

Evolution of the ABPA Subunit of Androgen-Binding Protein Expressed in the Submaxillary Glands in New and Old World Rodent Taxa

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Abstract The salivary androgen-binding proteins (ABPs) are members of the secretoglobin gene family present in mammals. Each ABP is a heterodimer assembled as an ABPA subunit encoded by an *Abpa* gene and linked by disulfide bridges to an ABPBG subunit encoded by an *Abpbg* gene. The ABP dimers are secreted into the saliva of mice and then transferred to the pelage after grooming and subsequently to the environment allowing an animal to mark territory with a biochemical signal. The putative role of the mouse salivary ABPs is that of pheromones mediating mate selection resulting in assortative mating in the *Mus musculus* species complex. We focused on comparing patterns of molecular evolution between the *Abpa* genes expressed in the submaxillary glands of species of New World and Old World muroids. We found that in both sets

of rodents the *Abpa* genes expressed in the submaxillary glands appear to be evolving under a similar evolutionary regime, with relatively high nonsynonymous substitution rates, suggesting that ABP might play a similar biological role in both systems. Thus, ABP could be involved with mate recognition and species isolation in New World as well as Old World muroids.

Keywords Synonymous substitution · Nonsynonymous substitutions · Rodentia · Positive selection · Salivary ABP

Introduction

Speciation can be driven by the isolation of two populations through geographic, temporal, ecological, or behavioral barriers (see Coyne and Orr 2004 for an extended discussion). In many mammals olfaction is a dominant sensory modality and chemical cues can be used to convey information about individuality. In the common house mouse (*Mus musculus*) subspecies complex, the salivary androgen-binding proteins (ABPs) are hypothesized to be a component of such a cue, as they are thought to mediate mate recognition (Laukaitis et al. 1997; Talley et al. 2001). ABPs are secretoglobins (Klug et al. 2000; Laukaitis et al. 2005; Laukaitis and Karn 2005; Mukherjee and Chilton 2000) present in the saliva following expression within the submaxillary, sublingual, and parotid glands (Dlouhy et al. 1986; Laukaitis et al. 2005). The putative biological function of mouse salivary ABPs is that of a pheromone, mediating mate selection resulting in assortative mating and incipient reinforcement at the edges of the house mouse hybrid zone in Europe (Bímová et al. 2005, 2011).

ABP is secreted into the saliva and transferred to the pelage and environment after grooming, allowing an

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animal to mark territory with a biochemical signal. From a genomic standpoint, the mouse ABP system was thought to consist of three single copy genes, *Abpa*, *Abpb*, and *Abpg*, encoding for the three separate subunits, ABPA, ABPB, and ABPG, respectively (Dlouhy et al. 1987; Karn and Laukaitis 2003). Genomic comparisons, however, revealed a more complex pattern (Emes et al. 2004; Laukaitis et al. 2008; Karn and Laukaitis 2009). Most mammals have a single *Abpa* gene encoding an ABPA protein and a single *Abpbg* gene encoding an ABPBG protein in the *Abp* locus, however, rat and house mouse possess multiple paralogs in the corresponding location (Laukaitis et al. 2008; Karn and Laukaitis 2009). In the case of house mouse there are 64 *Abp* paralogs (30 *Abpa* and 34 *Abpbg* genes) over a 3 megabase (Mb) region, whereas the rat genome has 6 paralogs (3 *Abpa* and 3 *Abpbg* paralogs). Analyses of intron sequences suggest that these expansions occurred independently in these two species (Laukaitis et al. 2008). In house mouse, the *Abpa27* paralog has been the most studied gene in the *Abp* locus. The translated gene ABPA27 forms a dimer with either ABPBG26 or ABPBG27 via disulfide bridges and is expressed in the submaxillary glands of both males and females. Evidence suggests the ABPA27 subunit plays a significant role in conspecific recognition and mate selection (Bímová et al. 2005; Hwang et al. 1997; Karn et al. 2002; Laukaitis et al. 1997; Talley et al. 2001). Interestingly, recent studies also suggest that additional *Abpbg* paralogs secreted into the saliva of house mouse, specifically *Abpbg26* and *Abpbg27* show patterns of molecular variation suggesting adaptive evolution (Laukaitis et al. 2012).

Protein coding genes associated with reproductive and chemosensory roles have a tendency to evolve rapidly and display signatures of positive selection (Duret and Mouchiroud 2000; Kosiol et al. 2008; Park et al. 2011; Swanson and Vacquier 2002; Torgerson et al. 2002). In line with these expectations, comparisons among different *Mus* sp. alleles revealed high rates of nonsynonymous to synonymous substitutions, and comparative analyses among lineages within the *Mus* species complex have shown that distinct *Abpa27* alleles are fixed in the different subspecies of *M. musculus*, and molecular evolution analyses of mouse *Abpa27* sequences detected strong signals of Darwinian selection (Emes et al. 2004; Hwang et al. 1997; Karn and Nachman 1999; Karn et al. 2002). Therefore the combination of controlled mate choice experiments and the observed genetic signatures consistent with positive selection suggests that ABPs probably play a role in olfactory communication, assortative mating, and incipient reinforcement of reproductive isolation (Bímová et al. 2005, 2011; Karn and Dlouhy 1991; Karn et al. 2002; Laukaitis et al. 2005). The expression and evolution of

ABP have been extensively studied in *Mus* (Dlouhy et al. 1987; Emes et al. 2004; Karn et al. 2010; Laukaitis et al. 2008; Laukaitis and Karn 2005; Karn and Laukaitis 2009). However, few data have been presented for other rodent genera: Karn and Dlouhy (1991) extracted and identified ABP in the saliva for New World rodents, and Laukaitis et al. (2008) identified two putative paralogs of *Abpa* in *Apodemus sylvaticus*.

In this study, our objective was to explore whether the patterns of variation in the *Abpa* genes that encode for the ABPA subunit expressed in the submaxillary glands are similar in New and Old World rodents. In particular, we focused on comparing patterns of molecular evolution between the *Abpa27* gene in house mouse, with their functional counterparts in other Old and New World rodent species. To do so, we analyzed partial sequence data from mRNA transcripts isolated from submaxillary glands of selected New and Old World muroids and used maximum likelihood methods to characterize patterns of molecular evolution. Our results indicate that the *Abpa* genes expressed in the submaxillary glands are evolving rapidly in both rodent groups, and that a similar set of codon positions appears to be under positive selection in the two systems. Given that proteins involved in species isolation tend to evolve rapidly, our findings would suggest that ABPs might play similar roles related to maintaining species boundaries in both sets of species.

Materials and Methods

Data Collection

Representatives of New World muroids were collected from West Texas and Ecuador and Old World muroid representative rodents were collected from northern Ukraine. Voucher specimens are stored in the Natural Science Research Laboratory collections at the Texas Tech Museum. The submaxillary gland was removed from the animal and stored at -70°C . Isolation of mRNA, cDNA preparation, PCR amplification of expressed *Abpa*, and sequencing of amplicons followed Wickliffe et al. (2002). In addition, we obtained additional known expressed *Abpa* sequences from *Spermophilus tridecemlineatus*, *Mus* sp., and *Rattus norvegicus* from Karn et al. (2002) and Laukaitis et al. (2008) (Supplementary File 1). *Mus spicilegus* was not included from Karn et al. (2002) given the exact sequence identity to *M. macedonicus*. In the case of heterozygous individuals, haplotypes were resolved using PHASE version 2.1 (Stephens and Donnelly 2003). Previously unpublished *Abpa* sequences were deposited in GenBank under the accession numbers JX275970–JX275986.

Sequence Alignment and Analysis

Partial *Abpa* cDNA sequences were aligned by codons with MUSCLE (Edgar 2004) as implemented in MEGA 5 (Tamura et al. 2011). We explored alignment sensitivity by comparing the MUSCLE alignment to the results of ClustalW (Thompson et al. 1994), MAFFT (Katoh et al. 2005), and PRANK (Löytynoja and Goldman 2005). Alternative alignment strategies yielded the same alignment, which was used for downstream analyses. The intraspecific number of haplotypes, nucleotide diversity, and the uncorrected average pairwise number of differences for nucleotide and amino acid changes were determined in MEGA 5. Intra-generic numbers of nonsynonymous and synonymous changes were counted within *Apodemus*, *Mus*, *Peromyscus*, and *Reithrodontomys*. Once redundant alleles were removed from the alignment, we reconstructed phylogenetic relationships using maximum likelihood as implemented in Treefinder version March 2011 (Jobb et al. 2004), and we evaluated support for the nodes with 1,000 bootstrap pseudoreplicates. We used the “propose model” tool of Treefinder to select the best-fit models of nucleotide substitution, with an independent model at each codon position in nucleotide analyses. Model selection was based on the Akaike information criterion with correction for small sample size.

We then estimated patterns of molecular evolution using the maximum likelihood approach described by Goldman and Yang (1994) as implemented in CODEML in PAML (Yang 2007). In order to estimate the putative role of negative and positive selection, we compared the rate of nonsynonymous substitution per nonsynonymous site (d_N) to the rate of synonymous substitution per synonymous site (d_S). The d_N/d_S ratio (also labeled as ω) can be used to measure the selective regime of a given codon, as similar rates of nonsynonymous and synonymous substitution ($\omega \approx 1$) are indicative of neutral evolution, an excess of synonymous mutations ($\omega < 1$) is indicative of purifying, or negative selection, and an excess of nonsynonymous mutations ($\omega > 1$) is indicative of positive Darwinian selection, or adaptive evolution. We compared models that allow ω to vary among codons in the alignment (M0 vs. M3, M1a vs. M2a, M7 vs. M8, and M8a vs. M8). In all cases, we used likelihood ratio tests (LRTs) to compare nested sets of model (Yang 1998). Bayesian Empirical Bayes (BEB) was used to calculate the posterior probabilities for sites under positive selection in models M2a and M8 (Yang et al. 2005). These analyses were performed separately for New World and Old World muroids, because orthology between *Abpa27* in *Mus* and our New World taxa cannot be guaranteed. To account for the potential problem generated by intraspecific polymorphism, these analyses were repeated using only one randomly selected

representative from each species. Phylogenetic trees were reconstructed from each subset under the same parameters as the full data set.

Residues were considered to be under selection if a position was predicted to be under positive selection with posterior probability >0.9 in one model and >0.5 in one other model. We predicted the *Mus* ABPA27 amino acid structure using Phyre2 (Kelly and Sternberg 2009). Residues under selection in New World rodents and Old World rodents were mapped onto the structure. Swiss-PDBviewer (Guex and Peitsch 1997) was used to manipulate the protein structure and the 3D image was rendered with POV-Ray (<http://www.povray.org>). Relative solvent accessibilities of residues were calculated based on criteria given in Emes et al. (2004). Relative accessibility values were divided into three categories, buried ($<9\%$ relative accessibility), intermediate ($9\text{--}35\%$ relative accessibility), and exposed ($>35\%$ relative accessibility) (Emes et al. 2004; Rost and Sander 1994).

PolyPhen-2 (Polymorphism Phenotyping ver. 2.0; <http://genetics.bwh.harvard.edu/pph2/index.shtml>), an in silico tool for predicting structural and functional effects of amino acid substitutions on proteins, was used to explore the possible impacts of amino acid variants that appeared to be under strong, positive selection within and among New World and Old World rodent lineages (Adzhubei et al. 2010). Default options were used for all analyses.

Results

Description of Data

We obtained sequences corresponding to 213 bp of the 279 nucleotides of coding sequence of *Abpa* transcripts from submaxillary glands from 34 rodent specimens. Twenty-seven samples were collected from New World species, 9 *Peromyscus leucopus*, 6 *Peromyscus maniculatus*, 3 *Reithrodontomys fulvescens*, 1 *Akodon aerosus*, 6 *Sigmodon hispidus*, 2 *Oligoryzomys microtis*; and six samples were collected from Old World species, 1 *Microtus oeconomus*, 1 *Apodemus agrarius*, 1 *Apodemus flavicollis*, and 3 *Apodemus sylvaticus* (Supplementary Table 1). The *Apodemus* sequences were previously isolated and described in Wickliffe et al. (2002). Even though *M. oeconomus* was collected in northern Ukraine, the genus is more closely related to New World muroid rodents. Therefore, the *M. oeconomus* *Abpa* cDNA sequence was compared to the remaining New World muroid rodents in our study. The *Abpa* sequence of *S. tridecemlineatus* reported by Laukaitis et al. (2008), which corresponds to a single-copy gene in the spetri2 assembly of the squirrel genome, was included as an outgroup for phylogenetic analyses. All alignment

strategies yielded similar results, with no gaps or premature stop codons found among cDNA-derived sequences (Supplementary File 1). Intraspecific pairwise comparisons of the number of nucleotide substitutions ranged from 0 to 14 and from 0 to 12 when comparing amino acid sequences (Table 1). Despite the relatively small sample sizes, we found that *A. sylvaticus*, *P. leucopus*, *P. maniculatus*, and *R. fulvescens* were polymorphic for the *Abpa* gene in our sample, whereas there was no sequence variation among the six specimens of *Sigmodon hispidus* surveyed (Table 1).

We then estimated phylogenetic relationships among the different alleles using maximum likelihood, excluding redundant haplotypes. In the resulting tree the New World and Old World muroid sequences fell in reciprocally monophyletic clades, as expected given current estimates of organismal phylogeny (Fig. 1; Jansa and Weksler 2004; Steppan et al. 2004). In general, the sequences of given genera were monophyletic, with the exception of the rat *Abpa* paralogs (Fig. 1). Phylogenies based on intronic sequence suggest that the presence of multiple paralogs of *Abpa* in rat and mouse derived from lineage-specific expansions (Laukaitis et al. 2008). Our results differ from those of Laukaitis et al. (2008) as the rat paralogs did not form a monophyletic clade. However, an approximately unbiased topology test (Shimodaira 2002) could not discriminate between the maximum-likelihood tree and a tree where the rat paralogs were constrained to monophyly. This issue warrants further attention once intronic sequences from the *Abp* genes of a wider selection of Old World muroid rodents become available.

Patterns of Molecular Evolution

As a starting point, we compared nonsynonymous and synonymous substitution rates in a pairwise manner. We detected an excess of nonsynonymous substitutions relative to synonymous substitutions in intrageneric and intraspecific comparisons within the Old World and New World muroid

groups. This is especially noticeable in comparisons within *R. fulvescens* (Supplementary Table 2). In line with these results, our estimates of $d_N/d_S (= \omega)$ for the whole fragment sequenced were high for both data sets, 0.9 for New World muroids and 1.18 for Old World rodents (Supplementary Table 3). In general, estimates of d_N/d_S averaged over all sites in excess of 0.5 have been considered as suggestive of positive Darwinian selection (Swanson et al. 2004).

We then compared different models of molecular evolution that explore variation in ω among codons in a tree-based approach. We first explored whether there was evidence of variation in ω among codons. We compared models M0, which assigns the same value of ω to all codons with M3, a model that groups codons in three separate classes with independent estimates of ω for each class. There was significant variation in ω , as the LRT-rejected M0 in favor of M3 in both cases (Table 2). We then looked for evidence of positive Darwinian selection among our samples by comparing models that allow a class of sites to have $\omega > 1$, M2a and M8, with the corresponding models that restrict all ω estimates to be ≤ 1 , M1a, M7, and M8a. In all comparisons, M1a versus M2a, M7 versus M8, and M8a versus M8, the models that allow a class of sites to have $\omega > 1$ were favored by the corresponding LRTs (Table 2). Under the criteria given, 5 and 12 sites were found to be evolving under positive selection in New World and Old World muroids, respectively (Table 2; Supplementary Table 3). Similar results were obtained when we reran the analyses using only one representative from each species (Table 2). Most of the sites were found to be in exposed regions of the ABPA27 structure in both the New World and Old World groups (Supplementary Table 4; Fig. 2). We then focused on amino acid variants at sites 68 and 69 [90 and 91 in the accessioned amino acid sequence for *M. musculus* (http://www.ncbi.nlm.nih.gov/protein/NP_033726.1)] as these sites exhibited marked positive selection in the New World and somewhat in the Old World muroids. In silico simulations with PolyPhen-2 suggest that changes in either of these sites do not appear to alter the structure or putative function of the *Abpa*.

Table 1 *Abpa* sequence statistics for intraspecific samples

Species	<i>N</i>	<i>h</i>	π	Distance (nuc.)	Distance (aa)
<i>A. sylvaticus</i>	4	4	0.043	9.3 (6–14)	7.5 (4–12)
<i>P. leucopus</i>	12	7	0.008	1.8 (0–4)	0.8 (0–2)
<i>P. maniculatus</i>	7	3	0.009	2.0 (0–4)	1.5 (0–3)
<i>O. microtis</i>	2	1	0	0	0
<i>R. fulvescens</i>	3	3	0.048	10 (1–15)	6.7 (1–10)
<i>S. hispidus</i>	6	1	0	0	0

The number of samples sequenced (*N*), number of unique haplotypes (*h*) and nucleotide diversity (π) are listed along with the average (and range in parentheses) pairwise number of differences (distance) for both nucleotide (nuc.) and amino acid (aa) sequences

Discussion

In house mouse, the ABP heterodimers present in the saliva appear to play a behavioral role in reproductive isolation (Laukaitis et al. 1997; Talley et al. 2001; Laukaitis et al. 2012). Most of these studies have focused on the *Abpa27* gene, which is expressed in the submaxillary glands in both male and female *Mus musculus* (Laukaitis et al. 2005). In the *M. musculus* subspecies complex, several lines of evidence suggest salivary ABP plays an important role in



Fig. 1 Phylogenetic reconstructions from the 210 bp fragment of *Abpa*. **a** Maximum likelihood phylogeny for all newly isolated *Abpa* cDNA sequences, and previously published Old World orthologs and paralogs, excluding redundant haplotypes. **b** Maximum likelihood

phylogeny for the Old World *Abpa* sequences included in the molecular evolution analyses and **c** maximum likelihood phylogeny for the New World *Abpa* sequences included in the molecular evolution analyses

assortative mating. First, alternative alleles are fixed in each *M. musculus* subspecies at the *Abpa27* locus (Hwang et al. 1997; Karn and Dlouhy 1991). Second, in laboratory experiments, female mice preferred to associate and mate with males of their own *Abpa27* genotype significantly more often than with males carrying a different allele (Laukaitis et al. 1997; Talley et al. 2001). Lastly, comparisons of evolutionary rates among *Abpa27* alleles in different species of mice revealed a large excess of

nonsynonymous substitutions over synonymous substitutions, consistent with positive Darwinian selection (Hwang et al. 1997; Karn and Nachman 1999; Karn et al. 2002).

Because the rat and mouse *Abpa* repertoires derived from largely independent sets of duplications, resolving orthology for the expressed genes and predicted proteins can be difficult. However, the evidence at hand indicates that all of the *Abpa* genes in mammals were derived from the single copy ancestral *Abpa* gene present in the common

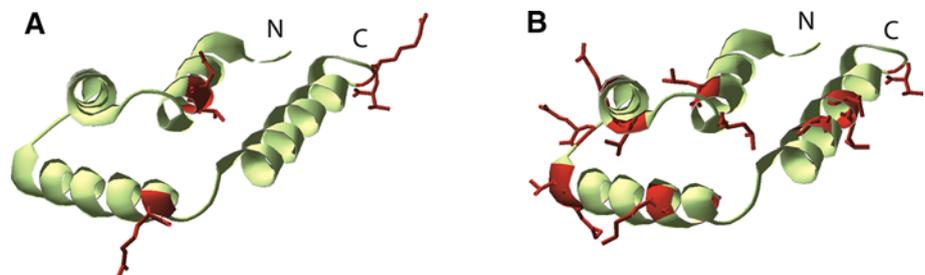
Table 2 Tests of positive selection for *n* sequences among sites for the New World and Old World groups

Group	<i>n</i>	2(<i>ΔL</i>)			M2a sites under selection	M8 sites under selection
		M2a versus M1a	M3 versus M0	M8 versus M8a		
New World	17	14.54*	67.06**	14.34*	10 11 47 68 69	7 <u>10 11</u> 12 16 23 32 33 40 43 <u>47</u> 61 62 65 66 <u>67 68 69</u>
New World (single sequence per sp.)	7	8.18*	51.20**	8.08*	11 40 47 62 68 69	7 10 <u>11</u> 15 16 18 23 25 26 27 29 30 32 33 <u>40</u> 43 47 55 57 58 61 <u>62</u> 66 68 69
Old World	16	18.96**	38.76**	18.90**	<u>12</u> 14 18 23 27 30 32 33 34 36 <u>43</u> 45 <u>55</u> 59 60 68 69	7 12 14 18 <u>23</u> 27 30 32 33 <u>34</u> 36 43 <u>45</u> 48 51 <u>55 59 60 68 69</u>
Old World (single sequence per sp.)	13	17.23**	31.12**	17.09**	<u>12</u> 14 18 23 30 32 33 36 43 45 <u>55</u> 59 60 68 69	12 14 18 <u>23</u> 27 30 32 33 36 43 45 51 <u>55 59</u> <u>60</u> 68 <u>69</u>

Sites listed had a posterior probability >0.5. Underlined positions were predicted to be under positive selection with a posterior probability 0.9–0.949 and bold sites had a posterior probability >0.95

* *p* < 0.001, ** *p* < 0.0001

Fig. 2 Sites found to be under positive selection for New World muroids (a) and Old World muroids (b) mapped to the *Mus* ABPA27 subunit. Codons and their side chains predicted to be under selection are colored red. See the electronic version for color



ancestor of placental mammals. The most recent assessments of variation in the gene complement of the *Abp* gene family (Emes et al. 2004; Laukaitis et al. 2008; Karn and Laukaitis 2009) indicate that the duplications that gave rise to the presence of multiple *Abpa* genes in mouse genome are specific to the mouse lineage (see Fig. 1), therefore, we infer all mouse *Abpa* paralogs are co-orthologs with the ancestral single copy *Abpa* gene. We have no evidence of the presence of multiple *Abpa* paralogs in New World rodents; thus, we assumed that *Abpa* gene from New World rodents is an ortholog of the ancestral single-copy gene. Because of this shared ancestry and tissue-specific patterns of expression, we assumed that the *Abpa27* genes of house mouse were functionally equivalent to the *Abpa* genes expressed in the submaxillary glands of other rodents. To ensure this, we extracted and sequenced cDNA from the salivary glands in New World rodent group using primers designed to amplify *Abpa27* in *Mus* (Hwang et al. 1997). If the proposed role of the APBA subunit of New World rodents is to act as a mate choice pheromone, as the ABPA27 subunit does in *Mus*, we would expect to observe similar patterns of molecular evolution in New and Old World rodents. In our case, we addressed this question by comparing patterns of molecular evolution between Old World and New World muroids.

Our results suggest that both the New and Old World muroid *Abpa* sequences share the signature of positive Darwinian selection. In both sets of sequences we found high rates of nonsynonymous substitutions within and among genera (Supplementary Table 2), consistent with positive selection. In particular, all analyses indicate that a similar subset of positions appears to be under positive selection within these two separate rodent lineages. These results suggest that changes in a few key residues might play a significant role in the evolution of this protein. Also, a prediction of solvent accessibility is consistent with previous results where the majority of sites under positive selection are mostly in exposed regions of the protein (Fig. 2; Supplementary Table 4). Because there is strong evidence that *Abpa27* plays an important role in speciation among Old World muroids, we speculate that the ABPA subunits might be playing a similar role among the New World rodents studied.

Interestingly, the patterns of intraspecific variation we identified are not entirely consistent with those reported for the *M. musculus* subspecies complex. The signal of positive selection detected among New World muroids is not as strong as the one detected among Old World rodents. There were five sites under selection inferred among New World rodents compared to the 12 sites detected in the Old World

muroids. In addition, despite our limited sample size, we found significant levels of intraspecific variation in species of the genera *Apodemus*, *Peromyscus* and *Reithrodontomys* (Supplementary Table 2). Thus, the data would suggest that the putative role of the *Abpa* gene in New World muroids might be slightly different relative to the *M. musculus* complex where different alleles segregate with different subspecies (Bímová et al. 2005; Bímová et al. 2011). The fact that alternative variants of the *Abpa27* paralog are fixed in the two subspecies *M. m. musculus* and *M. m. domesticus* and that the ABPA27 subunit is apparently involved with reinforcing reproductive barriers in the Central Europe hybrid zone between these two subspecies could account for the higher rate of molecular evolution in this system relative to New World rodents (Bímová et al. 2005; Bímová et al. 2011).

In rodents, chemical cues and pheromones play a central role in individual recognition as evidenced by the approximately 1,000 functional olfactory receptors and 212 vomeronasal receptors found in the mouse genome (Shi and Zhang 2009; Zhang and Firestein 2002). Currently, the primary hypotheses proposed to explain patterns of ABP evolution are related to assortative mating, and the coevolution of the ABP pheromone and vomeronasal (VNO) receptors of the V1R receptor family (Karn et al. 2010). As suggested by Karn et al. (2010), it may be that these two systems, ABP and V1R receptors, are coevolving to promote and maintain species boundaries. It would be interesting to identify the V1Rs involved in this putative interaction, and evaluate whether positive selection is also acting on their evolution.

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