonging to both families of VRs (family 1 and family 2), expression differentiation within *M. m. musculus* between 'Choosy' and 'Non-Choosy' samples only affects members of family 2 (V2R). This corresponds to a significant enrichment of this category of genes among all DE genes. Traditionally, V2Rs were thought to be involved primarily in pheromone recognition (as opposed to V1Rs that were thought to be involved in the detection of the physiological status of other animals). Recent findings have shown that individual V2Rs are specifically tuned to detect the nature of the signal emitter (e.g. conspecifics, heterospecifics, individual recognition), whereas individual V1Rs are activated by cues from multiple species (Isogai *et al.* 2011). Additionally, V2R-positive Vomeronasal Sensory Neurons detect members of several large peptide or protein families (in particular MHC class I peptides and some Major Urinary Proteins (MUPs) secreted in urine) that are thought to inform the receiver about the genetic identity of a signaler (Hurst *et al.* 2001; Leinders-Zufall *et al.* 2004; Papes *et al.* 2010; Chamero *et al.* 2012). In agreement with this, urines have been shown to diverge in their composition of MUPs between the two subspecies (Hurst *et al.* 2017; Stopková *et al.* 2007) and between 'Choosy' versus 'Non-Choosy' samples within *M. m. musculus* (Hurst *et al.* 2017), which establishes MUPs as potential candidates acting as subspecific signals and potentially recognised by V2Rs. Since our study also confirms previous findings that indicate that some MUPs are also expressed in the VNO, a future challenge will be to understand better the respective role of VNO-expressed MUPs and MUPs expressed in the liver and excreted in urine in mouse communication.

572

573 *Phylogenetic clustering and functional specialisation of differentially expressed vomeronasal*
574 *receptors*

575 We found that four out of five of the differentially expressed V2Rs belong to the same
576 phylogenetic cluster (*V2r69*, *V2r70*, *V2r73* and *V2r75* in clade 3 of the V2R family). Although
577 these four DE genes are in the same genomic cluster on chromosome 7 and share a
578 relatively high degree of sequence similarity, we have evidence to exclude the possibility of a
579 methodological artefact. We did not find any evidence for a bias when aligning these DE
580 genes with other closely-related paralogs and comparing coverage information in specific
581 versus shared positions along these genes. Moreover, some of the closely-related paralogs
582 belonging to the same genomic cluster as the DE genes did not show significant differential
583 expression. If this pattern of phylogenetic clustering of DE VRs is true, it suggests that the
584 evolution of assortative mate preference in *M. m. musculus* could involve the specialisation
585 of a group of closely-related receptors in the clade 3 of V2Rs. This hypothesis is consistent
586 with the study by Isogai *et al*. (2011) which showed that distinct mouse VR receptor
587 subfamilies have evolved towards the specific recognition of certain animal groups
588 (heterospecific signals from predators or sympatric closely-related species/subspecies, male
589 or female conspecific signals) or chemical structures (volatile steroids, non-volatile proteins).
590 Interestingly, the VNOs analysed in their study were from *M. m. domesticus* (laboratory
591 mice), and when tested using *M. m. musculus* scents, several V2Rs were activated including
592 *V2r17* (DE gene in our study) and its close relatives and *V2r66* (belonging to the same clade 3
593 as the four VRs differentially expressed in our study). Other receptors identified as
594 differentially expressed in our study could not be individually functionally characterised.
595 Based on these results, these authors and others (Chamero *et al.* 2012; Wynn *et al.* 2012)
596 hypothesized a role of these VRs in reproductive isolation. An interesting follow-up from our
597 work would be to apply the methods developed by Isogai et al. to test whether the same set

598 of closely-related V2Rs, identified by these authors as activated by *M. m. musculus* scents
599 when expressed in *M. m. domesticus* VNOs, would be activated by *M. m. domesticus* stimuli
600 when expressed by *M. m. musculus* VNOs, as our RNA-seq results suggest.
601
602 *Evolution of expression changes and the role of selection*
603 Regulatory changes in genes involved in reproduction are thought to be prime targets for
604 divergence during speciation. They are expected to evolve under natural and/or sexual
605 selection. Previous studies had shown that expression divergence in testis-associated genes
606 seems to establish during a later phase of the speciation process when reproductive
607 isolation is complete (Voolstra, Tautz, Farbrother, Eichinger, & Harr, 2007; Bryk, Somel,
608 Lorenc, & Teschke, 2013). In our study, we provide evidence for expression divergence in
609 VNO-related genes between populations within the *M. m. musculus* subspecies, in a context
610 of secondary contact with the subspecies *M. m. domesticus*. This result may suggest that
611 expression divergence at these genes has evolved rapidly in the 'Choosy' populations from
612 the border of the hybrid zone. Whatever the source of expression divergence (new
613 mutations, standing variation, hybridisation), changes in expression related to the evolution
614 of assortative mate preferences are expected, in the context of reinforcement, to evolve
615 rapidly under diverging selection (natural and sexual selection against hybridisation in the
616 house mouse system, Britton-Davidian *et al.* 2005; Latour *et al.* 2014). Although
617 accumulating evidence suggests a predominantly neutral model of gene expression
618 evolution (e.g. in house mice, Staubach, Teschke, Voolstra, Wolf, & Tautz, 2010), positive
619 selection has also been shown to shape changes in expression for some genes and lineages
620 (e.g. Wittkopp, Haerum, & Clark, 2008; Jeukens & Bernatchez, 2012; Chapman *et al*. 2013).
621 In a recent study, we identified several genomic regions surrounding VR genes in the house
622 mouse with signatures of positive selection specifically in populations from the border of the
623 hybrid zone that display assortative mate preference (same samples as the 'Choosy' *M. m.*

musculus samples analysed in the present study). Interestingly, one of these genomic regions corresponds to the downstream and putative regulatory region of the cluster containing the candidate VRs identified in the present study, in the vicinity of *V2r75* (Smadja *et al.* 2015). Another study on wild-derived strains of the two subspecies has suggested that V2Rs identified by Isogai *et al*. (2011) as detecting subspecific cues are prone to evolve under positive selection, in contrast to V2Rs detecting other classes of signals (Wynn *et al.* 2012). Analysing genome-wide patterns of nucleotide polymorphism and divergence in the same biological samples as the ones used in the present study should allow us to confirm whether some differentially expressed vomeronasal receptors or their *cis*-regulatory regions evolve under divergent selection in the populations displaying assortative mate preferences.

To conclude, this study suggests that changes in expression at key receptors for pheromone recognition may play an important role in the evolution of assortative mating and reinforced sexual isolation in the house mouse. Evidence for differential expression at some closely-related vomeronasal receptors between populations within *M. m. musculus* that do or do not display assortative mate preferences (close to or more distant from the hybrid zone, respectively) implicates them in this behavioural shift. This is consistent with previous findings suggesting the specialisation of this group of receptors in the recognition of odorant signals that may allow subspecies recognition and assortative mating.

644 ACKNOWLEDGEMENTS

645 We are very grateful to Ingrid Jakob, former engineer at Centre des Sciences du Goût et de
646 l'Alimentation (UMR 1324), University of Burgundy, Dijon, France, for her advice on
647 dissecting mouse vomeronasal organs. We would also like to thank very much Josette
648 Catalan, Marco Perriat-Sanguinet, Yamin Latour for their help in collecting mice in Denmark
649 and the Danish farmers for their hospitality, as well as Giveskud zoo personnel. We thank
650 Janice Britton-Davidian, Josette Catalan and Marco Perriat-Sanguinet for their help in
651 dissecting mice. We thank the platform "High throughput qPCR" of the University of
652 Montpellier for having given us access to an Agilent instrument to assess RNA sample
653 quality. Finally, we thank Roger Butlin for his comments on a previous version of this
654 manuscript. The research leading to these results has received funding from the European
655 Union's Seventh Framework Programme [FP7/2007-2013] – Marie Curie European
656 Reintegration Grant (ERG), under Grant agreement n°PERG06-GA-2009-251008, as well as
657 from the Agence Nationale pour la Recherche (ANR) under Grant agreement No.
658 2010BLAN171401-AssortMate. This is publication ISEM 2017-114.

659

660

661 REFERENCES

662 Albrechtova J, Albrecht T, Baird SJE *et al.* (2012) Sperm-related phenotypes implicated in
663 both maintenance and breakdown of a natural species barrier in the house mouse.
664 *Proceedings of the Royal Society B-Biological Sciences*, **279**, 4803–4810.

665 Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance.
666 *Austral Ecology*, **26**, 32–46.

667 Bailey RI, Innocenti P, Morrow EH, Friberg U, Qvarnström A (2011) Female Drosophila
668 melanogaster gene expression and mate choice: the X chromosome harbours
669 candidate genes underlying sexual isolation. *PloS one*, **6**, e17358.

670 Bear DM, Lassance J-M, Hoekstra HE, Datta SR (2016) The Evolving Neural and Genetic
671 Architecture of Vertebrate Olfaction. *Current Biology*, **26**, R1039–R1049.

672 Benjamini Y, Yekutieli D (2001) The control of the false discovery rate in multiple testing
673 under dependency. *Annals of Statistics*, **29**, 1165–1188.

674 Bhattacharyya T, Gregorova S, Mihola O *et al.* (2013) Mechanistic basis of infertility of mouse
675 intersubspecific hybrids. *Proceedings of the National Academy of Sciences*, **110**, E468–
676 E477.

677 Bimova B, Macholan M, Baird SJE *et al.* (2011) Reinforcement selection acting on the
678 European house mouse hybrid zone. *Molecular Ecology*, **20**, 2403–2424.

679 Boursot P, Din W, Anand R *et al.* (1996) Origin and radiation of the house mouse:
680 mitochondrial DNA phylogeny. *J. Evol. Biol.*, **9**, 391–415.

681 Britton-Davidian J, Fel-Clair F, Lopez J, Alibert P, Boursot P (2005) Postzygotic isolation
682 between the two European subspecies of the house mouse: estimates from fertility
683 patterns in wild and laboratory-bred hybrids. *Biol. J. Linn. Soc.*, **84**, 379–393.

684 Broad K, Keverne E (2012) The Post-Natal Chemosensory Environment Induces Epigenetic
685 Changes in Vomeronasal Receptor Gene Expression and a Bias in Olfactory Preference.

686 *Behavior Genetics*, **42**, 461–471.

687 Bryk J, Somel M, Lorenc A, Teschke M (2013) Early gene expression divergence between
688 allopatric populations of the house mouse (Mus musculus domesticus). *Ecology and*
689 *evolution*, **3**, 558–68.

690 Cadiou H, Aoudé I, Tazir B *et al.* (2014) Postnatal odorant exposure induces peripheral
691 olfactory plasticity at the cellular level. *The Journal of neuroscience : the official journal*
692 *of the Society for Neuroscience*, **34**, 4857–70.

693 Carroll SB, Grenier JK, Weatherbee SD (2005) *From DNA to diversity : molecular genetics and*
694 *the evolution of animal design*. Blackwell Pub.

695 Cassone BJ, Mouline K, Hahn MW *et al.* (2008) Differential gene expression in incipient
696 species of Anopheles gambiae. *Molecular ecology*, **17**, 2491–504.

697 Chamero P, Leinders-Zufall T, Zufall F (2012) From genes to social communication: molecular
698 sensing by the vomeronasal organ. *Trends in Neurosciences*, **35**, 597–606.

699 Chan YF, Marks ME, Jones FC *et al.* (2010a) Adaptive Evolution of Pelvic Reduction in
700 Sticklebacks by Recurrent Deletion of a Pitx1 Enhancer. *Science*, **327**, 302–305.

701 Chan YF, Marks ME, Jones FC *et al.* (2010b) Adaptive Evolution of Pelvic Reduction in
702 Sticklebacks by Recurrent Deletion of a Pitx1 Enhancer. *Science*, **327**, 302–305.

703 Chapman MA, Hiscock SJ, Filatov DA (2013) Genomic Divergence during Speciation Driven by
704 Adaptation to Altitude. *Molecular Biology and Evolution*.

705 Chung H, Loehlin DW, Dufour HD *et al.* (2014) A Single Gene Affects Both Ecological
706 Divergence and Mate Choice in Drosophila. *Science*, **343**, 1148–1151.

707 Cucchi T, Auffray J-C, Vigne J-D (2012) History of house mouse synanthropy and dispersal in
708 the near east and europe: a zooarchaeological insight. In: *Evolution of the house mouse*
709 (eds Macholan M, Baird SJE, P M, Pialek J), pp. 65–93. Cambridge University Press.

710 Dalton RP, Lomvardas S (2015) Chemosensory receptor specificity and regulation. *Annual*

review of neuroscience, **38**, 331–49.

Davidson JH, Balakrishnan CN (2016) Gene Regulatory Evolution During Speciation in a Songbird. *G3: Genes|Genomes|Genetics*, **6**, 1357–1364.

Dixon P (2003) VEGAN, a package of R functions for community ecology. *Journal of Vegetation Science*, **14**, 927–930.

Etges WJ (2014) No boundaries: genomes, organisms, and ecological interactions responsible for divergence and reproductive isolation. *The Journal of heredity*, **105 Suppl**, 756–70.

Eyres I, Jaquiéry J, Sugio A *et al.* (2016) Differential gene expression according to race and host plant in the pea aphid. *Molecular Ecology*, **25**, 4197–4215.

Fortes-Marco L, Lanuza E, Martinez-Garcia F (2013) Of Pheromones and Kairomones: What Receptors Mediate Innate Emotional Responses? *The Anatomical Record*, **296**, 1346–1363.

Francia S, Pifferi S, Menini A, Tirindelli R (2014) *Vomeronasal Receptors and Signal Transduction in the Vomeronasal Organ of Mammals*. CRC Press/Taylor & Francis.

Fujii T, Fujii T, Namiki S *et al.* (2011) Sex-linked transcription factor involved in a shift of sex-pheromone preference in the silkmoth Bombyx mori. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 18038–43.

Ganem G, Litel C, Lenormand T (2008) Variation in mate preference across a house mouse hybrid zone. *Heredity*, **100**, 594–601.

Haerty W, Singh RS (2006) Gene regulation divergence is a major contributor to the evolution of Dobzhansky-Muller incompatibilities between species of Drosophila. *Molecular biology and evolution*, **23**, 1707–14.

Hansen KD, Irizarry RA, Wu Z (2012) Removing technical variability in RNA-seq data using conditional quantile normalization. *Biostatistics (Oxford, England)*, **13**, 204–16.

736 Hoang K, Matzkin LM, Bono JM (2015) Transcriptional variation associated with cactus host
737 plant adaptation in Drosophila mettleri populations. *Molecular Ecology*, n/a-n/a.
738 Hoekstra HE, Coyne JA (2007) The locus of evolution: Evo devo and the genetics of
739 adaptation. *Evolution*, **61**, 995–1016.
740 Horth L (2007) Sensory genes and mate choice: Evidence that duplications, mutations, and
741 adaptive evolution alter variation in mating cue genes and their receptors. *Genomics*,
742 **90**, 159–175.
743 Huang DW, Sherman BT, Lempicki RA (2009) Systematic and integrative analysis of large
744 gene lists using DAVID bioinformatics resources. *Nature protocols*, **4**, 44–57.
745 Huestis DL, Marshall JL (2009) From Gene Expression to Phenotype in Insects: Non-
746 microarray Approaches for Transcriptome Analysis. *BioScience*, **59**, 373–384.
747 Hurst JL, Beynon RJ, Armstrong SD *et al.* (2017) Molecular heterogeneity in major urinary
748 proteins of Mus musculus subspecies: potential candidates involved in speciation.
749 *Scientific Reports*, **7**, 44992.
750 Hurst JL, Payne CE, Nevison CM *et al.* (2001) Individual recognition in mice mediated by
751 major urinary proteins. *Nature*, **414**, 631–634.
752 Ibarra-Soria X, Levitin M, Logan D (2013) The genomic basis of vomeronasal-mediated
753 behaviour. *Mammalian Genome*, 1–12.
754 Ibarra-Soria X, Levitin MO, Saraiva LR, Logan DW (2014) The Olfactory Transcriptomes of
755 Mice. (J Zhang, Ed,). *PLoS genetics*, **10**, e1004593.
756 Isogai Y, Si S, Pont-Lezica L *et al.* (2011) Molecular organization of vomeronasal
757 chemoreception. *Nature*, **478**, 241–245.
758 Kaler G, Truong DM, Sweeney DE *et al.* (2006) Olfactory mucosa-expressed organic anion
759 transporter, Oat6, manifests high affinity interactions with odorant organic anions.
760 *Biochemical and biophysical research communications*, **351**, 872–6.

761 Karn RC, Young JM, Laukaitis CM (2010) A Candidate Subspecies Discrimination System
762 Involving a Vomeronasal Receptor Gene with Different Alleles Fixed in M. m.
763 domesticus and M. m. musculus. *PLoS ONE*, **5**, 11.

764 Keller M, Baum MJ, Brock O, Brennan PA, Bakker J (2009) The main and the accessory
765 olfactory systems interact in the control of mate recognition and sexual behavior.
766 *Behavioural Brain Research*, **200**, 268–276.

767 King MC, Wilson AC (1975) Evolution at two levels in humans and chimpanzees. *Science*
768 *(New York, N.Y.)*, **188**, 107–16.

769 Latour Y, Perriat-Sanguinet M, Caminade P *et al.* (2014) Sexual selection against natural
770 hybrids may contribute to reinforcement in a house mouse hybrid zone. *Proceedings of*
771 *the Royal Society B: Biological Sciences*, **281**.

772 Laukaitis CM, Crister ES, Karn RC (1997) Salivary androgen-binding protein (ABP) mediates
773 sexual isolation in Mus musculus. *Evolution*, **51**, 2000–2005.

774 Leinders-Zufall T, Brennan P, Widmayer P *et al.* (2004) MHC class I peptides as chemosensory
775 signals in the vomeronasal organ. *Science*, **306**, 1033–1037.

776 Li B, Dewey CN (2011) RSEM: accurate transcript quantification from RNA-Seq data with or
777 without a reference genome. *BMC bioinformatics*, **12**, 323.

778 Liberles SD (2014) Mammalian Pheromones. *Annual Review of Physiology*, **76**, 151–175.

779 Logan DW, Marton TF, Stowers L (2008) Species Specificity in Major Urinary Proteins by
780 Parallel Evolution. *PLoS ONE*, **3**, e3280.

781 Mack KL, Campbell P, Nachman MW (2016) Gene regulation and speciation in house mice.
782 *Genome Research*, **26**, 451–461.

783 Martin M (2011) Cutadapt removes adapter sequences from high-throughput sequencing
784 reads. *EMBnet.journal*, **17**, 10.

785 McBride CS, Baier F, Omondi AB *et al.* (2014) Evolution of mosquito preference for humans

786 linked to an odorant receptor. *Nature*, **515**, 222–227.

787 Mombaerts P (2004) Genes and ligands for odorant, vomeronasal and taste receptors.
788 *Nature reviews. Neuroscience*, **5**, 263–78.

789 Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B (2008) Mapping and quantifying
790 mammalian transcriptomes by RNA-Seq. *Nat Meth*, **5**, 621–628.

791 Nosil P, Feder JL (2012) Genomic divergence during speciation: causes and consequences.
792 *Philosophical Transactions of the Royal Society B: Biological Sciences*, **367**, 332–342.

793 Nosil P, Schluter D (2011) The genes underlying the process of speciation. *Trends in Ecology*
794 *& Evolution*, **26**, 160–167.

795 Orr HA, Masly JP, Presgraves DC (2004) Speciation genes. *Current Opinion in Genetics &*
796 *Development*, **14**, 675–679.

797 Ortiz-Barrientos D, Counterman BA, Noor MAF (2007) Gene expression divergence and the
798 origin of hybrid dysfunctions. *Genetica*, **129**, 71–81.

799 Papes F, Logan DW, Stowers L (2010) The Vomeronasal Organ Mediates Interspecies
800 Defensive Behaviors through Detection of Protein Pheromone Homologs. *Cell*, **141**,
801 692–703.

802 Paradis E, Claude J, Strimmer K (2004) APE: Analyses of Phylogenetics and Evolution in R
803 language. *Bioinformatics*, **20**, 289–290.

804 Pavey SA, Collin H, Nosil P, Rogers SM (2010) The role of gene expression in ecological
805 speciation. In: *Year in Evolutionary Biology*, pp. 110–129. Blackwell Publishing, Oxford.

806 Pease JB, Guerrero RF, Sherman NA, Hahn MW, Moyle LC (2016) Molecular mechanisms of
807 postmating prezygotic reproductive isolation uncovered by transcriptome analysis.
808 *Molecular Ecology*, **25**, 2592–2608.

809 Phifer-Rixey M, Bomhoff M, Nachman MW (2014) Genome-Wide Patterns of Differentiation
810 Among House Mouse Subspecies. *Genetics*, **198**, 283–297.

811 Ragland GJ, Almskaar K, Vertacnik KL *et al.* (2015) Differences in performance and
812 transcriptome-wide gene expression associated with Rhagoletis (Diptera: Tephritidae)
813 larvae feeding in alternate host fruit environments. *Molecular ecology*, **24**, 2759–76.
814 Ranwez V, Harispe S, Delsuc F, Douzery EJP (2011) MACSE: Multiple Alignment of Coding
815 SEquences Accounting for Frameshifts and Stop Codons (WJ Murphy, Ed,). *PLoS ONE*, **6**,
816 e22594.
817 Raufaste N, Orth A, Belkhir K *et al.* (2005) Inference of selection and migration in the danish
818 house mouse hybrid zone. *Biological Journal of the Linnean Society*, **84**, 593–616.
819 Robinson MD, Oshlack A, Wang E *et al.* (2010) A scaling normalization method for
820 differential expression analysis of RNA-seq data. *Genome Biology*, **11**, R25.
821 Seehausen O, Butlin RK, Keller I *et al.* (2014) Genomics and the origin of species. *Nat Rev*
822 *Genet*, **15**, 176–192.
823 Sharrow SD, Vaughn JL, Zidek L, Novotny M V, Stone MJ (2002) Pheromone binding by
824 polymorphic mouse major urinary proteins. *Protein Science*, **11**, 2247–2256.
825 Smadja C, Catalan J, Ganem G (2004a) Strong premating divergence in a unimodal hybrid
826 zone between two subspecies of the house mouse. *Journal of Evolutionary Biology*, **17**,
827 165–176.
828 Smadja C, Catalan J, Ganem G (2004b) Strong premating divergence in a unimodal hybrid
829 zone between two subspecies of the house mouse. *Journal of Evolutionary Biology*, **17**,
830 165–176.
831 Smadja C, Ganem G (2002) Subspecies recognition in the house mouse: a study of two
832 populations from the border of a hybrid zone. *Behavioral Ecology*, **13**, 312–320.
833 Smadja C, Ganem G (2005) Asymmetrical reproductive character displacement in the house
834 mouse. *Journal of Evolutionary Biology*, **18**, 1485–1493.
835 Smadja CM, Loire E, Caminade P *et al.* (2015) Seeking signatures of reinforcement at the

genetic level: a hitchhiking mapping and candidate gene approach in the house mouse. *Molecular ecology*, **24**, 4222–4237.

Soneson C, Delorenzi M (2013) A comparison of methods for differential expression analysis of RNA-seq data. *BMC Bioinformatics*, **14**, 91.

Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics (Oxford, England)*, **30**, 1312–3.

Stapley J, Reger J, Feulner PGD *et al.* (2010) Adaptation genomics: the next generation. *Trends in ecology & evolution*, **25**, 705–12.

Staubach F, Teschke M, Voolstra CR, Wolf JBW, Tautz D (2010) A test of the neutral model of expression change in natural populations of house mouse subspecies. *Evolution*, **64**, 549–560.

Stopková R, Stopka P, Janotová K, Jedelský PL (2007) Species-specific expression of major urinary proteins in the house mice (Mus musculus musculus and Mus musculus domesticus). *Journal of Chemical Ecology*, **33**, 861–869.

Stopková R, Vinkler D, Kuntová B *et al.* (2016) Mouse Lipocalins (MUP, OBP, LCN) Are Co-expressed in Tissues Involved in Chemical Communication. *Frontiers in Ecology and Evolution*, **4**, 47.

Turner L, Harr B (2014) *Genome-Wide Mapping In A House Mouse Hybrid Zone Reveals Hybrid Sterility Loci And Dobzhansky-Muller Interactions*. Cold Spring Harbor Labs Journals.

Turner LM, Schwahn DJ, Harr B (2012) Reduced male fertility is common but highly variable in form and severity in a natural jouse mouse hybrid zone. *Evolution*, **66**, 443–458.

Uebbing S, Künstner A, Mäkinen H *et al.* (2016) Divergence in gene expression within and between two closely related flycatcher species. *Molecular Ecology*, **25**, 2015–2028.

Utsumi M, Ohno K, Kawasaki Y *et al.* (1999) Expression of major urinary protein genes in the

nasal glands associated with general olfaction. *Journal of Neurobiology*, **39**, 227–236.

Voolstra C, Tautz D, Farbrother P, Eichinger L, Harr B (2007) Contrasting evolution of expression differences in the testis between species and subspecies of the house mouse. *Genome Research*, **17**, 42–49.

Wang Z, Gerstein M, Snyder M (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetics*, **10**, 57–63.

Wang J, Kalyan S, Steck N *et al.* (2015) Analysis of intestinal microbiota in hybrid house mice reveals evolutionary divergence in a vertebrate hologenome. *Nature Communications*, **6**, 6440.

Wilkinson GS, Breden F, Mank JE *et al.* (2015) The locus of sexual selection: moving sexual selection studies into the post-genomics era. *Journal of Evolutionary Biology*, **28**, 739–755.

Wittkopp PJ, Haerum BK, Clark AG (2008) Regulatory changes underlying expression differences within and between Drosophila species. *Nat Genet*, **40**, 346–350.

Wolf JBW, Bayer T, Haubord B *et al.* (2010) Nucleotide divergence vs. gene expression differentiation: comparative transcriptome sequencing in natural isolates from the carrion crow and its hybrid zone with the hooded crow. *Molecular Ecology*, **19**, 162–175.

Wynn EH, Sanchez-Andrade G, Carss KJ, Logan DW (2012) Genomic variation in the vomeronasal receptor gene repertoires of inbred mice. *Bmc Genomics*, **13**, 13.

Yang H, Zhang YP (2007) Genomic organization and sequence analysis of the vomeronasal receptor V2R genes in mouse genome. *Chinese Science Bulletin*, **52**, 336–342.

Young JM, Shykind BM, Lane RP *et al.* (2003) Odorant receptor expressed sequence tags demonstrate olfactory expression of over 400 genes, extensive alternate splicing and unequal expression levels. *Genome Biology*, **4**, 15.

886 Zhang XM, Firestein S (2002) The olfactory receptor gene superfamily of the mouse. *Nature*
887 *Neuroscience*, **5**, 124–133.
888 Zhang XH, Marcucci F, Firestein S (2010) High-Throughput Microarray Detection of
889 Vomeronasal Receptor Gene Expression in Rodents. *Frontiers in Neurosciences*, **4**, 164.
890 Zhang XH, Zhang XM, Firestein S (2007) Comparative genomics of odorant and pheromone
891 receptor genes in rodents. *Genomics*, **89**, 441–450.
892
893

DATA ACCESSIBILITY

Raw reads have been archived in the NCBI SRA public database under the bioproject identifier PRJNA356041 and through the biosample identifiers SAMN06212561 to SAMN06212563 and SAMN06212565 to SAMN06212572. Expression values (raw and normalised counts) for each gene in each sample are provided in Table S3.

Author contributions

C.S., P.B. and G.G. conceived the experiment. C.S., G.G. and P.C. conducted fieldwork and dissections. P.C. and D.S. performed RNA sample and library preparation and sequencing. E.L. and S.T. analysed the data. C.S. wrote the manuscript and E.L., S.T. and G.G. provided comments and revisions on it.

MAIN FIGURES

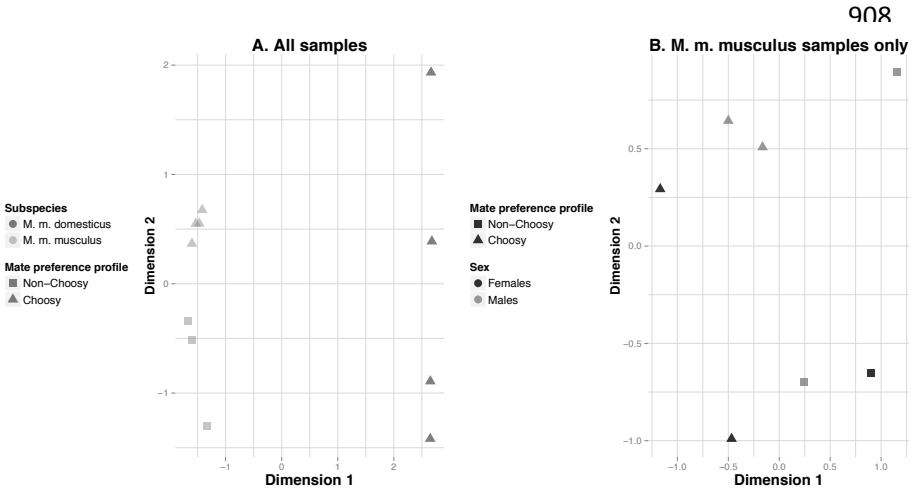
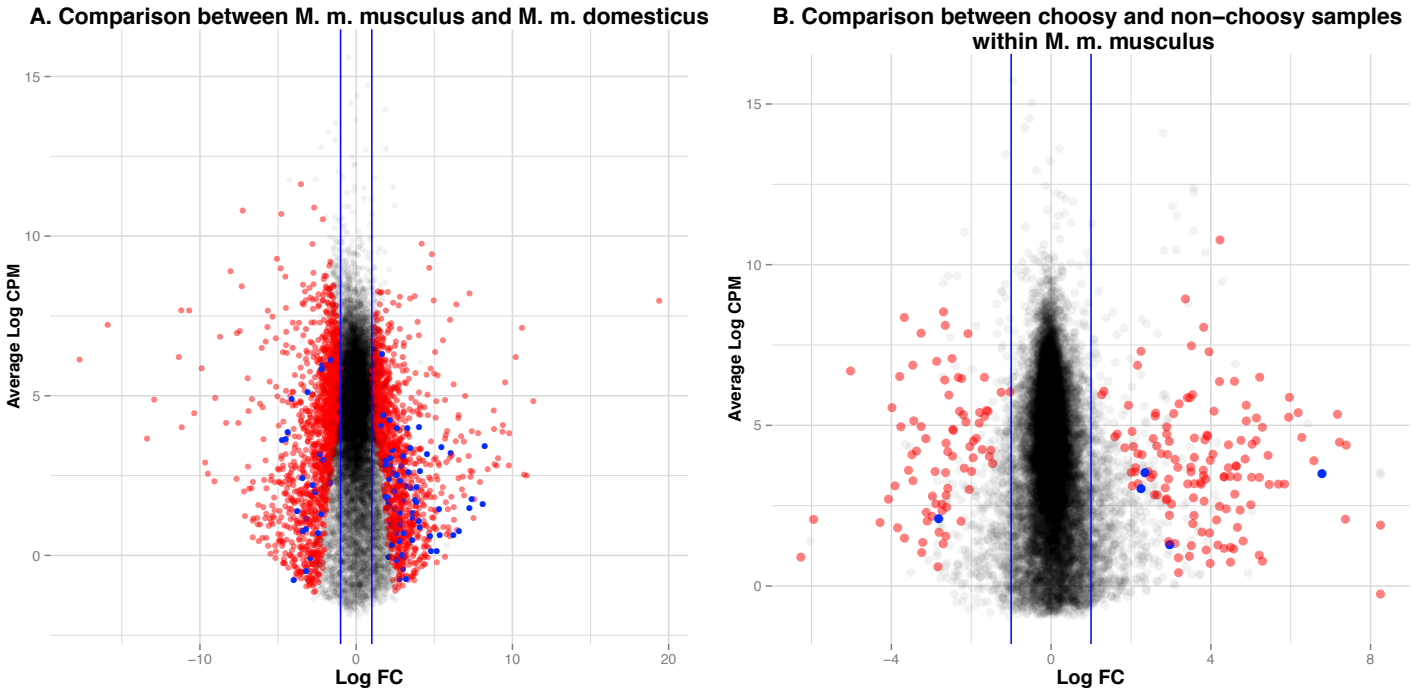


Figure 1: Multi-Dimensional Scaling (MDS) plots indicating variation in gene expression among (A) all M. *m. musculus* and *M. m. domesticus* samples and (B) *M. m. musculus* samples only.



932

933 **Figure 2**: Smear plots indicating significantly differentially expressed genes (red dots) and
934 vomeronasal receptors (blue dots) between (A) *M. m. musculus* 'Choosy' and *M. m.*
935 *domesticus* 'Choosy' samples; (B) 'Choosy' and 'Non-Choosy' samples within *M. m. musculus*.
936 Non-differentially expressed genes are shown as black dots. The blue vertical lines indicate 1
937 log fold change. The x-axis indicates genes that are up regulated in each sample (plot A: dots
938 on the right and on the left corresponding to genes up regulated in *M. m. musculus* and in
939 *M. m. domesticus* respectively; plot B: dots on the right and on the left corresponding to
940 genes up regulated in 'Choosy' *M. m. musculus* and in 'Non-Choosy' *M. m. musculus*
941 respectively). The y-axis indicates the level of average expression of the gene (highly
942 expressed with higher values). CPM: counts per million; FC: fold change.

943

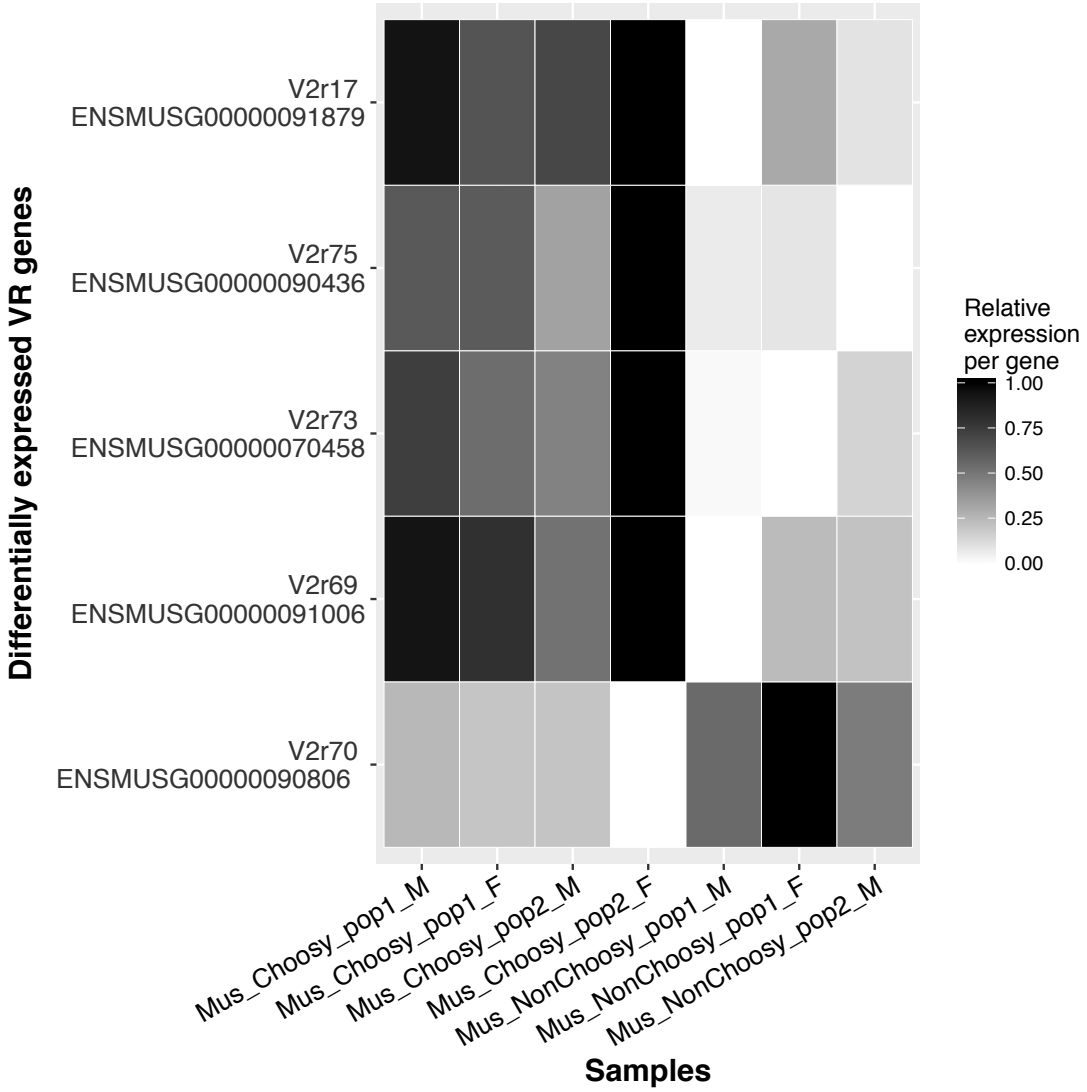


Figure 3: Heatmap plot of differentially expressed vomeronasal receptor (VR) genes in the comparison between 'Choosy' and 'Non-Choosy' *M. m. musculus* samples. The values are presented in log fold change (FC) per gene, in which the sample with the lowest expression value corresponds to 0.

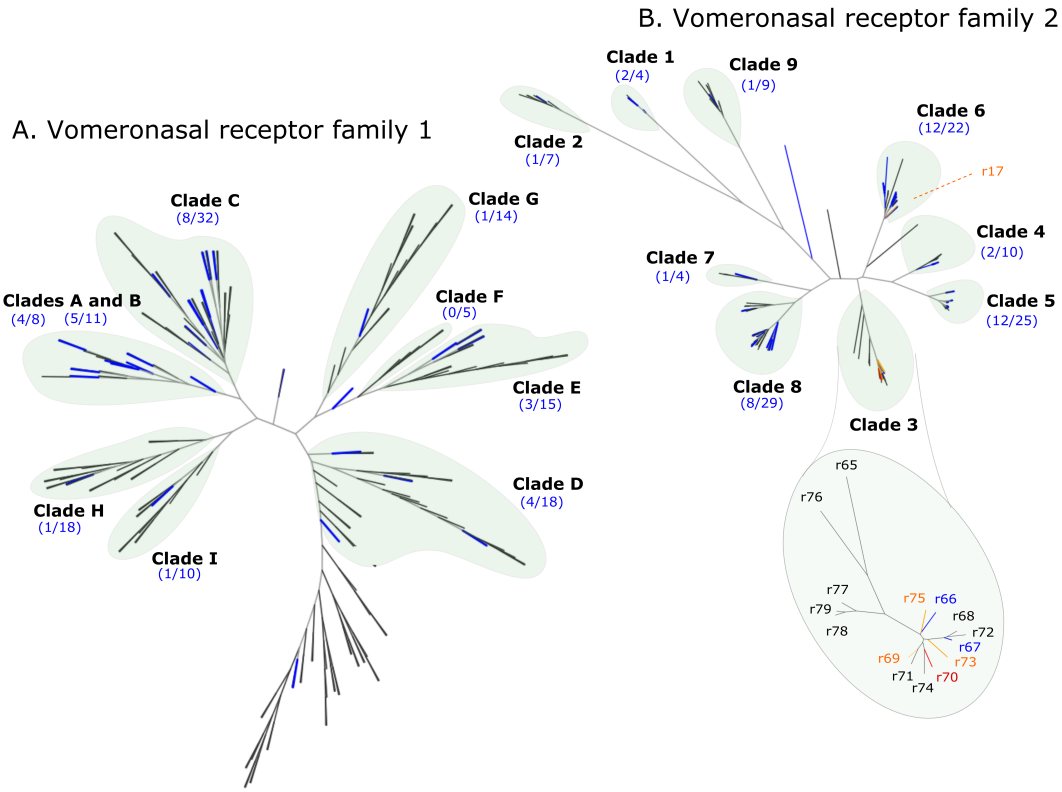


Figure 4: Phylogenetic trees of the two families of vomeronasal receptors (VR) (A: family 1; B: family 2). Branches coloured in blue indicate differentially expressed VR between *M. m. musculus* and *M. m domesticus.* The number of differentially expressed VR genes between the two subspecies and the total number of VR genes in each subfamily (clade) are presented between brackets (e.g. 8/29). Branches coloured in orange indicate differentially expressed VR between 'Choosy' and 'Non-Choosy' samples within *M. m. musculus*. Branches coloured in red indicate VR genes with significant differential expression in both comparisons. In family 2, clade 3 is shown in detail with receptor IDs (MGI nomenclature, see Methods).

Supplemental Information for:

Do changes in gene expression contribute to sexual isolation and reinforcement in the house mouse?

Etienne Loire, Sergio Tusso, Pierre Caminade, Dany Severac, Pierre Boursot, Guila Ganem and Carole M. Smadja

Table of Contents:

List of supplementary figures and tables	Page 2
Supplementary text	Page 3
Figure S1	Page 4
Figure S2	Page 5
Figure S3	Page 6
Figure S4	Pages 7-11

LIST OF SUPPLEMENTARY FIGURES AND TABLES:

Table S1: Sample description (separated Excel file)

Table S2: General mapping and gene expression statistics (separated Excel file)

Table S3: Raw and normalised (CPM) counts for all genes and all samples (separated Excel file, sheet 1: ALL_GENES_raw_counts; sheet 2: ALL_GENES_normalised_counts, sheet 3: OR_normalised_counts; sheet 4: VR_normalised_counts; sheet 5: MUP_normalised_counts; sheet 6: OBP_normalised_counts)

Table S4: Variation in mean count and in expressed gene number among samples (separated Excel file, sheet 1: Table S4a: number and percentage of expressed genes per sample; sheet 2: Table S4b: average counts per sample)

Table S5: List of differentially expressed genes (separated Excel file, sheet 1: Table S5a: Differentially expressed genes between "Choosy" *Mus musculus musculus* and "Choosy" *M. m. domesticus;* sheet 2: Table S5b: Differentially expressed genes between "Choosy" and "Non-Choosy" samples within *Mus musculus musculus*)

Table S6: Functional enrichment results (separated Excel file, sheet 1: Table S6a: enriched functional categories in differentially expressed genes between 'musculus Choosy' and 'domesticus Choosy' samples; sheet 2: Table S6b: enriched functional categories in differentially expressed genes between 'musculus Choosy' and 'musculus Non-Choosy' samples)

Figure S1: Map indicating the geographical origins of the mice used in this study.

Figure S2: Violin plots of log-transformed normalised gene coverage.

Figure S3: Distribution of phylogenetic distances among random sampling of five vomeronasal receptor genes.

Figure S4: Coverage per position (bp) for the five differentially expressed VR genes in the comparison between ‘Choosy’ and ‘Non-Choosy’ *M. m. musculus* samples.

Supplementary information

Discussion:

Robustness of differential expression inferences

RNA-seq on pooled samples is a cost-effective strategy widely used to assess variation in gene expression among experimental conditions or biological groups while producing transcriptome-wide data (reviewed in Todd *et al.* 2016). This pooling design offers an advantage by increasing sample size into the analysis, and when working with samples that may exhibit a large amount of biological variation, analysing several sample pools, rather than a few single samples per condition, also lessens the impact of single aberrant samples (Todd *et al.* 2016). Although sample pooling can theoretically result in lower precision in gene expression estimation (for lowly expressed transcripts) and increased false positive rates in differential expression (DE) analyses as compared to individual sequencing (Rajkumar *et al.* 2015), this limitation is overcome when the number of individuals per pool is sufficiently large (n > 5) and the number of biological replicates per group is maximized (Todd *et al.* 2016). By using eight individuals per pool and from three to four biological replicates per group for DE analyses, we placed ourselves in favourable conditions to reliably identify changes in gene expression between groups with a pooling design. To minimize further the risk of false positives in DE analyses, we also applied a stringent false discovery correction and selected the method for DE detection (*edgeR*) with the highest sensitivity and specificity compared to other methods (e.g. *DEseq2*, *Cuffdiff2*) (Rajkumar *et al.* 2015). Altogether, the stringent DE analysis and the consistency of differential expression among biological replicates strongly support the idea of divergence in gene expression between 'Choosy' and 'Non-Choosy' *M. m. musculus* samples.

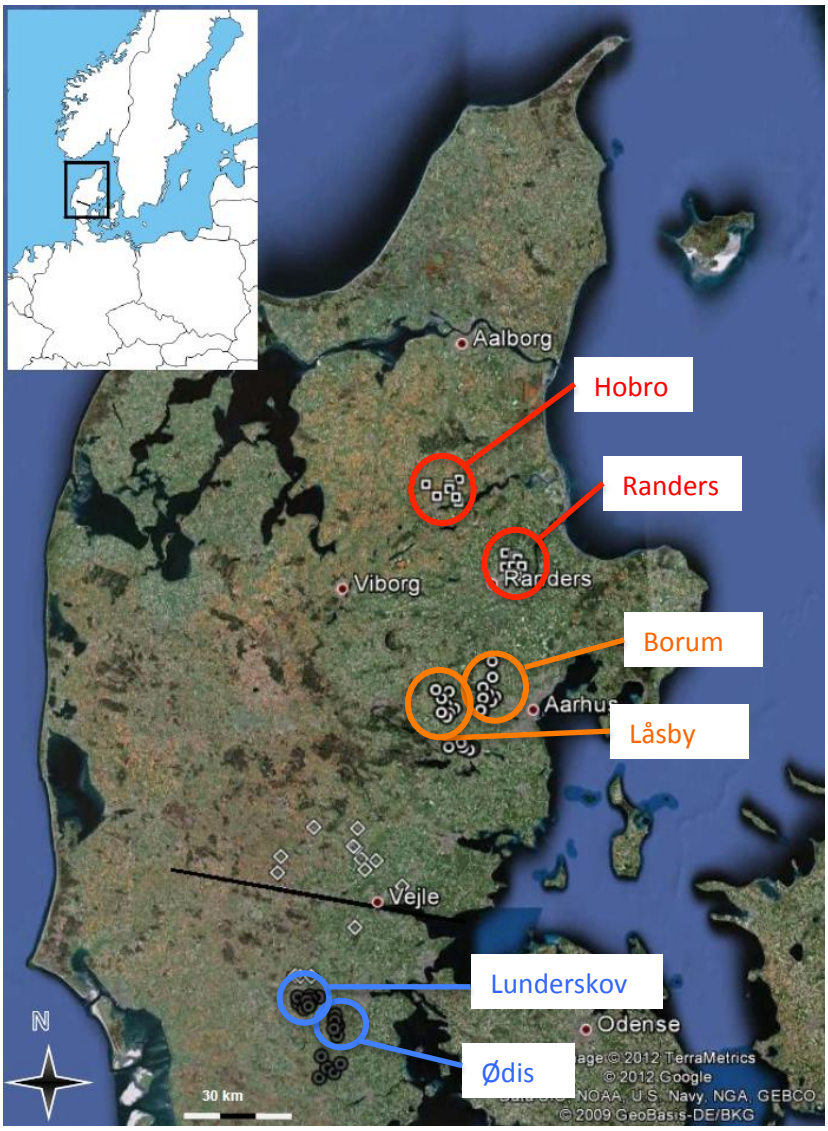


Figure S1: Map indicating the geographical origins of the mice used in this study (adapted from Latour et al. 2014).
Trapping sites in Jutland (Denmark) used in Latour et al. 2014 (the bold black line represents the genetic centre of the hybrid zone as defined in Raufaste et al 2005).
The colored circles indicate the trapping sites defining the populations analysed in this study (red: "Non-Choosy *M. m. musculus* populations"; orange : "Choosy *M. m. musculus* populations"; blue: "Choosy *M. m. domesticus* populations

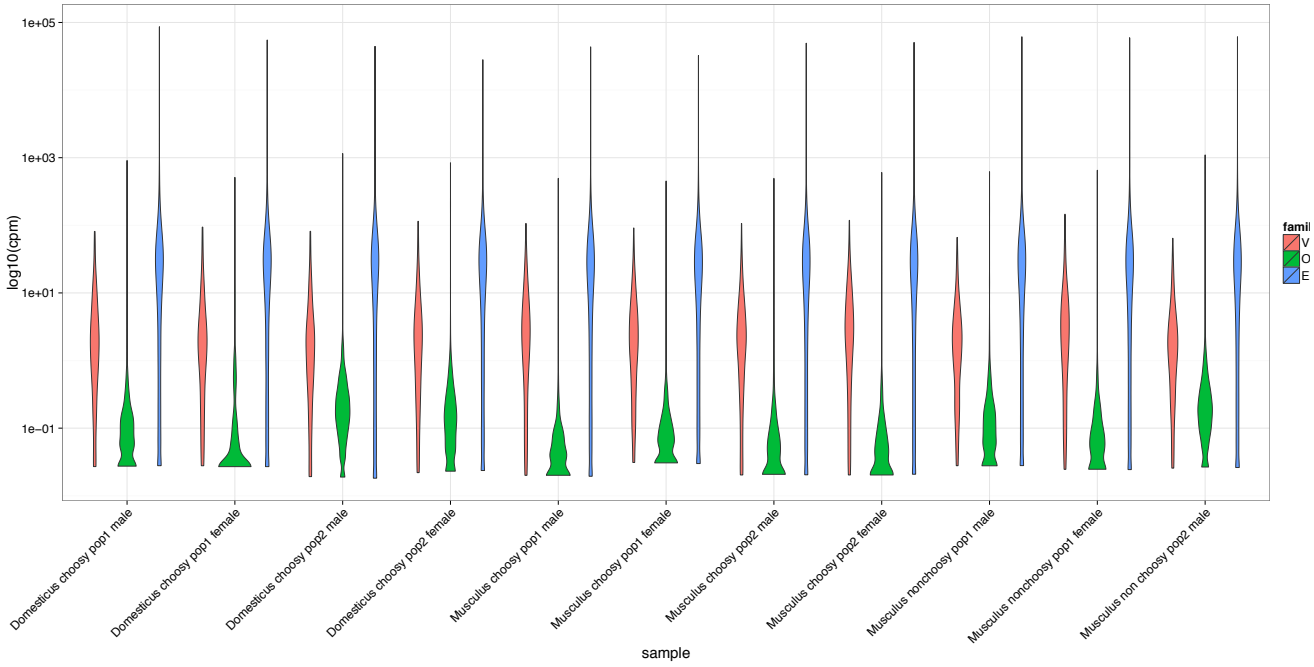


Figure S2: Violin plots of log-transformed normalised gene coverage (CPM) per population for vomeronasal receptor (VR) (red), olfactory receptor (OR) (green) and all other categories of genes (blue).

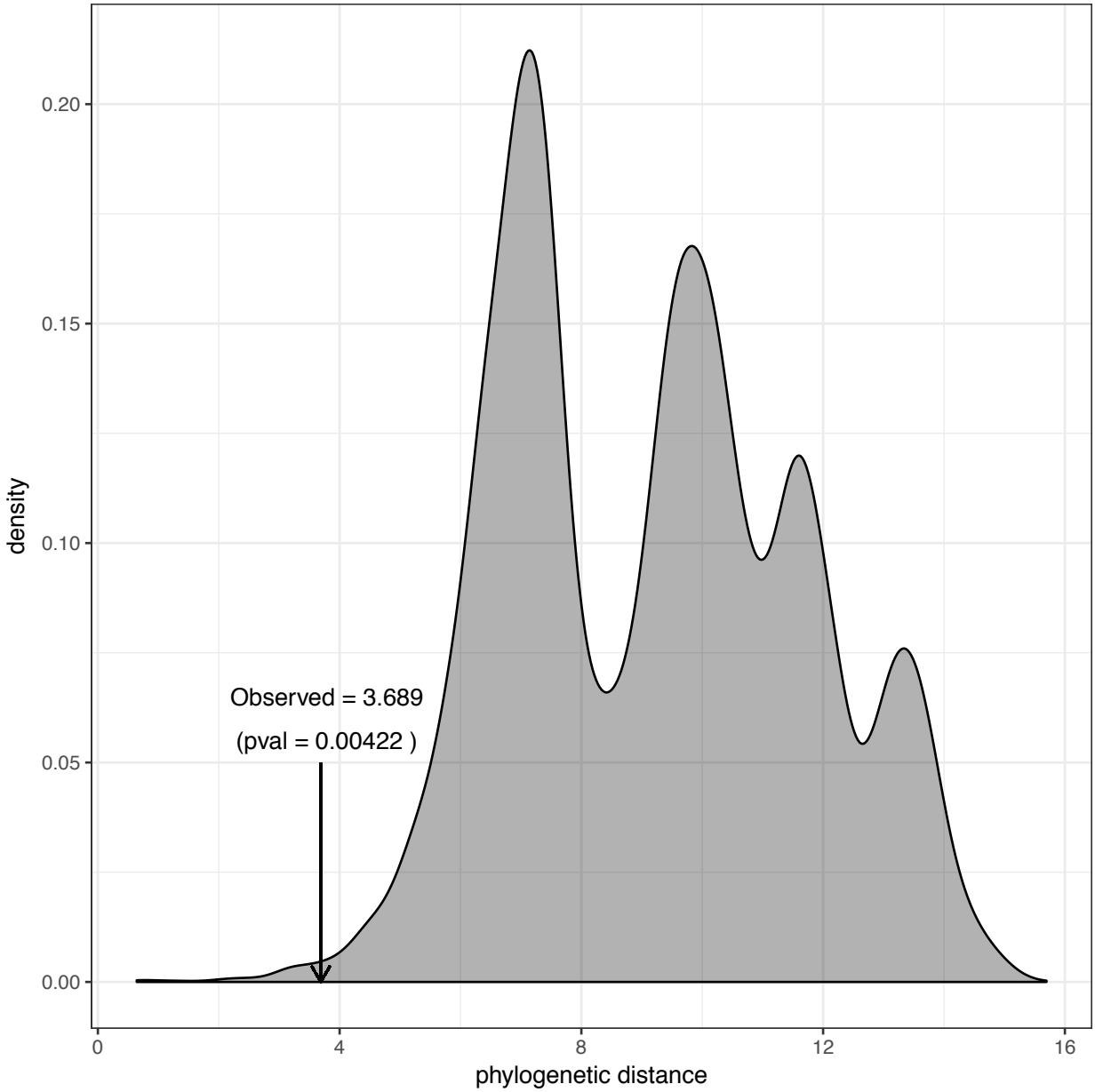
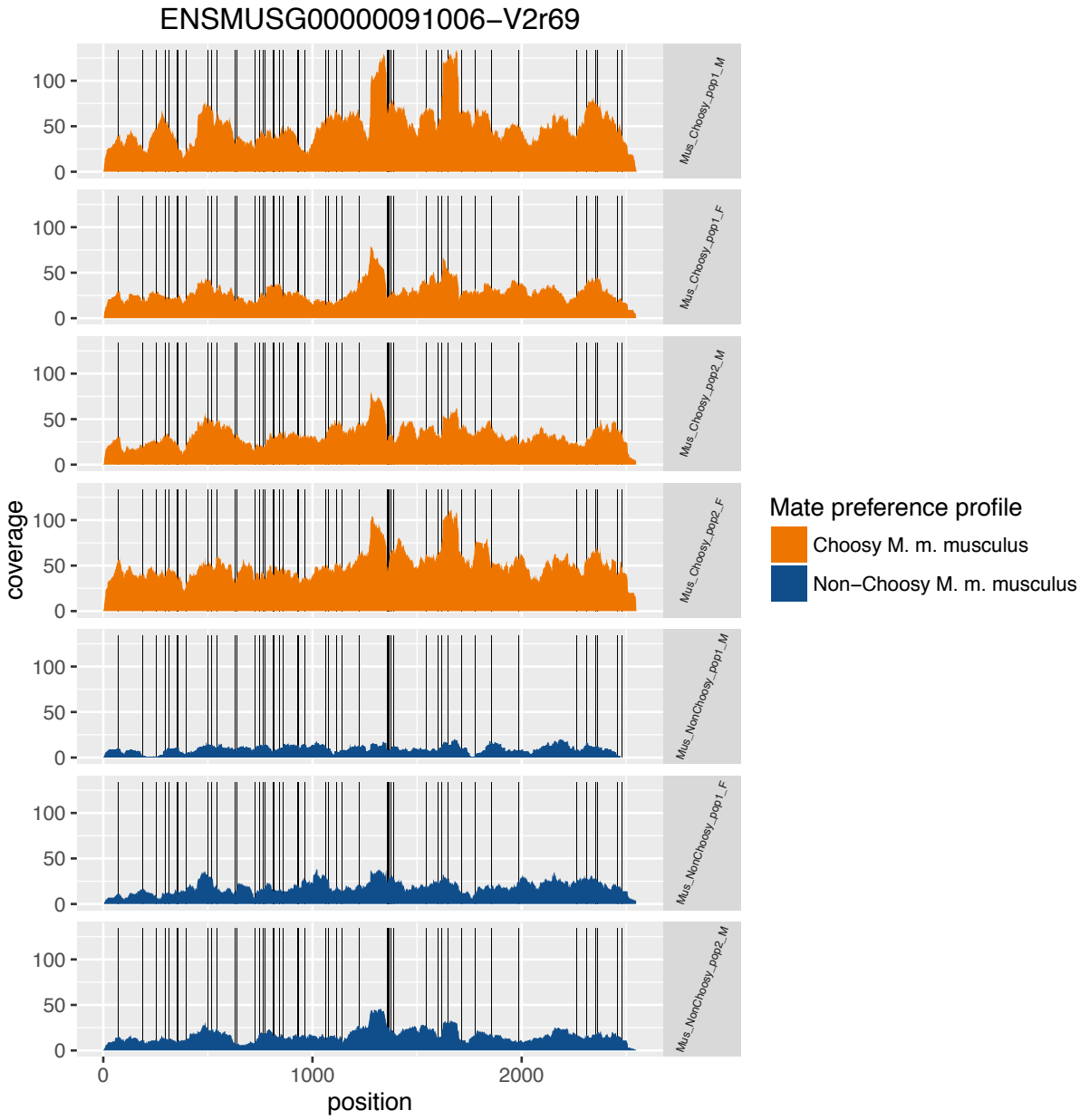
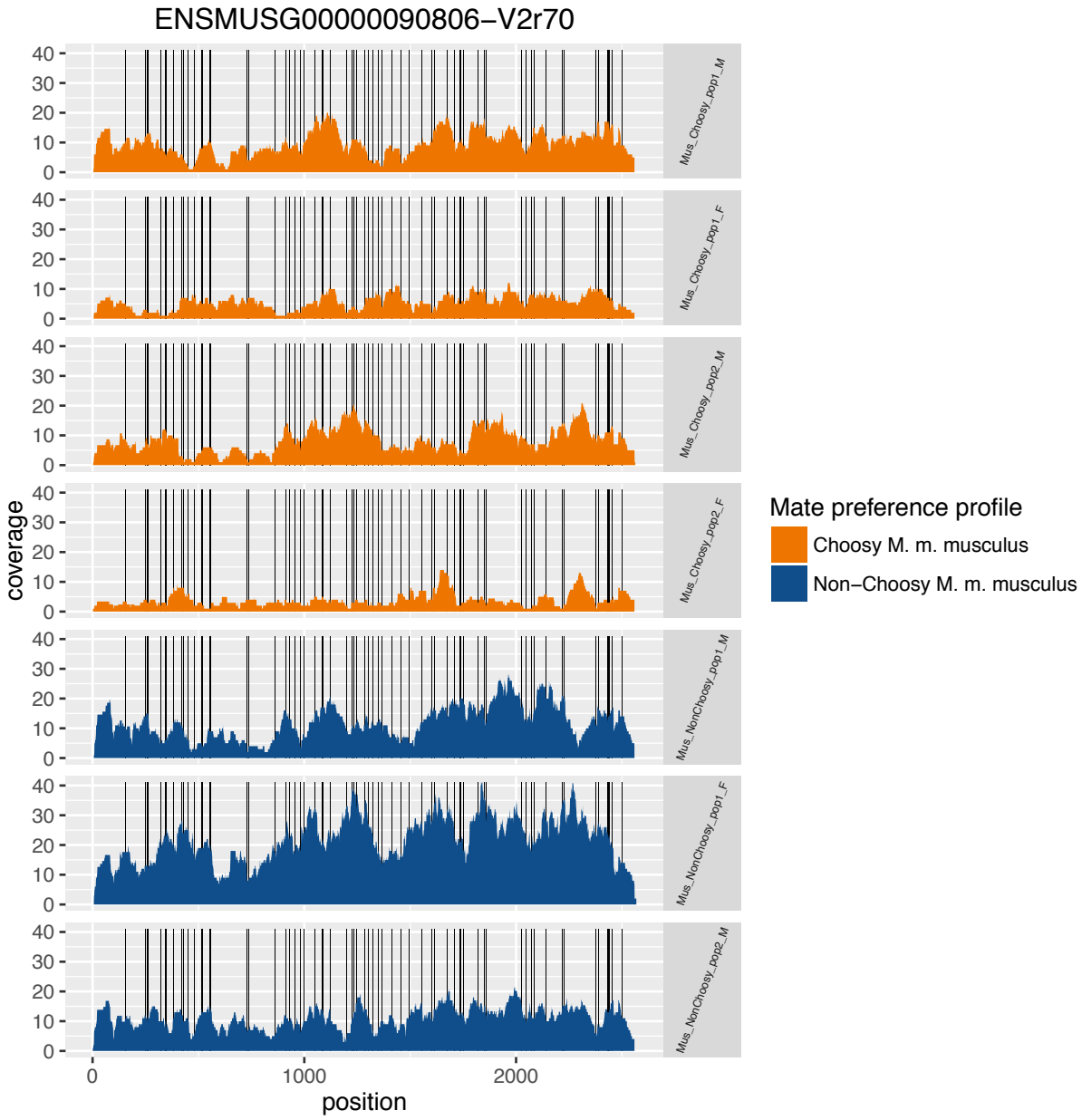
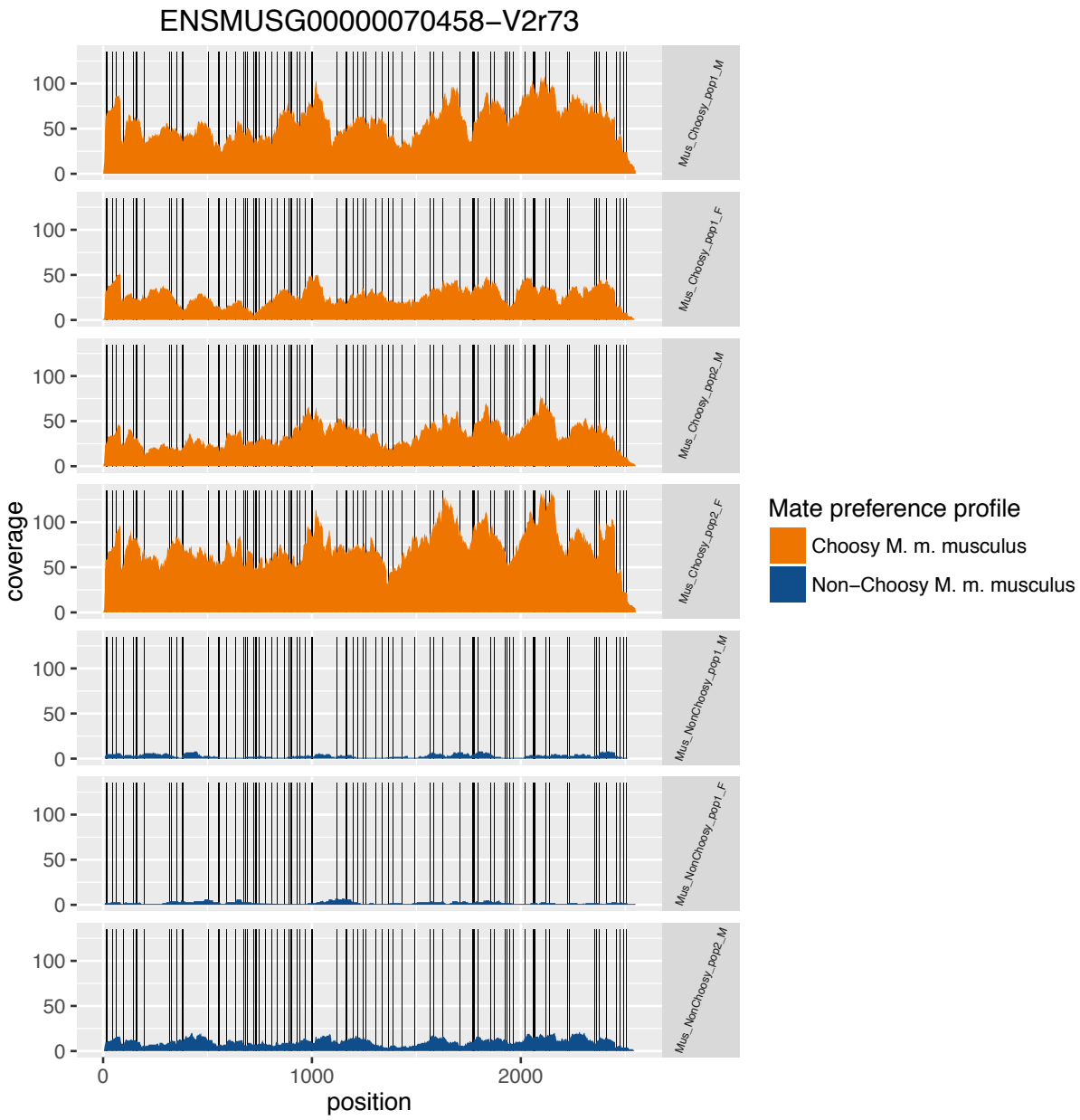


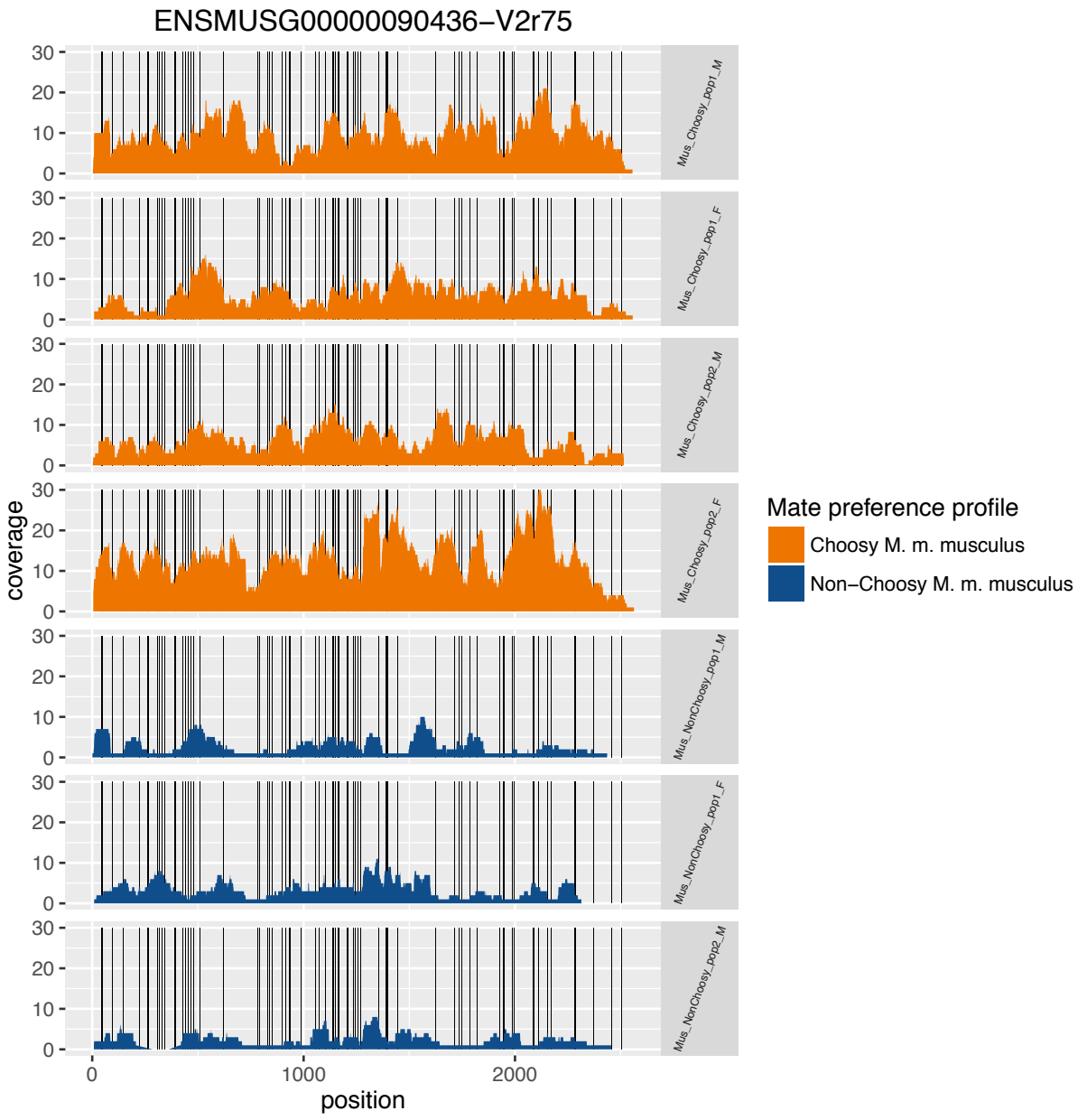
Figure S3: Distribution of phylogenetic distances among random sampling of five vomeronasal receptor genes. The arrow represents the observed distance between the five VRs found differentially expressed between 'Choosy' and 'Non-Choosy' samples, and its probability.

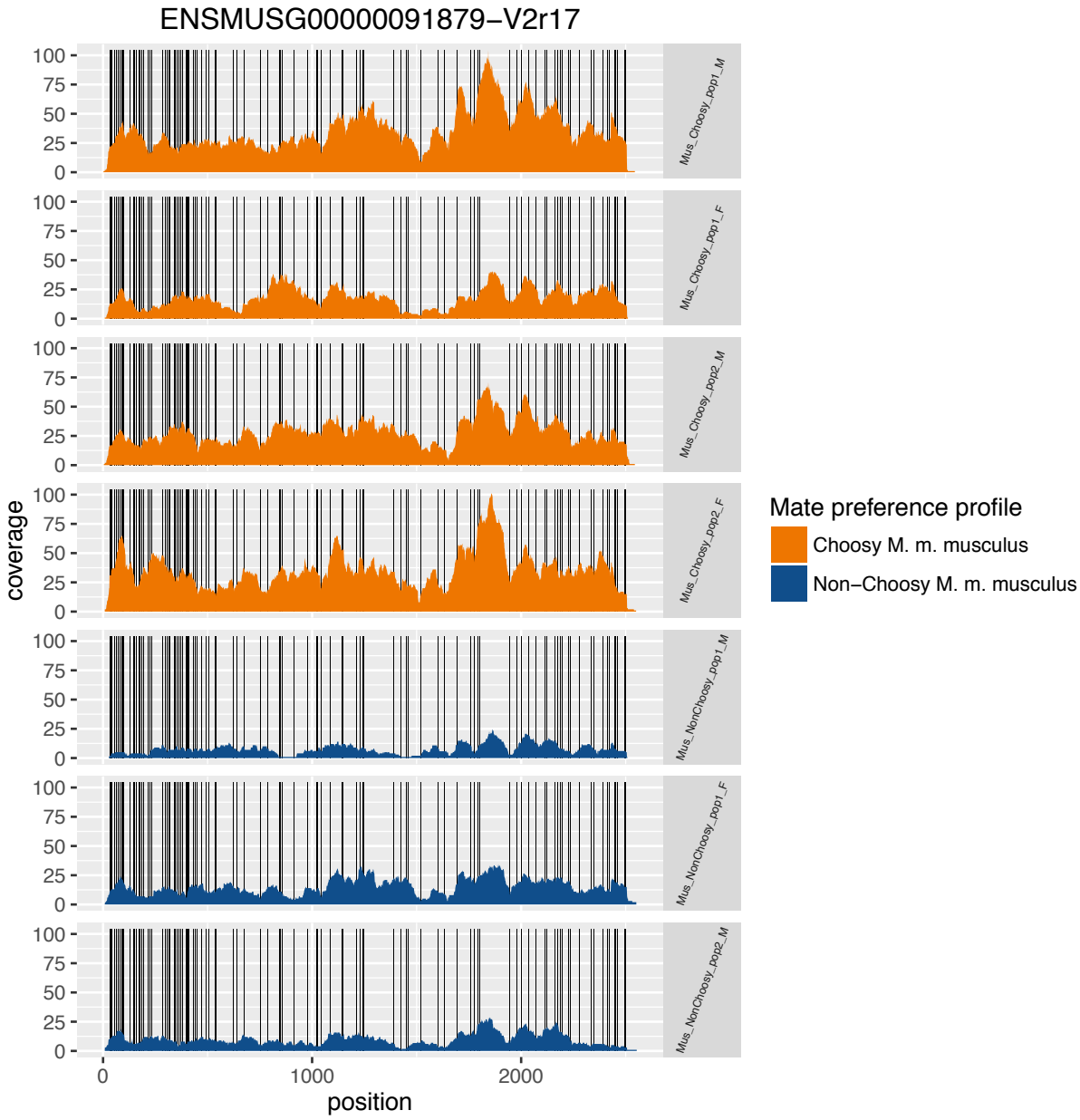
Figure S4: Coverage per position (bp) for the five differentially expressed VR genes in the comparison between 'Choosy' and 'Non-Choosy' *M. m. musculus* samples. Vertical lines represent variable sites of the gene.




ENSMUSG00000090806–V2r70
coverage
position
0
1000
2000
Mus_Choosy_pop1_M
Mus_Choosy_pop1_F
Mus_Choosy_pop2_M
Mus_Choosy_pop2_F
Mus_NonChoosy_pop1_M
Mus_NonChoosy_pop1_F
Mus_NonChoosy_pop2_M
Mate preference profile
Choosy M. m. musculus
Non–Choosy M. m. musculus


ENSMUSG00000070458–V2r73
coverage
100
50
0
Mus_Choosy_pop1_M
Mus_Choosy_pop1_F
Mus_Choosy_pop2_M
Mus_Choosy_pop2_F
Mus_NonChoosy_pop1_M
Mus_NonChoosy_pop1_F
Mus_NonChoosy_pop2_M
0
1000
2000
position
Mate preference profile
Choosy M. m. musculus
Non–Choosy M. m. musculus


ENSMUSG00000090436–V2r75
coverage
position
0
1000
2000
Mus_Choosy_pop1_M
Mus_Choosy_pop1_F
Mus_Choosy_pop2_M
Mus_Choosy_pop2_F
Mus_NonChoosy_pop1_M
Mus_NonChoosy_pop1_F
Mus_NonChoosy_pop2_M
Mate preference profile
Choosy M. m. musculus
Non–Choosy M. m. musculus


ENSMUSG00000091879−V2r17
coverage
position
0
1000
2000
100
75
50
25
0
Mus_Choosy_pop1_M
Mus_Choosy_pop1_F
Mus_Choosy_pop2_M
Mus_Choosy_pop2_F
Mus_NonChoosy_pop1_M
Mus_NonChoosy_pop1_F
Mus_NonChoosy_pop2_M
Mate preference profile
Choosy M. m. musculus
Non−Choosy M. m. musculus