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SHORT COMMUNICATION

Olfactory receptors and behavioural isolation: a study on *Microtus* voles

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Abstract Genetic variation in olfactory receptors may trigger mate choice, suggesting that olfaction has undergone diversifying selection in diverging populations and may contribute to premating reproductive isolation. In the present study, we analysed two olfactory receptor genes as candidate barriers of reproductive isolation between two recently divergent voles: *Microtus lusitanicus* and *Microtus duodecimcostatus*. In addition, evolutionary relationships and signs of positive selection were inferred in a European subgenera context, based on 76 samples from 14 species. DNA sequence analysis revealed the presence of shared haplotypes among various *Microtus* species. Tests of selection detected negatively selected amino acids in the extracellular loops of both olfactory receptors and a majority of negatively selected residues in the transmembrane helices, the most variable regions responsible for the reception of odorants. Our findings suggest that, for several *Microtus* species, including *M. lusitanicus* and *M. duodecimcostatus*, these proteins probably recognise conserved odour cues not related to behavioural isolation.

Keywords Behavioural isolation · Olfaction · Olfactory receptors · *Microtus* · Sister species

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Introduction

Behaviour is highly influenced by olfaction, the dominant sense in most mammals (reviewed in Arakawa et al. 2008). Behavioural interactions make often use of information on species, sex and identity that is provided to the receiving individual in polymorphic odour cues (e.g. Hurst and Beynon 2004; Petrulis 2013). These odour cues may come from a variety of sources (e.g. urine, faeces and specialised scent gland secretions) that are detected by elaborated olfactory systems mostly specialised in the detection of volatile molecules present in the nasal airstream (Brennan and Kendrick 2006). The main olfactory epithelium typically contains receiver proteins, such as olfactory receptors, which are expressed by olfactory sensory neurons (Zhang et al. 2004; Fleischer et al. 2009). Olfactory receptors are highly variable, consistent with the structural diversity of odour cue molecules (e.g. Emes et al. 2004; Ignatieva et al. 2014). In mammals, olfactory receptors have been mainly analysed not only in expression and repertoire studies (e.g. Feldmesser et al. 2006; Gilad and Lancet 2003; Rouquier et al. 2000; Young et al. 2003; Zhang et al. 2004) but also in evolutionary (e.g. Gaillard et al. 2004; Gilad et al. 2003; Li et al. 2015; Zhuang et al. 2009) and phylogenetic contexts (e.g. McGowen 2011). Furthermore, genetic variation in olfactory receptors may trigger mate

choice, suggesting that olfaction has undergone diversifying selection in diverging populations and may contribute to premating reproductive isolation (Li et al. 2015; Smadja and Butlin 2009).

Odour communication has been considered as part of a behavioural barrier of prezygotic reproductive isolation in rodents (e.g. Moore 1965; Nevo et al. 1976; Theiler and Blanco 1996; Kotenkova and Naidenko 1999; Stippel 2009), essential to speciation in the absence of other reproductive barriers. This seems to be the case of sister species Lusitanian pine vole *M. lusitanicus* Gerbe (1879) and Mediterranean pine vole *M. duodecimcostatus* de Selys-Longchamps (1839), two of the most recent *Microtus* species, estimated to have diverged only 60,000 years ago (Brunet-Lecomte and Chaline 1991). *M. lusitanicus* and *M. duodecimcostatus* share a considerable area of sympatry in the Iberian Peninsula (e.g. Santos 2009; Bastos-Silveira et al. 2012), and physical barriers, such as mountains and rivers, do not affect the distribution of these voles, because they inhabit low and high altitude locations (Cotilla and Palomo 2007; Mira and Mathias 2007) and are proficient swimmers (Giannoni et al. 1993, 1994). Regarding ecological/spatial isolation, *M. lusitanicus* and *M. duodecimcostatus* inhabit similar habitats, e.g. meadows, woods and agricultural areas (e.g. Cotilla and Palomo 2007; Mira and Mathias 2007), and can occur in syntopy (Duarte et al. 2015). These species present similar sexually active periods (Cotilla and Palomo 2007; Mira and Mathias 2007), indicating that temporal isolation does not play a role in preventing heterospecific copulation between both voles.

Odour cues appear to contribute to behavioural isolation between *M. lusitanicus* and *M. duodecimcostatus*. Two-way mate choice assays in a Y-shaped olfactometer with urine and faeces as stimuli revealed a preference for conspecific individuals in these sister species (Soares 2013). Using a similar methodology, odour communication was also associated to pair bonding behaviour in *M. lusitanicus* and *M. duodecimcostatus* (Duarte et al. 2015). Hence, olfactory discrimination is a potential premating reproductive isolation mechanism between these sister vole species as in other Cricetidae taxa (e.g. Moore 1965; Theiler and Blanco 1996) and rodents in general (e.g. Nevo et al. 1976; Pillay et al. 1995; Kotenkova and Naidenko 1999; Smadja and Ganem 2008; Stippel 2009).

In the present study, we chose a candidate gene approach as a first step for a molecular understanding of the potential contribution of olfactory receptors to reproductive isolation in the rapidly speciating *Microtus* genus (Fink et al. 2010; Beysard et al. 2012, 2015). Molecular data for olfactory receptors is not available for *Microtus sp.*; thus, we based our selection of candidate genes on information from *Mus musculus*, the closest animal model. We chose the class II olfactory receptors Olfr31 and Olfr57 (Glusman et al. 2000; Niimura and Nei 2007) because they are expressed in cell lines of the mouse olfactory placode, which gives rise to olfactory sensory neurons in the olfactory epithelium (Illing et al. 2002; Pathak et al. 2009). Genetic variation in these receptors may thus lead to functionally relevant variation in the body region where odour cues are primarily perceived.

Given very high levels of genetic polymorphism in the *Microtus* genus (Jaarola et al. 2004; Fink et al. 2007, 2010; Fischer et al. 2014; Lischer et al. 2014), we expected high variation in Olfr31 and Olfr57 and possibly segregating receptor types between sibling species such as *M. lusitanicus* and *M. duodecimcostatus*. If these receptors were involved in reproductive isolation through odour communication, this may lead to molecular signals of positive selection in the relevant peptides. In particular, we expected to detect positively selected amino acids in the extracellular loops and extracellular half of the transmembrane helices of Olfr31 and Olfr57, since these variable regions are responsible for the binding of odour molecules (Emes et al. 2004). Molecular signatures of adaptive evolution can be difficult to detect in very recently diverged species (e.g. Fink et al. 2007), thus we extended our analyses to cover a total of 14 European species which span most of the evolutionary divergence in the *Microtus* genus (see Fink et al. 2010).

Materials and methods

Seventy-six tissue samples from 14 European *Microtus* species (Online Resource 1) were stored in absolute ethanol at −20 °C. Genomic DNA was isolated using a phenol-chloroform extraction procedure (Sambrook et al. 1989).

Our molecular analyses targeted a part of the single exon each for Olfr31 and Olfr57 based on PCR primer pairs designed for *Mus musculus* (Pathak et al. 2009). Reactions contained 100 ng of template DNA, 0.3 mM of each primer, 1.25 U of GoTaq® Flexi DNA Polymerase (Promega), 1× buffer (Promega), 2.5 mM $MgCl_2$, 0.1 μg of BSA (New England Biolabs), and 0.2 mM of each dNTP (Thermo Scientific), to a final volume of 25 μl. PCR amplifications were performed in a MyCycler thermal cycler (Bio-Rad Laboratories Inc.) and consisted in denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min. An extension step at 72 °C for 10 min was added at the end. PCR products were verified on 1 % agarose gels and purified using ExoI/FastAP protocol (Fermentas). Sequencing using the amplification primers was carried out by Macrogen Inc. (South Korea and the Netherlands) and at the Institute of Ecology and Evolution, University of Bern, using ABI Prism® 3130 Genetic Analyzer (Applied Biosystems).

DNA sequences were aligned using Sequencher 4.8 (Gene Codes Corporation). JModelTest 0.0.1 (Posada 2008) was used to select the best-fitting model of nucleotide substitution

(TPM1uf+I, Kimura 1981) based on the Akaike information criterion (Akaike 1974). We applied a recent approach to integrate heterozygous information in existing phylogenetic programs by repeated random haplotype sampling (Lischer et al. 2014). This method generates haploid sequences for each individual by randomly selecting a haplotype from the detected alternative alleles at each position. A tree is then inferred and the process of haplotype generation and tree inference is repeated multiple times, from which a majority rule consensus tree is generated that covers the full extent of allelic and haplotypic variation. Thus, this approach tries to avoid an underestimation of sequence divergence and branch length in the constructed phylogenetic tree (see Lischer et al. 2014). Both Olfr31 and Olfr57 alignments were subjected to $n = 10.000$ replicates for the maximum likelihood analysis (RAxML) (Stamatakis 2014), and $n = 20$ replicates, nchains = 4, ngen = 2.000.000 and mcmc burn-in = 500.000 for the Bayesian inference analysis (MrBayes) (Ronquist and Huelsenbeck 2003). The outgroup chosen for both genes was *Mus musculus*. Consensus trees were edited using FigTree version 1.3.1.

DNA polymorphism parameters were estimated using DnaSP version 5.10.1 (Librado and Rozas 2009). Between species pairwise divergences were calculated using the TrN+I+G (Olfr31) and TrN models (Olfr57) (Tamura and Nei 1993) implemented in MEGA version 5.1 (Tamura et al. 2011), with standard deviations estimated from 10,000 bootstrap replicates. Recombination was inferred using methods implemented in the HyPhy package (Pond et al. 2005) web interface DataMonkey (Delport et al. 2010) and RDP 4 (Martin et al. 2010).

We tested for positive selection using the CodeML subroutine included in PAML 4.8 (Yang 1997, 2007). Maximum likelihood estimations of ω (non-synonymous/synonymous substitution rates) among codons were generated according to six models: M0 (one ω), M1 (nearly neutral), M2 (positive selection), M3 (discrete), M7 (nearly neutral with beta distribution approximating ω variation) and M8 (positive selection with beta distribution approximating ω variation) (Goldman and Yang 1994; Yang et al. 2000, 2005). Additionally, branch-site models were tested in order to allow ω variation among amino acids in the protein and across branches on the phylogenetic tree and thereby detect possible positive selection affecting a few sites along particular lineages (Yang 1998; Yang and Nielsen 1998). We compared the null (model = 2; NSsites = 2; $\omega = 1$) and neutral M1a (model = 0; NSsites = 1; $\omega = 1$) models to MA1 (model = 2; NSsites = 2; ω estimated). Likelihood ratio tests (LRT) of M0 vs. M3, M1 vs. M2, M7 vs. M8, null model vs. MA1 and M1a vs. MA1 were performed in order to determine the most likely model (Nielsen and Yang 1998; Yang et al. 2000). Positively selected sites under M2, M3, M8 and MA1 were identified using the Naive Empirical Bayes and the Bayes Empirical Bayes analysis (Yang et al. 2005). Since the power of CodeML can be affected by the accuracy of the input phylogenetic tree (Anisimova et al. 2003), we combined PAML results with HyPhy selection detection methods: SLAC (Pond and Frost 2005), FEL (Pond and Frost 2005), IFEL (Pond et al. 2006) and MEME (Murrell et al. 2012). Due to alignment size restrictions, it was not possible to test REL (Pond et al. 2005) and branch-site REL (Pond et al. 2011).

Results and discussion

Amplifications were successful for most species, with the exception of *M. gerbei* and *M. tatricus* for which Olfr57 could not be amplified (Online Resource 2). This positive outcome suggests that these olfactory receptors may be also fruitful as molecular markers for other *Microtus* taxa (Cricetidae), or even other eumuroids, considering that the primers used were designed for the mouse model (Pathak et al. 2009), which belongs to a different family (Muridae). For Olfr31, we obtained a 352-bp fragment corresponding to *Mus musculus* Olfr31 position 225–576. For Olfr57, we amplified a 488-bp fragment equivalent to *Mus musculus* Olfr57 position 324–811. A tight homology to *Mus musculus* DNA sequences, including two characteristic sequence motifs (transmembrane domain 3 MAYDRYVAIC for Olfr31 and Olfr57, and transmembrane domain 6 KAFSTCASH for Olfr57), and an absence of stop codons and indels indicate that these gene fragments are functional olfactory receptors and do not correspond to pseudogenes (e.g. Malnic et al. 2004). Olfr31 and Olfr57 sequences were collapsed into 31 and 16 unphased diploid genotypes, respectively (Online Resource 2). Considering the full European *Microtus* set, nucleotide diversity and number of variable and parsimony informative sites are higher for Olfr57 than for Olfr31 (Online Resource 2). The same does not apply when considering the *M. lusitanicus* and *M. duodecimcostatus* subsets alone (Online Resource 2). We have deposited the obtained genotypes into GenBank (accession numbers KU172584-KU172615 for Olfr31 and KU172616-KU172632 for Olfr57). These are the first contributions of DNA sequences of Olfr31 and Olfr57 from non-model vertebrates and of olfactory receptor genes in general for *Microtus* sp. The limited available data are only from mouse transcriptome repertoire studies (e.g. Young et al. 2003).

A total of seven haplotypes were shared by more than one *Microtus* species, four for Olfr31 and three for Olfr57 (Figs. 1 and 2). These repeated random haplotypes were generated in order to integrate Olfr31 and Olfr57 heterozygous sites in our phylogenetic analyses (see Lischer et al. 2014). Considering Olfr31, two haplotypes were shared by the sister species *M. lusitanicus* and *M. duodecimcostatus*, another one by *M. lusitanicus*, *M. duodecimcostatus* and the other *Terricola*

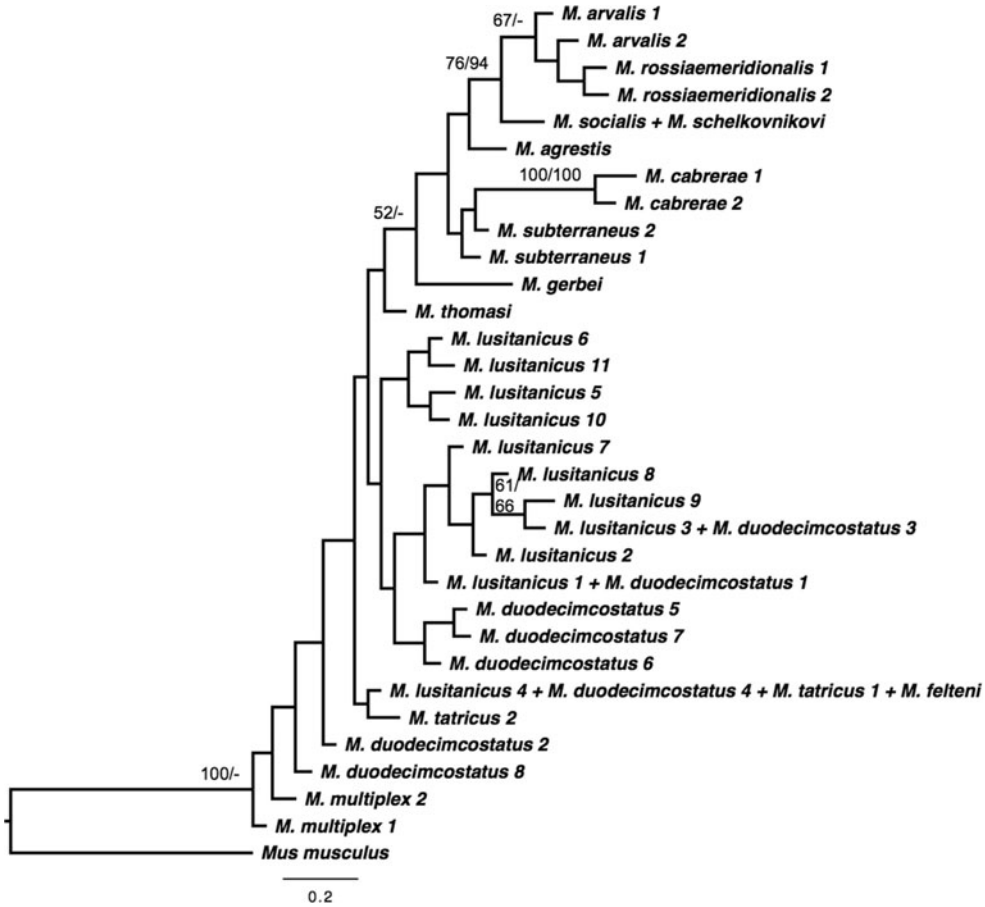

Fig. 1 Bayesian inference phylogenetic tree obtained for the Olfr31 gene fragment. Posterior probability (Bayesian inference) and bootstrap (maximum likelihood) values >50 % are indicated

voles *M. tatricus* and *M. felteni*, and the fourth by *M. socialis* (*Hyrcanicola*) and *M. schelkovnikovi* (*Microtus*) (Fig. 1). Analogously, for Olfr57, *M. lusitanicus* and *M. duodecimcostatus* shared two haplotypes, and *Terricola M. multiplex* and *M. subterraneus* presented a common haplotype (Fig. 2). The presence of shared haplotypes also supports the close evolutionary relationship between the species in the *Terricola* subgenus, particularly the recently diverged *M. lusitanicus* and *M. duodecimcostatus*. Consistent with previous studies (Jaarola et al. 2004; Bastos-Silveira et al. 2012; Barbosa et al. 2013), genetic divergence between both species was relatively low: 0.2 % for Olfr57 and 0.5 % for Olfr31 (Online Resource 3). The highest genetic divergence involved species from older *Microtus* lineages (Fink et al. 2010): *M. cabrerae* for Olfr31 (2–3.5 %) and *M. agrestis* for Olfr57 (3.5–4.6 %) (Online Resource 3).

Maximum likelihood and Bayesian inference tree topologies were congruent for each of the respective data sets (we only present the latter, Figs. 1 and 2). Phylogenetic trees did not reflect the taxonomy attributed at the subgenera level, nor the geographic origin.

We did not find signs of recombination in the analysed Olfr31 and Olfr57 gene fragments. Considering PAML and HyPhy branch-site models, branch-site REL was the only method that indicated a branch under episodic diversifying selection ($p<0.05$), corresponding to evolutionarily early divergent *M. agrestis* for the Olfr57 fragment gene. For both genes, LRTs of site and branch-site models supported equal substitution rates and ω ratios suggest that the analysed gene fragments are mostly under negative/purifying selection ($\omega<1$) (Online Resource 4). PAML and HyPhy detected more negatively than positively selected amino acids (Fig. 3; Online Resource 4). With the *Mus musculus* protein sequence as a reference, models M2, M8 and MEME indicated positive selection for amino acid 145 of Olfr31. For Olfr57, M2, M8 and MEME identified amino acid 220, plus 154 and 227 that were only observed in the M8 model (Online Resource 4). Amino acid 145 is located in the fourth transmembrane helix of Olfr31, while residues 154, 220 and 227 of Olfr57 are in the fourth and fifth transmembrane helices and third intracellular loop, respectively (Fig. 3). These sites were not significant for Naive Empirical Bayes or Bayes Empirical Bayes analysis. Regarding negatively selected sites in Olfr31, three were indicated by SLAC, seven by REL, 11 by FEL and two by IFEL; however, only amino acids 170 and 180 were

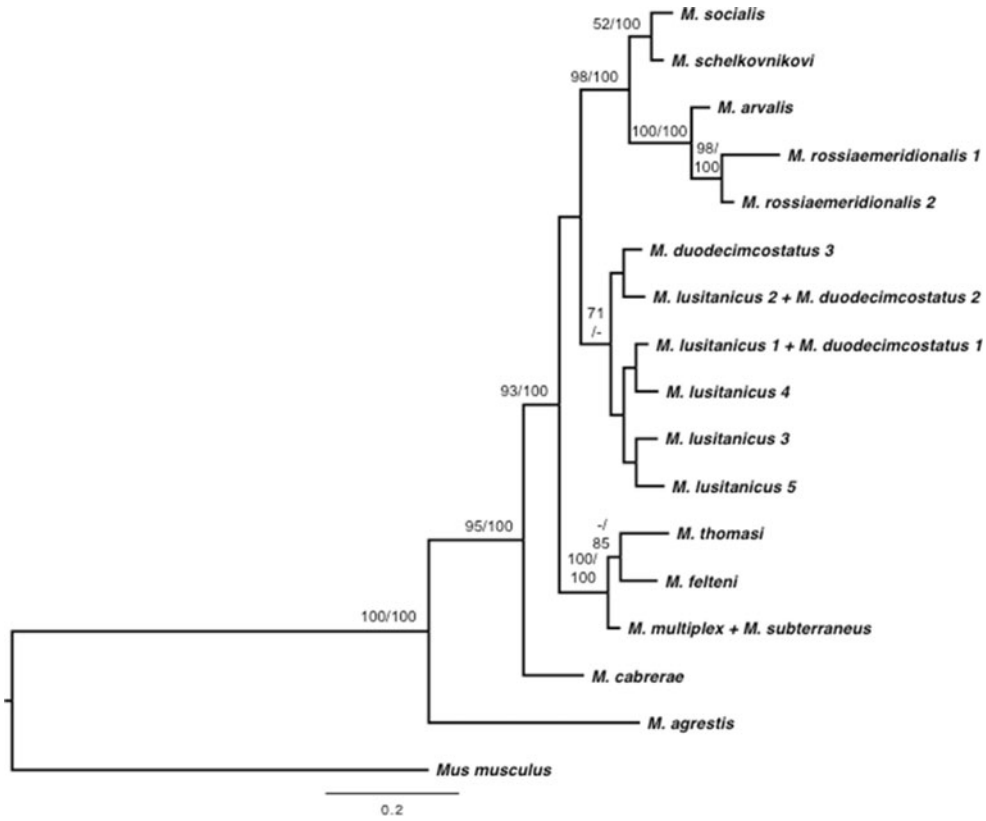

Fig. 2 Bayesian inference phylogenetic tree obtained for the Olfr57 gene fragment. Posterior probability (Bayesian inference) and bootstrap (maximum likelihood) values >50 % are indicated

common amongst all methods (Online Resource 4). Both residues are located in the second extracellular loop, and amino acid 180 is also in motif 3 of the olfactory receptor signature (Fig. 3). This signature is composed by five conserved motifs that provide a characteristic fingerprint for olfactory receptors. For Olfr57, one site was indicated by SLAC, 14 by FEL and one by IFEL, with amino acid 241 being detected by all tests (Online Resource 4). This residue is located in the sixth transmembrane helix, in motif 4 of the olfactory receptor signature (Fig. 3). In the extracellular loops of Olfr31 and Olfr57, only negatively selected sites were detected, whereas on the transmembrane helices, both positively and negatively selected amino acids were revealed (Fig. 3). Considering the intracellular loops, only negatively selected sites were identified for Olfr31, whilst for Olfr57, both positively and negatively selected amino acids were found (Fig. 3). Nevertheless, we have to consider that these selection tests may have a limited statistical power due to limited size of the DNA sequences analysed (e.g. Yang and dos Reis 2011; Jobling et al. 2014).

A comparison between *Mus musculus* and *Microtus* sp. amino acid sequences revealed a majority of conserved residues between mouse (Muridae) and *Microtus* voles (Cricetidae) (Online Resource 5). For Olfr31, only six polymorphic amino acids (with two being *Microtus*-specific), associated to five amino acid sequences, were detected in a total of 117. For Olfr57, we uncovered 18 variable residues (with 13 being *Microtus*-specific), linked to six amino acid sequences, out of 162 residues (Online Resource 5). All amino acid positions that are polymorphic for Olfr31, beside positively selected 145, and half of Olfr57 polymorphic residues (154—positively selected, 155, 161, 164, 166, 188, 195, 206 and 265) are located at the extracellular loops or extracellular half of the transmembrane helices (Online Resource 5).

Contrary to our expectation regarding the location of positive selection in the variable regions responsible for the binding of odour cue molecules (Emes et al. 2004), we only found negatively selected residues in the extracellular loops of Olfr31 and Olfr57, and more negatively selected amino acids than positively selected ones in the transmembrane helices. The present results suggest that Olfr31 and Olfr57 probably recognise conserved odour cues, with very low or inexistent interspecific variation among the analysed *Microtus* sp.

Our results seem to indicate that Olfr31 and Olfr57 are not related to premating behavioural isolation between *M. lusitanicus* and *M. duodecimcostatus*. Haplotype sharing between these two sister species reduces the chance that sequence polymorphisms in these

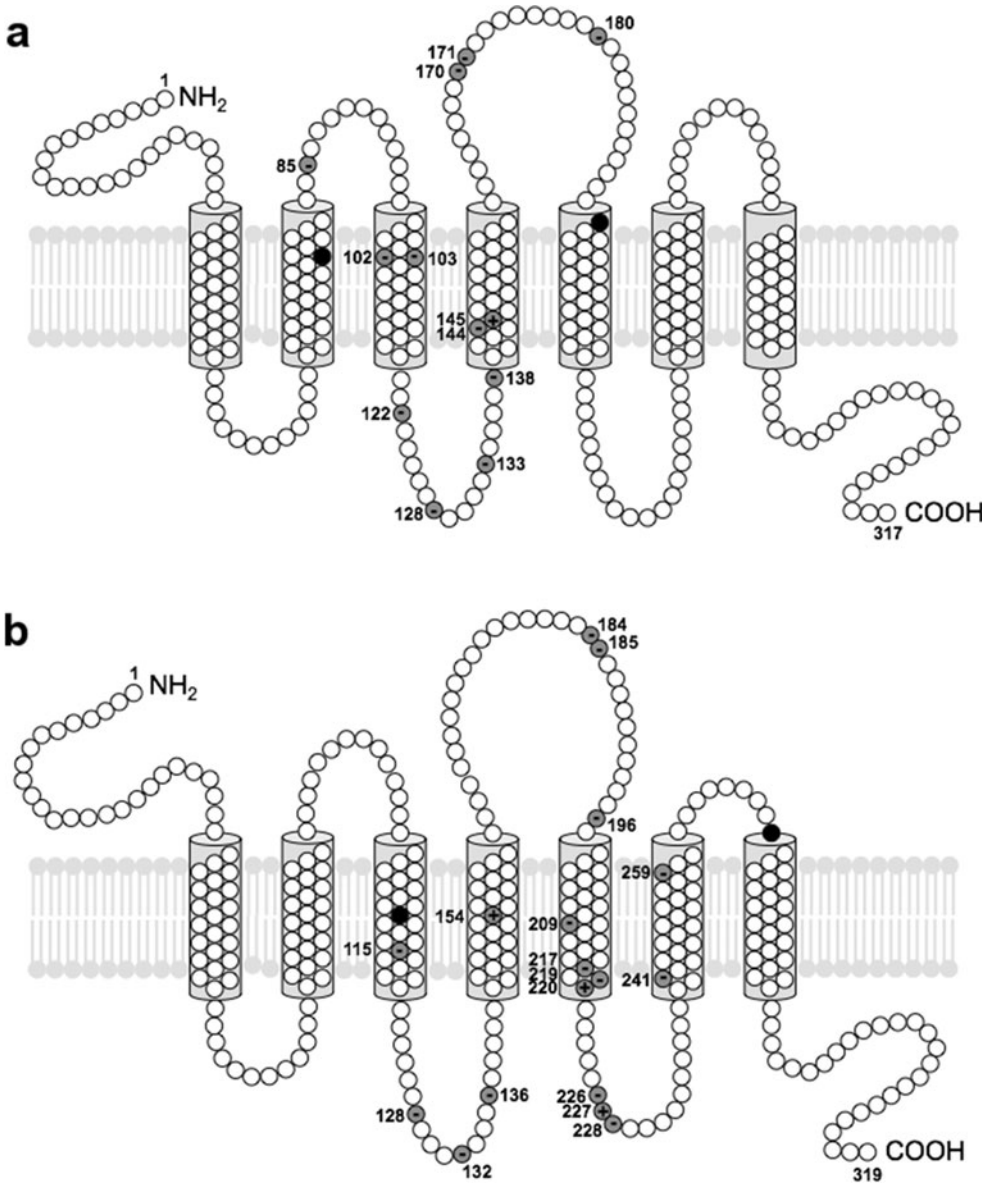

Fig. 3 Schematic amino acid model of Olfr31 (**a**) and Olfr57 (**b**) proteins, using *Mus musculus* as reference. Positively and negatively selected amino acids are highlighted as the respective position in the expressed proteins. Beginning and end of the amplified gene fragments (*black circle*), positively selected amino acid (*grey circle with a plus sign*), and negatively selected amino acid (*grey circle with a minus sign*)

markers could lead to subtle changes in olfactory perception and influence subsequent specific behaviours. These two markers did also not present species-specific polymorphisms for the other *Microtus* taxa analysed. Considering these results, two hypotheses emerge: (i) Olfr31 and Olfr57 may not contribute to behavioural barriers mediated via odour, and (ii) the expression of Olfr31 and Olfr57 may better reveal the contribution of these receptors than DNA sequence polymorphism data. Thus, considering that hundreds of olfactory receptor genes were detected in the olfactory epithelium of *Mus musculus* (Young et al. 2003), it is pertinent to consider a protein expression approach as the next step. This could be performed in the olfactory epithelium of different *Microtus* taxa, particularly those under ongoing speciation events (e.g. Gileva et al. 2000; Castiglia et al. 2008; Bastos-Silveira et al. 2012; Sutter et al. 2013; Beysard and Heckel 2014). Expression variation of the receptors in the olfactory epithelium under controlled conditions could indicate an interspecific difference of responsiveness of the transduction of chemosignals that are associated with reproductive behaviours, i.e., higher expression levels could indicate higher sensitivity to small changes in the quantity of odour cues. At present, such analyses are experimentally highly challenging (e.g. Rice et al. 2011; Hohenbrink et al. 2014), particularly if controlled laboratory experiments are combined with ecological testing, but they could provide major insights into the role of olfactory receptors on behavioural isolation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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