ORs and gustatory receptors (GRs) of insects were originally classified as GPCRs (Clyne et al. 1999, 2000). However, in Drosophila, it has recently been found that the vertebrate model of protein structure does not apply for ORs. These form dimers with a co-expressed receptor, OR83b, and the membrane orientation of the ORs and OR83b is reversed with the NH2-tail intracellular and the COOH-tail extracellular (Benton et al. 2006; Wistrand et al. 2006; Lundin et al. 2007). Dimerization of ORs occurs through coupling of the COOH-terminal region with OR83b, and interactions between the third cytoplasmic loop of ORs and OR83b imply that this is a conserved mechanism of the Drosophila OR family (Benton et al. 2006).

The insect GRs are closely related to the ORs (Robertson et al. 2003), with some GRs also expressed in the antenna (Jones et al. 2007). However, GR neurons do not express OR83b and responses to ligands are independent of OR83b expression (Benton et al. 2006). Recent studies of signal transduction in Drosophila ORs have shown that they serve as ion channels and as a GPCR, suggesting that the signal transduction consists of a fast and transient ion conductance and a long-lasting non-selective cation conductance (Sato et al. 2008; Smart et al. 2008; Wicher et al. 2008).

The mechanism of ligand recognition by insect ORs and GRs is unknown. By analogy with mammals, it was hypothesized that odorants are recognized at the sensillum pore and transported to underlying ORs by small soluble odorant-binding proteins, present in the lymph surrounding receptor neurons (Hekmat-Scafe et al. 2002).

Twelve Drosophila genome sequences are available (Consortium 2007), allowing analyses of the evolution of chemosensory genes across species that differ in chemosensory-driven behaviours such as oviposition and mate choice. Gene duplication and pseudogenization show patterns possibly related to the degree of ecological specialization (McBridge & Arguello 2007) or population biology (Gardiner et al. 2008). Which regions of the chemosensory receptors show evidence of evolutionary constraint or divergence? Functionally important regions may be inferred from sequence conservation. Divergence will be minimal in constrained regions, but relatively high functional divergence rates can occur through either relaxed constraints or positive diversifying selection (Nielsen 2005). Here, we investigate the distribution of amino acids that show accelerated rates of evolutionary divergence of ORs and GRs in the 12 Drosophila species.

2. MATERIAL AND METHODS

Homologues of the ORs and GRs of Drosophila melanogaster were identified and aligned as previously reported (Gardiner et al. 2008). Orthologues were tested for selection using the M1–M2 and M7–M8 models of the codeml program in PAML (Yang 1997). M1–M2 comparisons are very stringent and can lack power to detect signatures of diversifying selection; these were not significant for any locus. M7 and M8 impose less constraint on the distribution of \( \omega \) with M8 having the most power to detect significant signatures of diversifying selection on a fraction of sites (Anisimova et al. 2002). When M7–M8 comparisons were significant (\( p < 0.05 \)), we used M8a (\( \omega = 1 \)) and M8 (\( \omega \) free) models to investigate the potential roles of reduced purifying selection versus positive selection (Swanson et al. 2003). The Bayes empirical Bayes
(BEB) method was used to calculate the posterior probability (PP) that each codon is from a class of sites under diversifying selection in the M8 model (Yang et al. 2005).

Sites identified with PP > 0.50 were analysed; we refer to these as ‘candidate sites’ that show accelerated divergence, either under positive selection or relaxed purifying constraints (in cases when the evidence for selection was not significant in the M8a–M8 tests). We also compared the distribution of sites with a higher PP (above 0.80), increasing the stringency. Corrections for multiple tests have not been applied, so that these should be viewed as a class of candidate sites for which there is some evidence of divergence.

We used information on domain structure of *Drosophila* GRs and ORs at the BAPASy proteomics server (http://ca.expasy.org/) and predicted the secondary structure and membrane topology using TMHMM (Krogh et al. 2001) and Tmpred (Hofmann & Stofleth 1993).

3. RESULTS

Previously (Gardiner et al. 2008), we detected that there is evidence (p < 0.05) of positive selection overall for 10 Gr and 10 Or genes (based on significant M7–M8 comparisons; see also table 2 in the electronic supplementary material), containing 8141 amino acids. Within these, BEB identified 67 GR and 74 OR amino acids (see table 1 in the electronic supplementary material). Approximately 55 per cent of these sites have PP ranging from 0.50 to 0.70, and the remainder have PP higher than 0.70, approximately 9 per cent of sites have PP > 0.90.

Seven TM domains were identified in all ORs and GRs. The TMHMM and Tmpred algorithms predicted an intracellular orientation of the NH2-tails and an extracellular orientation of the COOH-tails, supporting the ‘reverse’ membrane topology of *Drosophila* receptors. The majority of candidate sites in ORs are found in loop regions (approx. 68%), approximately 16 per cent of sites are located in TM regions (mainly in TM1 and TM4), and 15 per cent of sites are located on the cytoplasmic NH2-termini (figures 1a and 2), in general agreement with Guo & Kim (2007). Sites mapped to the loops have a surprising distribution; approximately 13 per cent of candidate sites were found in the ELs and 55 per cent mapped to the ILs, where we found them mostly in the IL1 (17 sites) and IL2 (18 sites).

Candidate sites in GRs show a different pattern with fewer found in the loop regions (43%) and more on the NH2 (22%) and COOH (21%) tails of the protein (figures 1a and 2). The sites mapped to the loops are nearly equally distributed between the extracellular (19% of sites) and intracellular surfaces (24% of sites); most sites were found in the EL2 (7 sites) and IL2 (10 sites). Approximately 13 per cent of sites mapped to TMs, mostly in TM1. The distribution of candidate sites among regions differed between the receptor types (G4 = 31.43, p < 0.0001).

Because the number of amino acids differs between regions and loci, we also analysed the proportion of sites per locus showing evidence of selection, using a binomial logistic regression, effectively controlling for the number of amino acids within a region. The incidence of potential selection varied between regions (deviance ratio = 9.51, $\chi^2_{4,0.05} < 0.001$), and there was a significant interaction between region and receptor type (deviance ratio = 10.08, $\chi^2_{4,0.05} < 0.001$). The main differences were that amino acids were more likely to be identified in the COOH-tail of GRs and in the IL1–3 regions of ORs (figure 1b).

We analysed the distribution of sites with PP > 0.80 (19 OR and 16 GR sites). Again, the majority of sites (approx. 53%) mapped to the ILs in the ORs, but only approximately 15 per cent of sites mapped to the GRs. We did not find candidate sites on the COOH-tail in the ORs, but approximately 31 per cent of GR candidate sites mapped here.

After testing the strength of selection with the stringent M8a model, we find that only six genes (four Ors and two Grs) show significant evidence of diversifying selection (see table 2 in the electronic supplementary material). More than half of the candidate sites in these OR loci mapped to the ILs.

4. DISCUSSION

We detected evidence of increased functional divergence at 20 loci, but only six showed a signal of diversifying selection. Thus, a proportion of the candidate sites identified for these six loci have diverged under positive selection, but, for the rest of the genes, rapid divergence was probably due to relaxed purifying constraints. In *Drosophila* ORs, the majority of candidate sites were in the ILs. This is unexpected because these regions are likely to interact with secondary messengers and other proteins involved in signal transduction. We predicted these to be under constraint rather than diversifying or relaxed selection. The COOH-tail of ORs is essential for coupling with OR83b, and highly divergent OR proteins share the strongest similarity within the
COOH-termini (Benton et al. 2006). None of the candidate sites mapped to the COOH-tail. Only a few mapped to IL3, which interacts with IL3 of OR83b during dimerization (Benton et al. 2006), and therefore is expected to be under constraint. The majority of candidate sites mapped to IL1 and IL2, which do not interact with OR83b, and their role in odour recognition or signal transduction are unknown. The prevalence of divergent sites here may mean that relaxed constraints act on these regions; however, Or genes that showed the strongest signal of positive selection also had most sites within IL1 and IL2.

Surprisingly, the distribution of candidate sites in GRs differed from ORs, despite similar secondary structures. More sites mapped on the COOH-tail and fewer in the ILs in GRs. Evidence of positive selection was weakest for Gr genes (M8a and M8 comparisons were significant only for two Gr genes). Thus, more rapid divergence was probably due to relaxed purifying constraints. In this case, the prevalence of the candidate sites on the COOH-tail in GRs indicates that this domain is subject to less constraint than in ORs. Despite Drosophila GRs being evolutionarily related to the ORs, there are significant functional differences between these receptors. The olfactory system recognizes volatile ligands through the expression of ORs in the antenna and maxillary palps. GRs mediate response to soluble compounds and pheromonones (Thorne et al. 2004), CO₂ (Jones et al. 2007), and can be expressed in the central nervous system, possibly indicating non-gustatory roles (Thorne & Amrein 2008). The Drosophila olfactory system functions through OR/OR83b complexes, while GRs function independently of OR83b (Benton et al. 2006), suggesting that ORs and GRs have independent molecular properties as well as different functions.

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