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DOI: 10.1534/genetics.107.079731

Association of Polymorphisms in Odorant-Binding Protein Genes With Variation in Olfactory Response to Benzaldehyde in Drosophila

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Manuscript received July 30, 2007
Accepted for publication August 23, 2007

ABSTRACT

Adaptive evolution of animals depends on behaviors that are essential for their survival and reproduction. The olfactory system of *Drosophila melanogaster* has emerged as one of the best characterized olfactory systems, which in addition to a family of odorant receptors, contains an approximately equal number of odorant-binding proteins (OBPs), encoded by a multigene family of 51 genes. Despite their abundant expression, little is known about their role in chemosensation, largely due to the lack of available mutations in these genes. We capitalized on naturally occurring mutations (polymorphisms) to gain insights into their functions. We analyzed the sequences of 13 *Obp* genes in two chromosomal clusters in a population of wild-derived inbred lines, and asked whether polymorphisms in these genes are associated with variation in olfactory responsiveness. Four polymorphisms in 3 *Obp* genes exceeded the statistical permutation threshold for association with responsiveness to benzaldehyde, suggesting redundancy and/or combinatorial recognition by these OBPs of this odorant. Model predictions of alternative pre-mRNA secondary structures associated with polymorphic sites suggest that alterations in *Obp* mRNA structure could contribute to phenotypic variation in olfactory behavior.

INTERACTIONS with the chemical environment provide a sensitive target for forces of natural selection, as evident from the rapid evolution of odorant receptors (Young *et al.* 2002; Robertson *et al.* 2003). Drosophila provides an excellent model system for studies of olfaction due to its well-established genetics and the relative simplicity of its olfactory system. Although numerically simpler than the mammalian olfactory system in terms of the number of olfactory sensory neurons, the functional organization of the olfactory system of Drosophila is similar (Hallem *et al.* 2004). Olfactory sensory neurons in sensilla of the third antennal segments or maxillary palps form convergent projections onto output neurons in ∼43 glomeruli in the antennal lobe (Vosshall *et al.* 2000). Individual neurons express one, or rarely two, odorant receptors from a repertoire of 62 *Or* genes (Clyne *et al.* 1999; Vosshall *et al.* 1999). Uniquely expressed odorant receptors dimerize with the common *Or83b* receptor, which is essential for transport and insertion of odorant receptors in the chemosensory dendritic membrane (Larsson *et al.* 2004; Benton *et al.* 2006). Olfactory sensory neurons that express the same odorant receptor converge on the same antennal lobe glomerulus (Gao *et al.* 2000; Vosshall *et al.* 2000; Bhalerao *et al.* 2003).

Odorants must dissolve in the aqueous perilymph to reach their cognate membrane-associated odorant receptors. Their solubilization and transport is thought to be mediated by odorant-binding proteins (OBPs) that are secreted by support cells. The Drosophila genome encodes 51 OBPs with different spatial patterns of expression (McKenna *et al.* 1994; Pikielny *et al.* 1994; Galindo and Smith 2001), which contain a characteristic structural signature of conserved cysteines (Graham and Davies 2002; Hekmat-Scafe *et al.* 2002).

Altered regulation of expression of different subsets of OBPs has been observed following mating (McGraw *et al.* 2004), exposure to starvation stress (Harbison *et al.* 2005), during the development of alcohol tolerance after exposure to alcohol (Morozova *et al.* 2006), as a correlated response to artificial selection for divergent levels of copulation latency (Mackay *et al.* 2005) and aggression (Edwards *et al.* 2006), and as a consequence of pleiotropic effects arising from single *P*-element-induced mutations that affect olfactory behavior (Anholt *et al.* 2003).

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. EU088428–EU089648 for *Drosophila melanogaster* sequences and EU089649–EU089661 for *D. simulans* sequences.

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Whereas the role of OBPs in pheromone recognition has been clearly defined for several insect systems, the precise functions of these abundantly expressed proteins in olfaction remain obscure. Ligand specificities (whether broadly or narrowly tuned), interactions with odorant receptors (for which there remains scant evidence to date), interrelationships among OBPs with overlapping molecular-response profiles and their functional correspondence (if any) with odorant receptors, the significance of altered expression of some OBPs in aggression (Edwards *et al.* 2006), mating behavior (McGraw *et al.* 2004; Mackay *et al.* 2005), and alcohol sensitivity (Morozova *et al.* 2006), all pose as yet unresolved questions. To date, only one OBP in *Drosophila melanogaster*, encoded by *Lush*, has been characterized functionally (Kim *et al.* 1998; Xu *et al.* 2005). Flies homozygous for a deletion of the *Lush* gene do not avoid repellant concentrations of short-chain alcohols (Kim *et al.* 1998) and do not respond behaviorally or electrophysiologically to the aggregation pheromone 11-*cis*-vaccenyl acetate (Xu *et al.* 2005). The Or67d receptor, expressed in a subset of trichoid sensilla, has been identified as the receptor for 11-*cis*-vaccenyl acetate (Ha and Smith 2006; Kurtovic *et al.* 2007). Lush appears to be essential for delivering this pheromone to its receptor. Other insights into the functions of OBPs come from a recent study reporting that a polymorphism in *Obp57e* in *D. sechellia* determines preference for its host plant, *Morinda citrifolia*, and that *D. melanogaster* knock-out flies for *Obp57e* and *Obp57d* showed altered behaviors to hexanoic and octanoic acid produced by this plant (Matsuo *et al.* 2007).

Functional studies on OBPs have been hampered by the lack of *Obp* mutants, with the exception of *Lush*. Furthermore, if odorant recognition by OBPs is combinatorial, as is the case for odorant recognition by mammalian odorant receptors (Malnic *et al.* 1999), functional redundancy may render a laborious "one-gene-at-a-time" approach less than satisfactory, as it would provide only partial insights into the role of any one member of this multigene family in mediating olfactory behavior.

We devised a strategy to overcome these challenges by taking advantage of naturally occurring mutations that have arisen during evolution and that segregate as polymorphic variants in nature. We established isofemale lines from a natural population and inbred them for 20 generations, thus minimizing genetic variation within lines while retaining naturally occurring variation among the lines. We sequenced 13 *Obp* genes, located in two chromosomal clusters on the second and third chromosome, and used statistical tests for deviations from neutrality to assess patterns of selection. We then assessed whether polymorphisms in these 13 *Obp* genes were associated with naturally occurring variation in olfactory response to a standard test odorant, benzaldehyde. Finally, we show that single nucleotide polymorphisms (SNPs) in regulatory and coding regions that are associated with variation in chemosensory behavior can impact the predicted structure of pre-mRNA.

MATERIALS AND METHODS

Drosophila stocks: Isofemale lines were created from flies collected from a natural Raleigh (NC) population in 2002 and inbred by 20 generations of full sib mating to create 193 inbred lines. Flies were reared on cornmeal-molasses-agar medium under standard culture conditions of 25°, 70% humidity, and a 12-hr light/dark cycle.

OBP sequences: Genomic DNA was extracted with the Puregene DNA extraction kit (Gentra, Minneapolis) and PCR primers were designed to amplify overlapping coding regions and 5′- and 3′-untranslated regions of the *Obp* genes (*Obp56a–i* and *Obp99a–d*) for *Drosophila melanogaster* and for a *D. simulans* line originally collected in Florida City (FL) by Jerry Coyne. PCR products were purified using Qiaquick columns (QIAGEN, Valencia, CA) and amplified samples were sequenced. Sequences were aligned with the Vector NTI Suite 9.0 program (Informax, Frederick, MD) to identify polymorphic sites. Singletons were excluded from the association and linkage disequilibrium (LD) analyses.

Molecular population genetics: Neutrality tests were performed using the DnaSP 4.10.3 program (Rozas *et al.* 2003) (http://www.ub.es/dnasp). *D. simulans* sequences were compared to the sequences from the *D. melanogaster* population for the HKA test (Hudson *et al.* 1987) and the McDonald–Kreitman test (McDonald and Kreitman 1991). Estimates of Tajima's D (Tajima 1993), Fu and Li's D^* and F^* (Fu and Li 1993), and Fay and Wu's H (Fay and Wu 2000) take into account the calculated population recombination rate (Hudson 1987). Coalescent simulation was used to estimate P-values (two-tailed tests) with 10^3 coalescent simulations of an infinite site locus conditioned on the sample size; these simulations are implemented for a fixed number of segregating sites. LD between SNPs was analyzed using TASSEL 2.0 software (http://sourceforge.net/projects/tassel). Fisher's exact test was used to determine whether the pairs of sites were in significant LD.

Behavioral assays: Olfactory behavior was quantified by measuring responses to the standard odorant, benzaldehyde, in a well established "dipstick" assay that we (Anholt *et al.* 1996; Mackay *et al.* 1996; Fanara *et al.* 2002) and others (Devaud 2003; Stockinger *et al.* 2005) have used previously. Pilot experiments on 5 of the lines over a range of benzaldehyde concentrations established that a concentration of 3.5% (v/v) provided optimal resolution for evaluating variation in olfactory behavior in these lines. We measured olfactory behavior of 4–10 day-old non-virgin flies from 193 wild-derived inbred lines in single-sex groups of five flies/replicate and 10 replicates/sex. All assays were conducted between 2:00 and 4:00 pm in a behavioral room at 25° and 70% humidity under white light. The experimental design was randomized such that measurements on individual lines were collected over several days to average environmental variation. Theoretically a score of 2.5 reflects indifference to the odorant. Note, however, that the precise determination of the boundary between indifference and attraction or avoidance is determined statistically when for example the distribution of scores from mutants is compared to that of a control.

Locomotor reactivity was assessed by Jordan *et al.* (2007) by subjecting single flies to a mechanical disturbance by tapping the vial twice against a table and recording the amount of time the fly is active in the 45 sec immediately following the disturbance.

Quantitative genetic analysis of olfactory behavior: We used ANOVA to partition sources of variation in olfactory behavior according to the model $Y = \mu + L + S + L \times S + E$, where μ is the overall mean, L is the random effect of line, S is the fixed effect of sex, $L \times S$ is the random effect of line by sex, and E is environmental error. The total genotypic variance among lines was estimated as $\sigma^2_G = \sigma^2_L + \sigma^2_{LS}$, where σ^2_L is the among-line variance component and σ^2_{LS} is the variance for the line-by-sex interaction. The total phenotypic variance was estimated as $\sigma^2_P = \sigma^2_L + \sigma^2_{LS} + \sigma^2_E$, where σ^2_E is the environmental variance component. Broad-sense heritability was estimated as $H^2 = (\sigma^2_L + \sigma^2_{LS})/(\sigma^2_L + \sigma^2_{LS} + \sigma^2_E)$ (Carbone *et al.* 2006). Narrow-sense heritability was estimated as $h^2 = \sigma^2_A/(\sigma^2_A + \sigma^2_E)$, where $\sigma^2_A = 0.5(\sigma^2_L + \sigma^2_{LS})$ (Falconer and Mackay 1996). The genetic correlation between males and females was calculated as $r_{MF} = \mathrm{cov}_{MF}/\sigma_M\sigma_F$, where cov_{MF} is the covariance of line means for the two sexes and σ_M and σ_F are the square roots of the genotypic variances for each sex. Analyses of variance and tests of significance were calculated using the Proc GLM procedure, and variance components were estimated using the Proc VARCOMP procedure in SAS (SAS Institute, Cary, NC).

Genotype–phenotype associations: Association between polymorphisms and line means for olfactory behavior were analyzed using two way factorial ANOVA with the model $Y = \mu + S + M + M \times S + E$, in which μ is the overall mean, marker (M) and sex (S) are fixed effects, and E indicates error variance. We used permutation tests to determine random distributions under the null hypotheses of no association between *Obp* genotypes and olfactory behavior in response to benzaldehyde (Churchill and Doerge 1994). We performed two different permutation tests. To assess whether we observed more significant associations with olfactory behavior for each gene than expected by chance, we permuted the phenotypes among the markers 1000 times and recorded the number of significant associations at $P < 0.05$ for each permuted data set. To identify particular polymorphic sites with significant associations with behavior, we similarly permuted the data 1000 times and recorded the lowest P-value of each permuted data set. In both cases we used the 5% significance threshold of the permuted data sets to give an empirical type I error rate that accounts for multiple tests.

In cases where more than one polymorphism in a gene was associated with olfactory behavior, we tested for associations between haplotypes of these variants and line means for olfactory behavior by two-way ANOVA with the model $Y = \mu + S + H + H \times S + E$, in which haplotype ($H$) and sex ($S$) are fixed factors and E indicates error. We conducted *post hoc* analyses using least-square means to assess the effect of haplotypes and Tukey tests to control the experimentwise error rate.

The additive variance attributable to a marker polymorphism (σ^2_{AM}) was estimated as $\sigma^2_{AM} = 2pqa^2$, where a is one-half the difference in mean olfactory behavior between homozygous genotypes for the marker, q is the frequency of the rare marker allele, and $p = 1 - q$ (Falconer and Mackay 1996).

Prediction of RNA secondary structure: Secondary structures of the full length mRNA and pre-mRNA molecules transcribed from *Obp99a*, *Obp99c*, and *Obp99d* genes were predicted using Mfold and Afold, as described previously (Nackley *et al.* 2006; Shabalina *et al.* 2006). Energy minimization was performed by a dynamic programming method that finds the secondary structure with the minimum free energy with sums composed of factors that include stacking and loop length (Nackley *et al.* 2006; Shabalina *et al.* 2006). The RNA folding parameters were published by the Turner group (Mathews *et al.* 1999). Suboptimal stem-loop structures were analyzed by the Hybrid program (Nazipova *et al.* 1995).

RESULTS

Molecular population genetics of *Obp* genes: Odorant receptors evolve rapidly to adapt to changes in the chemical environment (Young *et al.* 2002; Robertson *et al.* 2003). Since OBPs are the first components of the insect olfactory system to encounter odorants, they might also be expected to undergo rapid adaptive evolution. The interaction between the chemical environment and an organism, however, is not constant, as different chemical signals become relevant during different developmental stages, under different physiological conditions, and during different social interactions (*e.g.*, courtship and mating). This raises the question whether different members of the *Obp* gene family follow similar or diverse evolutionary trajectories.

To address this question, we sequenced alleles of 13 *Obp* genes organized in two representative chromosomal clusters, *Obp56a–i* on the second chromosome, and *Obp99a–d* on the third chromosome, in wild-derived inbred lines of *D. melanogaster.* We initially sequenced 50 alleles of each gene. Preliminary analyses suggested associations with members of the *Obp99* gene family and olfactory behavior (see below); therefore, we obtained additional sequences of 143 alleles for the *Obp99* genes. We observed 299 SNPs and 18 insertion/deletion (indel) polymorphisms in this sample of *Obp* genes, with 154 and 163 polymorphic sites in the *Obp56* and *Obp99* gene clusters, respectively (Table 1). SNP numbers were highly variable, ranging from only a single SNP in *Obp56f* to 76 SNPs in *Obp99*c (Table 1).

Consistent with the large variation in SNP numbers among genes of similar size in the same genomic regions, estimates of nucleotide diversity [the average number of pairwise differences between sequences (π) and the number of segregating sites (θw)] vary over an order of magnitude among the *Obp* genes (Table 1), suggesting that OBPs have experienced different evolutionary histories. We applied tests for deviation from selective neutrality to members of the *Obp56* and *Obp99* gene clusters (Hudson *et al.* 1987; McDonald and Kreitman 1991; Fu and Li 1993; Tajima 1993; Fay and Wu 2000) [corrected for the estimated recombination rate, R, (Hudson 1987)] that are based on detecting reduction in genetic diversity over different evolutionary time scales. Null alleles of *Obp56c* that contain a premature stop codon at predicted amino acid position 17 segregate in this population with an allele frequency of 0.06. Since we expected these null alleles not to be under selection, we did not include them in tests for deviation from neutrality (our results, however, do not change when the null alleles are included in the analysis, because the software performing the tests treats the missing sequence as missing data in any case). Seven of the 13 *Obp* genes exhibited signatures of departure from neutrality (*Obp56a*, *Obp56c*, *Obp56g*, *Obp56h*, *Obp99b*, *Obp99c*, and *Obp99d*; Tables 1 and 2), although none of

TABLE 1

Population genetic parameters[a]

Genes	Length[b] (bp)	Polymorphisms		π	θ_w	Divergence	*D*	*D**	*F**	*HKA*	*H*	*R*
		Indel	SNPs									
Obp56a[c]	789	4	19	0.004	0.006	0.047	−0.911	−1.029	−1.172	0.054	−3.546	0.001
Obp56b[c]	476	0	9	0.004	0.004	0.050	−0.271	0.693	0.446	0.861	−2.371	21.90
Obp56c[c]	896	0	23	0.004	0.006	0.040	−0.257[d]	−0.136[d]	−0.210[d]	0.117[d]	−4.860[d,e]	12.40[d]
Obp56d[f]	666	0	23	0.011	0.008	0.041	1.327	1.077	1.381	1.742	−1.716	7.600
Obp56e[c]	617	1	16	0.008	0.006	0.060	0.897	0.366	0.648	0.052	0.865	11.50
Obp56f[c]	432	0	1	0.001	0.001	0.017	0.622	0.543	0.654	3.197	0.245	—
Obp56g[c]	464	1	16	0.011	0.008	0.119	1.240	1.160[e]	1.405	0.175	−0.607	11.30
Obp56h[c]	463	3	19	0.008	0.010	0.018	−0.708	1.316[e]	0.731	0.188	−1.747	4.700
Obp56i[c]	481	0	19	0.008	0.009	0.066	−0.348	−1.029	−0.941	0.663	0.297	14.80
Obp99a[g]	676	1	34	0.008	0.008	0.038	0.183	1.228	0.958	0.212	−0.039	77.70
Obp99b[g]	560	0	16	0.004	0.005	0.021	−0.371	−0.253	−0.358	0.082	−1.596	39.20
Obp99c[g]	1049	8	76	0.018	0.012	0.054	1.446[e]	0.895	1.373[e]	0.395	−2.489	28.80
Obp99d[g]	414	0	28	0.015	0.012	0.074	0.810	1.554	1.502[e]	0.074	−1.216	20.80

[a] Parameters for *Obp* genes that show significant deviation from neutrality are underlined.
[b] Gene length includes 5′ UTR, introns, exons, and 3′ UTR.
[c] Denotes values obtained from 50 lines.
[d] Denotes values obtained from 47 lines, in which three lines with premature stop codons were removed from the analyses.
[e] $P < 0.05$.
[f] Forty-nine lines were used for the analysis of *Obp56d*.
[g] Denotes values obtained from 193 lines. Analyses were based on total DNA sequence, including both coding and noncoding sequences.

the *P*-values of these tests survives a strict Bonferroni correction for multiple tests. Tajima's *D* was significant for *Obp99c*, Fu and Li's D* for *Obp56g* and *Obp56h*, F* for *Obp99c* and *Obp99d*, and Fay and Wu's *H* test statistic indicated a recent selective sweep for *Obp56c* (Table 1). No significant deviation from neutral expectations was detected by the HKA test (Table 1), but the McDonald–Kreitman test showed deviations from neutrality for *Obp56a*, *Obp99b*, and *Obp99d* (Table 2). For 5 *Obp* genes (*Obp56g*, *Obp56h*, *Obp99b*, *Obp99c*, and *Obp99d*) the departure from neutrality was such that there were more polymorphisms segregating at intermediate frequency than expected under neutral mutation-drift balance, which could be attributable to balancing selection. In contrast, *Obp56a* and *Obp56c* show evidence of rapid evolution. Since there is no LD between *Obp56a* and *Obp56c*, the signatures of positive selection experienced by these 2 genes are likely not due to hitchhiking (supplemental Table S1 at http://www.genetics.org/supplemental/).

Pairwise comparisons of LD between polymorphic sites for the *Obp56* and *Obp99* genes (except the highly conserved *Obp56f*) show evidence of extensive historic recombination within each gene cluster. However, there are regions exhibiting LD between genes within each cluster, *e.g.*, *Obp99c* and *Obp99d* (Figure 1; supplemental Table S1 at http://www.genetics.org/supplemental/), although long range LD is not common. We did not observe significant LD between polymorphisms in the *Obp56* cluster and those in the *Obp99* cluster (the Bonferroni corrected significance threshold is $P <$ 2.68 *E*-6). The diagrams in Figure 1 also illustrate the differences in SNP densities among these genes.

The pattern of polymorphisms in *Obp99d* contains an unusually large number of nonsynonymous substitutions (23 of 28), many of which occur at intermediate frequencies at adjacent nucleotide positions, generating extensive amino acid diversity (supplemental Table S2 at http://www.genetics.org/supplemental/). This

TABLE 2

McDonald–Kreitman tests[a]

Genes	Synonymous substitutions		Nonsynonymous substitutions		*P*-value
	Between species	Within species	Between species	Within species	
Obp56a	2	7	10	3	0.0274
Obp56b	8	7	4	2	0.6594
Obp56c	16	9	2	6	0.1015
Obp56d	5	8	6	3	0.3870
Obp56e	6	4	5	4	1.0000
Obp56f	3	0	4	0	—
Obp56g	16	7	24	3	0.1548
Obp56h	1	5	2	11	1.0000
Obp56i	13	7	14	11	0.7600
Obp99a[b]	8	9	2	2	1.0000
Obp99b[b]	6	5	0	6	0.0427
Obp99c[b]	2	17	2	7	0.5741
Obp99d[b]	15	8	8	22	0.0064

[a] *Obp* genes that show significant deviation from neutrality are underlined.
[b] Denotes values obtained from all 193 lines.

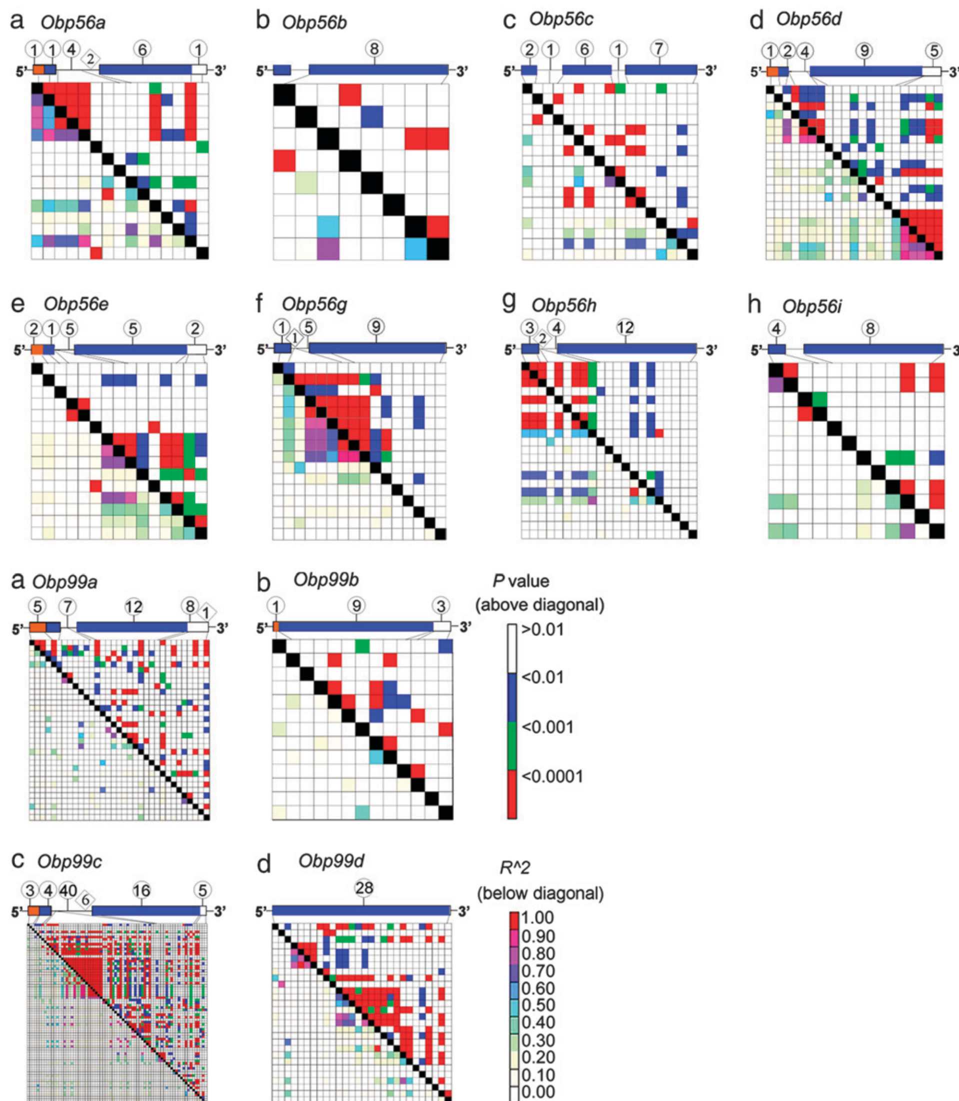

FIGURE 1.—LD in the Obp56 and Obp99 clusters. Boxes below the diagonal reflect R^2 values for all possible marker combinations and boxes above the diagonal indicate the corresponding P-values. *Obp* gene structures are denoted at the top by the horizontal line with exons represented by blue boxes, 5′-untranslated regions by orange boxes, and 3′-untranslated regions by white boxes. Introns are represented by the intervening line. The numbers in the circles indicate the number of polymorphisms contained in individual introns, exons, and untranslated regions. Since singletons have been excluded from the LD analysis, the number of polymorphisms for each gene indicated is sometimes lower than the number listed in Table 1.

unusual pattern of SNPs is not caused by a tandem polymorphic duplication of *Obp99d* (data not shown), and is consistent with the inference of balancing selection acting on this gene (Tables 1 and 2).

Quantitative genetics of olfactory behavior: We quantified naturally occurring variation in olfactory behavior among the 193 Raleigh inbred lines and observed broad variation in responsiveness to benzaldehyde, which appeared normally distributed and ranged from attractant (<<2.5) to repellant responses (>>2.5) (Figure 2a). To test whether the low responses were specific to olfactory behavior or due to a general deficit in locomotion, we assessed the correlation between locomotor behavior in response to a mechanical stimulus, which exhibits considerable inbreeding depression (JORDAN *et al.* 2007) and olfactory behavior in these lines. The correlation between locomotion scores and olfactory response scores was not significantly different from zero (Figure 2b). Thus, the variation in olfactory behavior cannot be explained by inbreeding depression for general locomotor impairment.

ANOVA shows significant variation in olfactory behavior between males and females, among lines, and for the sex-by-line interaction (Table 3). The environmental error variance component (σ^2_E) is high (0.621), demonstrating the sensitivity of olfactory behavior to uncontrollable environmental variance. The line variance component (σ^2_L) is also high (0.435), reflecting substantial genetic variation among lines. The estimate of broad-sense heritability (H^2) is $H^2 = 0.441$. Assuming strict additivity, the estimate of narrow-sense heritability (h^2) is $h^2 = 0.283$. This value is larger than previously estimated in a different population (MACKAY *et al.* 1996; $h^2 = 0.084$ and 0.134 for chromosome *1* and chromosome *3* substitution lines, respectively). The substantial genetic component to variation in olfactory behavior provides a favorable scenario for association analysis. The significant sex-by-line interaction term indicates that there is variation in sexual dimorphism in the response to benzaldehyde, although the cross-sex genetic correlation ($r_{MF} = 0.893$; Table 3) is higher than

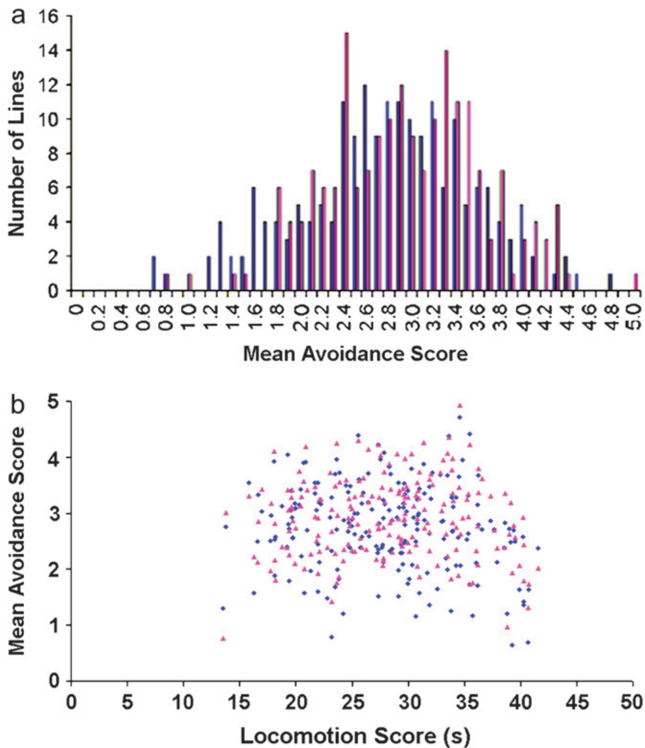

FIGURE 2.—Distribution of mean olfactory response scores for male and female flies of 193 wild-derived inbred lines (a) and lack of correlation between olfactory response scores and locomotion scores (b). Olfactory responses were measured at an odorant concentration of 3.5% (v/v) benzaldehyde. Blue and pink bars indicate scores for males and females, respectively. The correlation between olfactory responses and locomotion scores, shown in b is $r = 0.144$ for males (blue symbols) and $r = 0.020$ for females (pink symbols).

observed in a previous study of chromosome substitution lines (MACKAY *et al.* 1996).

Association of polymorphisms in *Obp* genes with olfactory behavior: We used a two-step strategy to identify polymorphisms in *Obp* genes that are associated with olfactory responsiveness to benzaldehyde. First, we conducted association tests with 50 lines, and used permutation analysis to estimate the number of associations one would observe by chance for each gene. We then compared the expected number of random associations with the observed number of associated polymorphic markers to identify *Obp* genes for further analysis. *Obp99a* and *Obp99d* showed a higher number of associations than expected by chance (supplemental Table S3 at http://www.genetics.org/supplemental/). Therefore, we focused our efforts on the *Obp99* cluster, and obtained DNA sequences for an additional 143 alleles from the same population.

Association analyses with 193 alleles for each of the four *Obp99* genes revealed individual genotype–phenotype associations with olfactory responsiveness that exceeded the permutation threshold for multiple testing in *Obp99a*, *Obp99c*, and *Obp99d* (Table 4, Figure 3). Since we found a significant line-by-sex interaction when we analyzed phenotypic variation in olfactory behavior in this population, we included a marker-by-sex interaction term in the association model. However, none of the significant SNPs had significant marker-by-sex interactions. The effects of the SNPs on olfactory behavior are quite large, ranging from 0.40 to 1.33 genetic standard deviation units and 0.26–0.88 phenotypic standard deviation units in the population of inbred lines. However, if we assume strict additivity, the individual polymorphisms explain only 3–6% of the total additive variance in olfactory behavior (Table 4).

Polymorphic markers C75G in *Obp99a* and C141G in *Obp99c* are located in introns, whereas markers G67A and T78G in *Obp99d* are in the coding region. The G67A SNP results in a glutamine to lysine substitution, whereas the T78G SNP is synonymous. These SNPs are in LD ($P < 0.0001$). In addition, the C29A SNP in *Obp99d* is near the permutation threshold for statistical significance (Figure 3). This nonsynonymous substitution changes a cysteine into tyrosine, thereby eliminating a cysteine that could contribute to the formation of a structurally important disulfide bond. This SNP is in substantial LD ($P < 0.01$) with T78G. Haplotype analysis of the G67A and T78G polymorphisms in *Obp99d* shows a significant difference in phenotypic values between

TABLE 3

Variance components and quantitative genetic parameters from ANOVA of olfactory behavior in response to benzaldehyde

	Source	d.f.	MS	F-value	Pr > F	Variance components
Sexes pooled	Sex	1	30.0540	48.37	<0.0001	
	Line	192	9.8724	15.89	<0.0001	0.435
	Sex × line	192	1.1698	1.88	<0.0001	0.055
	Error		0.6214			0.621
Female	Line	192	4.9498	7.70	<0.0001	0.431
	Error	1737	0.6431			0.643
Male	Line	192	6.0925	10.16	<0.0001	0.549
	Error	1737	0.5996			0.600
COV_{MF}						0.434
H^2						0.441
r_{MF}						0.893

TABLE 4

Associations of *Obp99* genotypes with olfactory behavior

Parameter estimate	*Obp99a* C75G	*Obp99c* C141G	*Obp99d* G67A	*Obp99d* T78G
q^a	0.036	0.161	0.057	0.280
$2a^b$	0.93	0.35	0.51	0.28
$2a/\sigma_G{}^c$	1.33	0.50	0.73	0.40
$2a/\sigma_P{}^d$	0.88	0.33	0.48	0.26
$\sigma^2_{AM}{}^e$	0.015	0.008	0.007	0.008
$\sigma^2_{AM}/\sigma^2_A{}^f$	0.061	0.034	0.028	0.032

[a] Frequency of rare allele.
[b] Difference in mean olfactory behavior between homozygous genotypes for common and rare alleles.
[c] Absolute values of the effects, expressed in genetic standard deviation units.
[d] Absolute values of the effects, expressed in phenotypic standard deviation units.
[e] Additive genetic variance attributable to the marker.
[f] Fraction of total additive genetic variance attributable to the marker.

haplotypes GT (71% frequency) and AG (5% frequency) in *Obp99d* (Figure 3). The observed differences in phenotypic values between these haplotypes were the same for both sexes.

Our results implicate *Obp99a*, *Obp99c*, and *Obp99d* in the recognition of benzaldehyde and suggest that these OBPs are either redundant for the recognition of this odorant, or more likely, that OBPs recognize odorants in a combinatorial manner similar to odorant recognition by odorant receptors (Malnic *et al.* 1999). Thus, a given odorant would interact with multiple OBPs with different affinities in a concentration-dependent manner and a given OBP would recognize multiple odorants on the basis of its molecular response profile.

Effects of polymorphisms associated with olfactory behavior on predicted secondary pre-mRNA structure: To gain insights into the mechanism by which synonymous or noncoding SNPs could affect phenotypic variation in olfactory behavior, we predicted secondary structures for pre-mRNA molecules transcribed from alternative SNP genotypes. The C75G polymorphism in *Obp99a* is located in an intron. Frequent local secondary structures predicted for sequences with C and G in position 75 are very similar except for the pairing in position 75, where C is paired and G is not paired (Figure 4). Position 75 in *Obp99a* pre-mRNA is close to the splice site (35 bp upstream from the exon/intron boundary), and the different predicted RNA structures could potentially affect splicing (Solnick 1985).

The C141G polymorphism in *Obp99c* is also located in an intron. Different stable structures are predicted for *Obp99c* sequences with nucleotides C and G in position 141, where C is paired with a higher probability ($P = 0.56$) than G ($P = 0.18$) (Figure 4; supplemental Table S4 at http://www.genetics.org/supplemental/). It is interesting that G in position 141 is frequently unpaired in suboptimal secondary structures, although G has the highest potential for base pairing among all four nucleotides.

The three common haplotypes formed by SNPs at positions 67 and 78 of *Obp99d* produce different optimal local secondary structures (Figure 4), although the free energy for the local secondary structures in this region does not differ dramatically (from −133.9 to −126.5 kcal/mol, Figure 4). Structure predictions for *Obp99d* mRNAs showed that nucleotides in positions 67 (G67A, a nonsynonymous polymorphism) and 78 (T78G, a synonymous polymorphism) are frequently involved in the neighboring stem-loop structures and modulate base pairing of neighboring nucleotides (Figure 4). Nucleotides G and A in position 67 have different potentials for base pairing for the different haplotypes ($P = 0.7$ for G67/T78, $P = 0.61$ for G67/G78, and $P = 0.05$ for A67/78G, respectively, supplemental Table S4 at http://www.genetics.org/supplemental/). The rarest haplotype (A67/T78) has a dramatically different potential for base pairing in position 78 ($P = 0.2$ for T78, supplemental Table S4) compared to the three more common haplotypes. Since these polymorphisms are in strong LD, we cannot infer whether the effect on olfactory behavior is due to a structural change in the protein or the mRNA.

DISCUSSION

We have used a population genetics approach in conjunction with behavioral measurements to identify OBPs that recognize benzaldehyde, while at the same time gaining insights in the history of natural selection, mutation, and recombination of members of the OBP multigene family. Sequence analyses showed that not all OBPs share the same evolutionary history. While patterns of polymorphism in six OBPs do not depart from those expected under neutral mutation–random drift balance, statistical tests for deviations from neutrality identify signatures of positive selection or balancing selection for seven OBPs. These diverse evolutionary trajectories may result from the diversity of biological functions influenced by OBPs. Differential expression of OBPs has been observed in lines artificially selected for aggression (Edwards *et al.* 2006), alcohol sensitivity (Morozova *et al.* 2006), copulation latency (Mackay *et al.* 2005), and starvation stress resistance (Harbison *et al.* 2005).

Sequence analyses showed that OBPs have different evolutionary histories and statistical tests for deviations from neutrality identify different signatures of selection for eight OBPs. The neutrality tests used detect signatures of selection over different evolutionary time scales (Sabeti *et al.* 2006); this is most likely the reason that inferences regarding selection were not consistent for the different tests. Tajima's D (Tajima 1993) and Fu and Li's D^* (Fu and Li 1993) tests are based on different sensitivities of summary measures of nucleotide diversity within a species to a selective sweep and have different

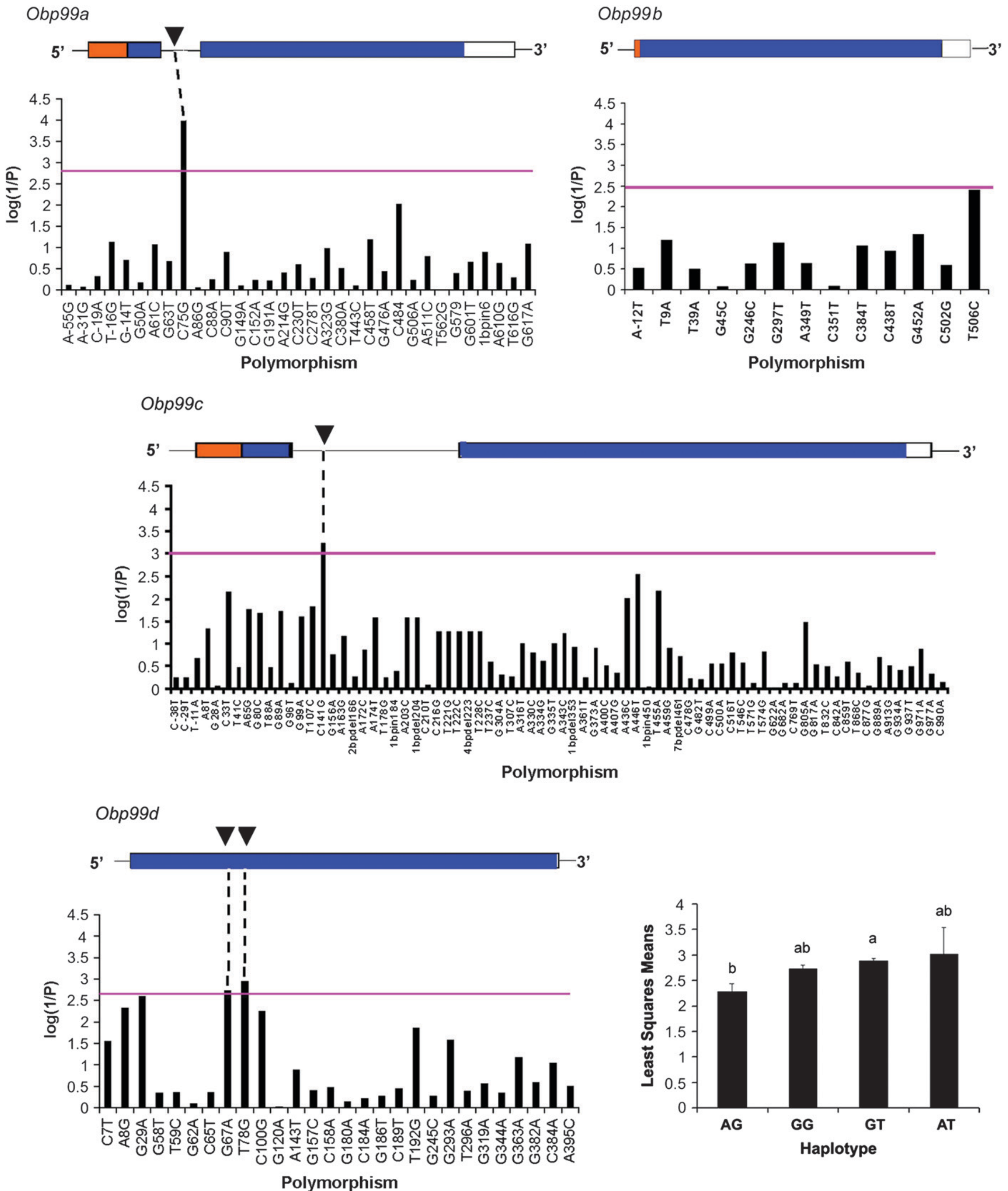

Figure 3.—Associations between polymorphisms in the *Obp99* cluster with variation in behavioral responses to benzaldehyde. The *Obp99a*, *b*, *c*, and *d* gene structures are schematically represented at the top of each graph with blue boxes representing exons, orange boxes 5′-untranslated regions, white boxes 3′-untranslated regions, and the intervening black line introns. The purple horizontal line indicates the significance threshold determined by permutation tests. Arrowheads indicate the locations of SNPs with significant phenotypic associations. The bar graphs show variation in olfactory behavior in response to benzaldehyde associated with four haplotypes corresponding to the two associated markers in *Obp99d*. Data were analyzed by ANOVA and haplotypes that differ significantly in olfactory behavior in response to benzaldehyde were identified by Tukey's test and are indicated with different letters at the top of the bars.

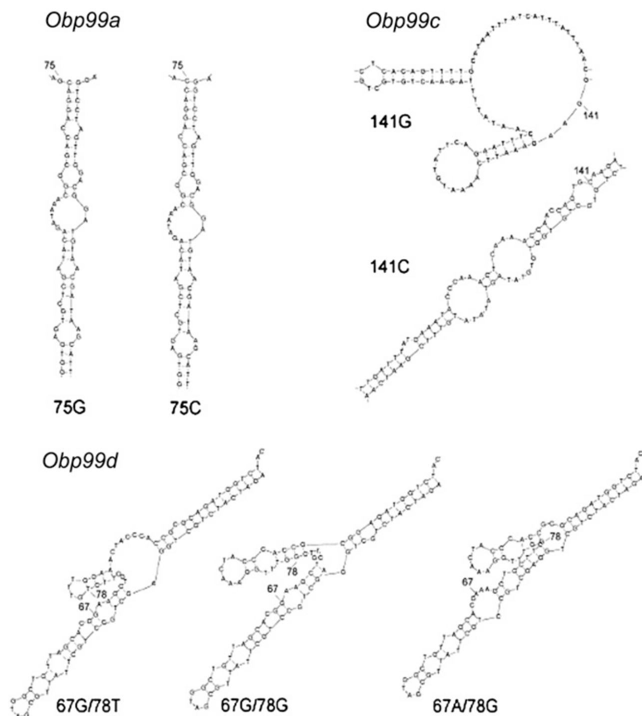

Figure 4.—Predicted local stem-loop structures associated with polymorphic markers C75G in *Obp99a*, C141G in *Obp99c*, and G67/T78, G67/G78, and A67/G78 in *Obp99d*. The local stem-loop structures in *Obp99d* modulate base pairing of their neighbors in an ∼100-nt window around positions 67 and 78. Free energies of local secondary structures for G67/T78 = −132.5 kcal/mol, for G67/G78 = −133.9 kcal/mol, and for A67/G78 = −126.5 kcal/mol.

powers to detect departure from neutrality. Fay and Wu's *H* test (Fay and Wu 2000) analyzes high-frequency-derived alleles as a signature of a recent selective sweep and, thus, extends over a recent evolutionary period as high-frequency-derived alleles rapidly reach fixation (Sabeti *et al.* 2006). The results of these tests may be confounded by demographic history. Changes of population size can affect Tajima's *D* (Tajima 1993) and Fu and Li's *D** (Fu and Li 1993) tests. Populations that have undergone a recent bottleneck in population size have a similar compressed genealogy to populations that are under positive selection; Tajima's *D* (Tajima 1993) and Fu and Li's *D** (Fu and Li 1993) statistics are expected to be positive in both cases. Negative Tajima's *D* (Tajima 1993) and Fu and Li's *D** (Fu and Li 1993) statistics are expected for populations undergoing recent expansion of population size, which increases in θw. Population subdivision can confound Fay and Wu's test by generating a nonsignificant *H* statistic. In contrast, the McDonald–Kreitman test (Mcdonald and Kreitman 1991) compares the ratios of nonsynonymous and synonymous substitutions within and among species and detects deviations from neutrality that persist over a long evolutionary time. Perhaps the best indicators of the diverse patterns of evolution of *Obp* genes are the different levels of nucleotide diversity in closely linked genes that presumably experience similar mutation and recombination rates (Figure 1).

Behavioral responses to benzaldehyde showed a greater range of phenotypic variation than observed previously with standard inbred laboratory stocks, and were elicited at a higher concentration of benzaldehyde. Olfactory responses were not correlated with locomotor reactivity scores, indicating that low olfactory-response scores did not result from locomotor impairments due to inbreeding depression. ANOVA showed significant variation in sexual dimorphism but a high genetic correlation between the sexes ($r_{\mathrm{MF}} = 0.893$; Table 3). Variation in sexual dimorphism in avoidance responses to benzaldehyde has also been observed previously in chromosome substitution lines (Mackay *et al.* 1996) and among co-isogenic *P*-element insertion lines that affect olfactory behavior (Anholt *et al.* 1996; Sambandan *et al.* 2006).

The extensive phenotypic variation in our wild-derived inbred population provided a favorable scenario for association analyses. We detected associations with SNPs in *Obp99a*, *Obp99c*, and *Obp99d* which implicate these OBPs in recognition of benzaldehyde. However, we examined only ∼25% of the *Obp* gene family and it is likely that additional OBPs would contribute to phenotypic variation in the response to this odorant. It should be noted that OBPs that interact with odorants, such as benzaldehyde, but for which there is no segregating variation in the population under study, would go undetected by our approach. Furthermore, the detection power for associations depends on the sample size, and additional associations that make a smaller contribution to the observed phenotypic variation might be detected if the population size were expanded. Similarly, our analysis to date has focused on only the commonly used test odorant benzaldehyde. Expanding this analysis to include the entire family of *Obp* genes with a battery of odorants would enable a comprehensive characterization of ligand specificities of the OBP family. However, our experiments with a single odorant and a limited number of *Obp* genes already show that the recognition of benzaldehyde by OBPs is redundant and likely combinatorial, reminiscent of odorant recognition by odorant receptors (Malnic *et al.* 1999). Functional redundancy may allow the persistence of segregating null alleles in the population, observed by us and others (Takahashi and Takano-Shimizu 2005).

Previous association studies in Drosophila have implicated SNPs in noncoding regions of *Catsup* in phenotypic variation in sternopleural bristle number, environmental plasticity of abdominal bristle number, and starvation resistance (Carbone *et al.* 2006). SNPs associated with variation in longevity, locomotor behavior, starvation resistance, and bristle number have been identified also in functional regions of the protein (Carbone *et al.* 2006). Tests for association of SNPs in *Obp* genes and

responsiveness to benzaldehyde revealed four polymorphisms implicating three OBPs, all within the *Obp99* cluster, in the recognition of this odorant. Whereas nonsynonymous SNPs in coding regions of *Obp* genes can affect ligand binding by introducing variation in protein structure, the most parsimonious explanation for the phenotypic effects of synonymous SNPs and SNPs in regulatory regions would be alterations in mRNA structure. Indeed, structure predictions of mRNAs encoded by alternative haplotypes of *Obp99d* show that a single base substitution can have a profound effect on secondary mRNA structure (Figure 4), which could affect its transport, splicing, ribosome binding, or translation efficiency (Kimchi-Sarfaty *et al.* 2007). Our theoretical predictions of causal effects of altered mRNA structures on the behavioral phenotype, however, will need to be supported experimentally in the future.

Elegant electrophysiological studies have generated molecular response profiles of odorant receptors in the Drosophila antennae and maxillary palps and shown that a single odorant can activate multiple odorant receptors (De Bruyne *et al.* 1999; Hallem *et al.* 2004; Carlson and Hallem 2006). Thus far, the function of OBPs in odorant recognition has remained enigmatic, as there is no clear correlation between expression patterns of OBPs and odorant receptors. The population genetics approach described here represents a first step toward defining molecular recognition profiles of the OBP family. Such information together with the expression patterns of odorant receptors of known response profiles will, ultimately, clarify how these olfactory gene families interact in enabling the fly to sense its chemical environment.

We thank Philip Awadalla for helpful discussions, Theodore J. Morgan for assistance with the permutation tests, Katherine Jordan for sharing locomotion data prior to publication, and Aleksey Y. Ogurtsov for assistance with the secondary structure predictions. This work was supported by National Institutes of Health (NIH) grant GM-059469 (to R.R.H.A.) and by the Intramural Research Program of the NIH, National Library of Medicine (S.A.S.).

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Communicating editor: L. G. Harshman