

Comprehensive, structurally-curated alignment and phylogeny of vertebrate biogenic amine receptors

Stephanie J. Spielman^{1,2,3}, Keerthana Kumar^{1,2,3}, and Claus O. Wilke^{1,2,3}

¹Department of Integrative Biology, The University of Texas at Austin, Austin, U.S.A.

²Institute of Cellular and Molecular Biology, The University of Texas at Austin, Austin, U.S.A.

³Center for Computational Biology and Bioinformatics, The University of Texas at Austin, Austin, U.S.A.

ABSTRACT

Biogenic amine receptors play critical roles in regulating behavior and physiology, particularly within the central nervous system, in both vertebrates and invertebrates. These receptors belong to the G-protein coupled receptor (GPCR) family and interact with endogenous bioamine ligands, such as dopamine, serotonin, and epinephrine, and they are targeted by a wide array of pharmaceuticals. Despite these receptors' clear clinical and biological importance, their evolutionary history remains poorly characterized. In particular, the relationships among biogenic amine receptors and any specific evolutionary constraints acting within distinct receptor subtypes are largely unknown. To advance and facilitate studies in this receptor family, we have constructed a comprehensive, high-quality, structurally-curated sequence alignment of vertebrate biogenic amine receptors. We demonstrate that aligning GPCR sequences without considering structure produces an alignment with substantial error, whereas a structurally-aware approach greatly improves alignment accuracy. Moreover, we show that phylogenetic inference with our structurally-curated alignment offers dramatic improvements over a structurally-naïve alignment. Using the structural alignment its corresponding phylogeny, we deduce novel biogenic amine receptor relationships and uncover previously unrecognized lineage-specific receptor clades. Moreover, we find that roughly 1% of the 3039 sequences in our structurally-curated MSA were either misannotated or unclassified, and we propose updated classifications for these receptors. We release our comprehensive alignment and its corresponding phylogeny as a resource for any research group interested in studying the dynamic evolution and sequence patterns in to the biogenic amine receptors.

Keywords: biogenic amine receptors, G-protein coupled receptors, multiple sequence alignment, phylogenetics, protein evolution

INTRODUCTION

Biogenic amines, such as the molecules serotonin and dopamine, play critical roles in virtually all Metazoans and exert significant influence on both behavior and physiology. In vertebrates, the biogenic amine receptor family, which includes dopamine (DRD), histamine (HRH), trace (TAAR), adrenergic (ADR), muscarinic cholinergic (mAChR), and most serotonin (5HTR) receptors, primarily mediates biogenic amine activity. Biogenic amine receptors belong to the broad family of G protein-coupled receptors (GPCRs), one of the largest and most diverse eukaryotic receptor families. Indeed, due to the extensive diversity of biological functions they direct and the ongoing expansion of their ligand repertoire, GPCRs are considered one of the most evolutionarily innovative and successful gene families (Bockaert and Pin, 1999; Lagerstrom and Schioth, 2008).

Biogenic amine receptors form a clade within the large Rhodopsin-like GPCR family (Fredriksson et al., 2003; Kakarala and Jamil, 2014), whose emergence likely accompanied that of the Opisthokont (Fungi and Metazoa) lineage (Krishnan et al., 2012). The Rhodopsin-like family expanded substantially in Metazoa, and the specific diversification of biogenic amine receptors has contributed significantly to central nervous system functioning (Callier et al., 2003; Nichols and Nichols, 2008). Like all GPCRs, biogenic amine receptors have a characteristic, highly-conserved structure of seven transmembrane (TM) domains separated by three extracellular (ECL) and three intracellular (ICL) loops, and they propagate intracellular signaling through a G protein-mediated pathway. Moreover, these receptors are prominent targets for a wide range of pharmaceuticals aimed to treat myriad diseases such as schizophrenia, migraines, hypertension, allergies and asthma, and stomach ulcers (Schoneberg et al., 2004; Evers et al., 2005; Mason et al., 2012).

In spite of these receptors' biological and clinical importance, studies on their evolution are limited and have predominantly focused on individual receptor subtypes, namely TAAR (Gloriam et al., 2005; Lindemann et al., 2005; Hashiguchi and Nishida, 2007), DRD (Callier et al., 2003; Yamamoto et al., 2013), and 5HTR (Anbazhagan et al., 2010). Moreover, many of these studies, and indeed studies on the general evolution of the Rhodopsin-like family, have examined very narrow species distributions, for instance specifically teleosts (Gloriam et al., 2005), primates (Anbazhagan et al., 2010), humans and mice (Vassilatis et al., 2003; Kakarala and Jamil, 2014), or even strictly humans (Fredriksson et al., 2003). Thus, virtually no studies accounting for the full breadth of vertebrate biogenic amine receptor sequences have been conducted.

To gain a comprehensive understanding of this receptor family's evolution, a high-quality multiple sequence alignment (MSA) is needed. MSAs provide the foundation for nearly all comparative sequence analyses, and they are commonly used to locate conserved sequence motifs, identify functionally important residues, and investigate evolutionary histories. As constructing an MSA represents the first step in any sequence analysis, MSA errors are known to bias these downstream analyses (Ogden and Rosenberg, 2006; Wong et al., 2008; Jordan and Goldman, 2012). It is therefore crucial to ensure accuracy in MSAs to the extent possible.

For GPCR sequences, in particular, any MSA should recapitulate the canonical seven-TM structure, which a naive alignment of sequences cannot necessarily accomplish. While there are certain MSA software platforms that explicitly incorporate structural information into the alignment algorithm [e.g. 3DCoffee (O'Sullivan et al., 2004), PROMALS3D (Pei et al., 2008), and HMMALIGN (Eddy, 1998)], these programs are fairly computationally-intensive and thus ill-suited for large-scale applications. Furthermore, such programs require the use of a single crystal structure or HMM profile to guide sequence alignment. While all GPCRs contain seven TM domains, different GPCR subfamilies, particularly the biogenic amine receptors, feature a wide variety of ICL and ECL sizes. For example, human HRH1 and DRD3 contain roughly 27 and 117 residues, respectively, in their ECL3 domains, and roughly 68 and 14 residues, respectively, in their ICL3 domains [as predicted by GPCRHMM (Wistrand et al., 2006)]. Thus, aligning diverse sequences using a single structure may not effectively capture the domain variability across biogenic amine receptor subtypes. A desirable alignment strategy would instead anchor all sequences by their conserved TM domains without inappropriately constraining the heterogeneous ECL and ICL domains.

Here, we have constructed such an MSA using a novel iterative strategy to ensure that all seven TM domains aligned correctly across all sequences, thus yielding the most comprehensive (3039 sequences) and structurally-curated vertebrate biogenic amine receptor MSA to date. We used this MSA to construct a structurally-aware maximum likelihood (ML) phylogeny of vertebrate biogenic amine receptors, and we found that our structurally-curated MSA offered dramatic improvements in phylogenetic fit relative to a structurally-naive MSA. Using this structurally-aware phylogeny, we were able to discern relationships among biogenic amine receptor subtypes with a far increased level of sensitivity relative to previous studies, as well as identify novel lineage-specific receptor clades and clarify NCBI annotations for over 30 sequences.

We present this vertebrate biogenic amine receptor MSA and its corresponding phylogeny as a resource for any group interested in studying the dynamic evolutionary processes, structural, and/or functional constraints operating within this class of GPCRs. All data, including MSAs, phylogenies, and sequence descriptions, as well as all code used to generate these data, are freely available from

https://github.com/sjspielman/amine_receptors. We expect that these data will prove extremely useful for studying both the broad patterns governing biogenic amine receptor sequence evolution and the evolutionary trends specific to certain receptor subtypes. Further, our curated MSA should serve as a helpful resource in the ongoing development of homology models and pharmaceutical therapeutics targeting these receptors (Kristiansen, 2004; Ishiguro, 2004; Evers et al., 2005; Mason et al., 2012).

RESULTS AND DISCUSSION

Constructing a structurally-curated MSA of biogenic amine receptors

We collected all sequences using PSI-BLAST from the RefSeq (Pruitt et al., 2013) database and filtered data to exclude poor-quality sequences and/or sequences with excessive ambiguities (see *Methods* for details). We additionally retained only sequences that the program GPCRHMM (Wistrand et al., 2006) could confidently classify as GPCRs, leaving a total dataset of 3464 receptor sequences to align. Before aligning sequences, we again used the program GPCRHMM (Wistrand et al., 2006) to assign every residue in all protein sequences to its respective structural domain (extracellular, transmembrane, or intracellular). GPCRHMM uses a hidden markov model approach to predict GPCR domains from a given protein sequence alone with high accuracy. Indeed, previous work has shown that GPCRHMM's domain predictions map exceptionally well to resolved Rhodopsin-like GPCR crystal structures (Spielman and Wilke, 2013).

We then built a structurally-curated MSA from these 3464 protein sequences according to the iterative strategy outlined in Figure 1. For each iteration, we aligned protein sequences with MAFFT (Katoh and Standley, 2013). Using the residue

domain assignments computed with GPCRHHM, we determined the consensus domain for each column in this MSA. Next, we discarded all sequences for which $\geq 5\%$ of residues did not correspond to their respective column's consensus domain. We realigned the remaining sequences with MAFFT and continued in this manner until no more sequences were discarded. Importantly, this strategy did not require any manual data filtering or visual data inspection, thus avoiding any confounding subjectivity in MSA processing. The final structurally-curated MSA contained a total of 3039 sequences, which were broadly distributed across receptor subtypes and vertebrate taxa (Figure 2).

From this final MSA, we additionally created a masked MSA, in which protein residues which did not conform to their respective consensus domains were replaced with a "?". By masking these positions with an ambiguous character, we ensured that each MSA column strictly contained residues belonging to the same structural domain. In total, 2.69% of all MSA positions were masked for this MSA.

We found that our structurally-curated MSA featured far less error than did a structurally-naïve MSA, built in MAFFT (Kato and Standley, 2013) from all 3464 GPCR sequences without undergoing the process in Figure 1. A graphical representation of the naïve and structurally-curated MSA, focused specifically on the MSA regions containing the TM domains, is shown in Figure 3. Clearly, many sequences in the naïve MSA do not conform to the overarching GPCR structure. Instead, many of these sequences' domains are shifted out of structural frame, leading TM domains to inappropriately align with loop domains, or vice versa, ultimately preventing MSA columns from being truly homologous. Moreover, the mere presence of these misaligned sequences in the naïve MSA introduced a substantial amount of gaps, often times within a single TM domain (notably TM1 and TM7, as seen in Figure 3). By removing such misaligned sequences through the strategy outlined in Figure 1, many of these gaps subsequently disappeared, leading to the alignment integrity of individual TM domains. Indeed, the naïve MSA contained roughly 23% more columns than did the structurally-curated MSA, and the vast majority of gaps in the structurally-curated MSAs occurred within the highly heterogeneous N-term, C-term, and ICL3 domains.

Structurally-aware MSA strongly improves phylogenetic inference

To demonstrate the utility of analyzing biogenic amine receptors with a structurally-curated MSA, we constructed five distinct maximum likelihood (ML) phylogenies, as detailed in Table 2, in RAxML. We built a phylogeny using the naïve MSA, and we built two phylogenies each from the structural unmasked and masked MSAs. Previous work has shown that combined structural and functional constraints impose differing selection pressures in TM vs. extramembrane (EM) domains, in turn producing distinct amino-acid frequency distributions in each domain class (Tourasse and Li, 2000; Stevens and Arkin, 2001; Julenius and Pedersen, 2006; Oberai et al., 2009; Spielman and Wilke, 2013; Franzosa et al., 2013). As our structurally-curated MSAs allowed us to precisely identify each MSA column as either TM or EM, we were able to conduct far more rigorous phylogenetic inference using a partitioned analysis. Therefore, for each of our two structurally-curated MSAs (masked and unmasked), we inferred two ML phylogenies: one with two partitions representing TM and EM columns and one with a single partition for the entire MSA.

As assessed by AIC scores, the structurally-curated masked MSA yielded a far superior phylogeny compared to all other MSAs (Table 2), highlighting the benefits of analyzing GPCRs in a structurally-aware context. This finding also underscores that any structurally-aware study must be undertaken cautiously. While partitioning the MSA based on structural domains was clearly beneficial, ensuring that each MSA column strictly contained residues belonging to the same domain was critical. Having even a few TM residues in a column assigned to the EM partition, or vice versa, strongly hindered phylogenetic fit.

That the masked structural MSA produced a phylogeny with far better fit than did the unmasked structural MSA additionally reveals the potential benefits to MSA filtering, which entails removing putatively unreliable positions and/or columns from MSAs. In spite of a large body of literature investigating the utility of MSA filtering, the particular circumstances under which filtering is beneficial remain ambiguous (Castresana, 2000; Talavera and Castresana, 2007; Schloss, 2010; Penn et al., 2010; Jordan and Goldman, 2012; Privman et al., 2012; Wu et al., 2012; Spielman et al., 2014). Here, we find that masking residues based on biological information regarding protein structure can greatly improve phylogenetic fit (Table 2), indicating that residue filtering, at least in this circumstance, successfully increased phylogenetic signal. However, we caution that standard MSA filtering algorithms may not always lead to such improvements. For instance, the underlying problem with our structurally-naïve MSA was likely not misaligned residues or columns, but rather the presence of confounding sequences which would not align properly with the overarching GPCR structure. Masking particular MSA regions would not have addressed this issue, as confounding sequences would have remained in the MSA even after filters were applied. Therefore, while MSA filtering may be beneficial, residue masking alone cannot fix a poorly-constructed MSA.

Structurally-aware phylogeny reveals unknown biogenic amine receptor relationships and clades

Our resulting phylogeny, shown in Figure 4, represents the most comprehensive and curated vertebrate biogenic amine receptor phylogeny to date. This tree broadly captures many known features of biogenic amine receptor evolution, in

particular that these receptors do not cluster based on ligand-binding but rather have undergone extensive functional convergent evolution. Indeed, our phylogeny reveals that only two ligand-based receptor classes, mAChR and TAAR, are truly monophyletic.

Our phylogeny features remarkably high bootstrap support for each distinct clade of receptor subtypes. We additionally find very strong support for three deeper nodes in the phylogeny that reveal the relationships among distinct receptor subtypes. The first contains the three clades HRH1, mAChR, and HRH-3,4, the second contains the clades 5HTR-1, 5HTR-5, and 5HTR-7, and the third contains the 5HTR-4 and TAAR clades. Previous studies have yielded conflicting phylogenetic placements for the 5HTR-7 clade; some have argued that 5HTR-7 is phylogenetically distinct from all other 5HTR sequences (Kakarala and Jamil, 2014), while others have found that evidence for a single clade containing 5HTR-5,7 as a sister taxa to a clade containing ADRA1 sequences (Fredriksson et al., 2003). Alternatively, we find moderate-to-strong support for the 5HTR-7 clade having originated before subsequent diversification into 5HTR-5 and 5HTR-1, and we find full support showing that ADRA1 forms an entirely distinct monophyletic group outside all other vertebrate biogenic amine receptors. In addition, as previously mentioned, our phylogeny reveals that HRH-3,4 is actually a single monophyletic group. While the HRH-4 clade contains strictly mammalian sequences, including monotreme (platypus) sequences, HRH-3 sequences are broadly distributed across vertebrate taxa. We therefore hypothesize that HRH-4 arose from an HRH-3 duplication concurrent with mammalian origins.

In addition, among the 3039 sequences in structurally-curated MSA, we identified 31 sequences (1%) that we considered misannotated (Table 3), either because the NCBI annotation did not match the sequences' phylogenetic placement or the sequences did not cluster with known biogenic amine receptor types. Several NCBI annotations identified the correct receptor class but the incorrect receptor subtype, whereas other sequences were entirely uncharacterized. We additionally uncovered an entirely unknown clade of biogenic amine receptors. This unknown clade, sister to HRH2, only contains avian sequences and a single *Xenopus tropicalis* (western-clawed frog) sequence. Two evolutionary scenarios may explain this taxonomic distribution: either this clade emerged concurrently with tetrapods and was secondarily lost in reptiles/birds and mammals, or this clade represents an avian-specific diversification which the *Xenopus tropicalis* sequence resembles only convergently. Interestingly, all but one of this clade's sequences were annotated in NCBI as either octopamine or No9-like receptors, both of which are insect-specific biogenic amine receptors that do not occur in vertebrate taxa (Roeder, 2005). The last sequence, alternatively, was annotated as 5HTR-7-like. Taken together, these sequence misannotations suggest an intriguing hypothesis that this clade evolved from an ancestral 5HTR sequence, and subsequent convergent evolution to insect-specific biogenic amine receptors has allowed these receptors to interact with atypical ligands for vertebrates.

Dynamic evolution of the trace-amine associated receptors

Of particular interest in our phylogeny are the unique evolutionary patterns revealed within the TAAR clade. While all TAAR sequences do cluster together, the relationships among TAAR subtypes are highly dynamic, reflecting the extensive expansion and contraction events characterizing this receptor family's evolution (Lindemann et al., 2005; Hashiguchi and Nishida, 2007; Stäubert et al., 2010, 2013). In fact, the TAAR subtree, displayed in Figure 5, differs somewhat from previously proposed TAAR phylogenies (Lindemann et al., 2005; Hashiguchi and Nishida, 2007). In particular, the presence of several lineage-specific subclades as well as unresolved subclades generate novel hypotheses regarding TAAR subtype origins. While TAAR-2, -3, and -4 form a well-resolved monophyly, its sister clade that contains the subtypes TAAR-5, -6, -7, -8, and -9 is less straight-forward to interpret. Indeed, the TAAR subtypes -6, -7, and -9 do not constitute distinct monophyletic groups, suggesting either poor NCBI sequence annotation or rampant diversification within this subclade. If we assume that these NCBI annotations are reasonably correct, we can deduce that this clade's ancestral sequence was most similar to TAAR-7 and subsequently diversified independently into TAAR-9 and TAAR-6, which in turn gave rise to the monophyletic TAAR-8.

Furthermore, several lobe-finned fish (coelacanth) sequences are scattered across the TAAR tree and do not clearly cluster with any TAAR subtypes, likely reflecting this lineage's ancient divergence and unique evolutionary trajectory (Amemiya et al., 2013). The phylogenetic distribution of lobe-finned fish sequences may aid future endeavors to tease apart evolutionary origins of certain TAAR subtypes, specifically whether they represent teleost-specific duplications (Gloriam et al., 2005) or whether they represent ancient TAARs that emerged before teleost divergence but were secondarily lost in lobe-finned fish and/or tetrapods.

In addition, a small clade sister to TAAR (labeled in Figures 4 and 5 as TAAR*) strictly contains sequences annotated by NCBI as "5HTR-4-like." At first glance, these annotations might suggest that 5HTR-4 is in fact paraphyletic, diversifying gradually before giving rise to TAARs. However, as all sequences in TAAR* belong taxonomically either to teleost or *Xenopus tropicalis*, we suspect that this clade actually corresponds to the so-called TAAR-V cluster identified by Hashiguchi and Nishida (2007). Indeed, the TAAR-V cluster contains a similar taxonomic distribution to our TAAR* and constitutes an outgroup to all other vertebrate TAAR sequences, as our phylogeny similarly displays.

Phylogenetic methods alone do not suffice to infer the evolutionary history of biogenic amine receptors

Although we were able to identify several new features of biogenic amine receptor evolution, the majority of deeper splits in the phylogeny had very low bootstrap support. Therefore, many of the broader relationships among biogenic amine receptors remain unresolved, and therefore this phylogeny alone is not sufficient for fully resolving the relationships among biogenic amine receptors. This result highlights the limitations of using a strictly phylogenetic approach to elucidate the complex evolutionary histories of expanding gene families. Indeed, modern phylogenetic inference methods are ill-suited for deducing such relationships, particularly because MSA gaps are treated simply as missing data. In reality, gaps represent the evolutionary events of insertion and deletions (indels). Current phylogenetic methods, however, focus solely on the substitution process and ignore the evolutionary information contained in gaps, ultimately hindering phylogenetic accuracy (Morrison, 2008; Loytynoja and Goldman, 2008; Warnow, 2012; Luan et al., 2013).

This limitation is especially problematic for GPCRs. Following duplication events, GPCRs appear to experience major indel events in their ICL and/or ECL domains, leading to dramatic shifts in loop domain sizes during the sub/neofunctionalization process. Unfortunately, the evolutionary intermediates that existed during these domain transitions have long-since disappeared from genomes, and there is no obvious way to infer the sequences of these missing links. Although the substitution process is key for understanding GPCR evolution, fully classifying relationships among GPCR families requires some understanding of how these radical domain changes occur. Therefore, additional approaches, such as syntenic analyses (Sundstrom et al., 2010; Widmark et al., 2011; Yegorov and Good, 2012; Hwang et al., 2013), combined with the phylogeny presented here should prove useful towards resolving the complete evolutionary history of vertebrate biogenic amine receptors.

CONCLUSIONS

We have established a comprehensive, high-quality, structurally-curated MSA of vertebrate biogenic amine receptors. We hope that this MSA, along with its ML phylogeny, will serve as a robust resource for future studies investigating the evolutionary dynamics as well as structural/functional constraints operating within distinct receptor clades or indeed universal patterns that generally govern biogenic amine receptor evolution. Future work may seek to combine the analyses we have accomplished here with syntenic or molecular clock approaches to elucidate receptors' origin and precise evolutionary trajectories. Moreover, our curated MSA should prove useful in increasing accuracy in homology modeling and/or pharmaceutical development for these clinically important receptors (Kristiansen, 2004; Ishiguro, 2004; Evers et al., 2005; Mason et al., 2012).

METHODS

Sequence Collection and Processing

We collected protein sequences using PSI-BLAST (Altschul et al., 1997), specifically from the RefSeq (v2.2.29+) database (Pruitt et al., 2013), for 42 distinct human biogenic amine receptor sequences representing the full range of known receptors in the human genome. To obtain distant yet well-supported orthologs, we ran each PSI-BLAST search for 5 iterations with an e-value cutoff of 10^{-20} , a sequence identity threshold of 25%, and a length difference of $\pm 50\%$ relative to the seed sequence. After combining all sequences recovered from the individual PSI-BLAST searches, we discarded duplicate sequences, leaving a total of 4232 PSI-BLAST results. We then filtered this sequence set to remove sequences from non-vertebrate taxa, sequences annotated as low-quality, pseudogene, and/or partial, and sequences which contained more than 1% ambiguous residues (i.e. B, X, or Z). We additionally used the program GPCRHMM (Wistrand et al., 2006) to determine whether a given sequence was indeed a GPCR. We discarded sequences which had either a local or global GPCRHMM score less than 10, both extremely conservative thresholds. Thus, while it is possible that some true GPCRs were discarded, these stringent thresholds for both local and global scores provide high confidence that all retained sequences were indeed GPCRs. Together, these filters left a total of 3464 receptor sequences.

Sequence Alignment and Phylogenetic Reconstruction

Before aligning sequences, we used the program GPCRHMM (Wistrand et al., 2006) to assign each residue in all protein sequences to its respective structural domain (extracellular, transmembrane, or intracellular) using a 0.5 posterior probability cutoff. We then aligned and filtered sequences according to the strategy outlined in Figure 1, which specifically employed MAFFT v7.149b (Kato and Standley, 2013).

All phylogenies were created using RAxML v8.1.1 (Stamatakis, 2014) using the LG+F (Le and Gascuel, 2008) amino acid exchangeability matrix with empirical amino acid frequencies and the CAT model of site heterogeneity (Stamatakis, 2006), with the default 25 rate categories. For inferences incorporating structural partitions, we assigned each partition a

unique evolutionary model using these settings. Final parameter values for all phylogenetic inferences were optimized with the GAMMA model of heterogeneity. We performed 200 bootstrap replicates for each phylogeny.

ACKNOWLEDGMENTS

This work was supported in part by NIH grant R01 GM088344, ARO grant W911NF-12-1-0390, DTRA grant HDTRA1-12-C-0007, and NSF Cooperative Agreement No. DBI-0939454 (BEACON Center). Computational resources were provided by the University of Texas at Austin's Center for Computational Biology and Bioinformatics (CCBB). We would like to thank Ahmad R. Sedaghat, MD, PhD for suggesting amine-receptor evolution as a worthwhile study system.

REFERENCES

- Akaike, H. (1974). A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, 19:6:716 – 723.
- Altschul, S., Madden, T., Schäffer, A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res*, 25:3389 – 3402.
- Amemiya, C. T., Alföldi, J., Lee, A. P., Fan, S., Philippe, H., MacCallum, I., Braasch, I., Manousaki, T., Schneider, I., Rohner, N., Organ, C., Chalopin, D., Smith, J. J., Robinson, M., Dorrington, R. A., Gerdol, M., Aken, B., Biscotti, M. A., Barucca, M., Baurain, D., Berlin, A. M., Blatch, G. L., Buonocore, F., Burmester, T., Campbell, M. S., Canapa, A., Cannon, J. P., Christoffels, A., De Moro, G., Edkins, A. L., Fan, L., Fausto, A. M., Feiner, N., Forconi, M., Gamielien, J., Gnerre, S., Gnirke, A., Goldstone, J. V., Haerty, W., Hahn, M. E., Hesse, U., Hoffmann, S., Johnson, J., Karchner, S. I., Kuraku, S., Lara, M., Levin, J. Z., Litman, G. W., Mauceli, E., Miyake, T., Mueller, M. G., Nelson, D. R., Nitsche, A., Olmo, E., Ota, T., Pallavicini, A., Panji, S., Picone, B., Ponting, C. P., Prohaska, S. J., Przybylski, D., Saha, N. R., Ravi, V., Ribeiro, F. J., Sauka-Spengler, T., Scapigliati, G., Searle, S. M. J., Sharpe, T., Simakov, O., Stadler, P. F., Stegeman, J. J., Sumiyama, K., Tabbaa, D., Tafer, H., Turner-Maier, J., van Heusden, P., White, S., Williams, L., Yandell, M., Brinkmann, H., Volff, J.-N., Tabin, C. J., Shubin, N., Schartl, M., Jaffe, D. B., Postlethwait, J. H., Venkatesh, B., Di Palma, F., Lander, E. S., Meyer, A., and Lindblad-Toh, K. (2013). The african coelacanth genome provides insights into tetrapod evolution. *Nature*, 496:311 – 316.
- Anbazzhagan, P., Purushottam, M., Kiran Kumar, H. B., Mukherjee, O., Jain, S., and Sowdhamini, R. (2010). Phylogenetic analysis and selection pressures of 5-HT receptors in human and non-human primates: Receptor of an ancient neurotransmitter. *J Biomol Struct Dyn*, 27(5):581 – 598.
- Bockaert, J. and Pin, J. P. (1999). Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J*, 18(7):1723 – 1729.
- Burnham, K. P. and Anderson, D. R. (2004). Multimodel inference: understanding AIC and BIC in model selection. *Sociol Method Res*, 33:261 – 304.
- Callier, S., Snapyan, M., Crom, S., Prou, D., Vincent, J.-D., and Vernier, P. (2003). Evolution and cell biology of dopamine receptors in vertebrates. *Biol Cell*, 95(7):489 – 502.
- Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution*, 17(4):540–552.
- Eddy, S. (1998). Profile hidden Markov models. *Bioinformatics*, 14:755 – 763.
- Evers, A., Hessler, G., Matter, H., and Klabunde, T. (2005). Virtual screening of biogenic amine-binding G-protein coupled receptors: Comparative evaluation of protein- and ligand-based virtual screening protocols. *J Med Chem*, 48(17):5448 – 5465.
- Franzosa, E., Xue, R., and Y, X. (2013). Quantitative residue-level structure-evolution relationships in the yeast membrane proteome. *Genome Biol Evol*, 5:734 – 744.
- Fredriksson, R., Lagerstrom, M., Lundin, L., and Schioth, H. (2003). The G-protein-coupled receptors in the human genome form five main families. phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol*, 63(6):1256 – 1272.
- Gloriam, D. E. I., Bjarnadóttir, T. K., Yan, Y.-L., Postlethwait, J. H., Schioth, H. B., and Fredriksson, R. (2005). The repertoire of trace amine G-protein-coupled receptors: large expansion in zebrafish. *Mol Phylogenet Evol*, 35(2):470 – 482.
- Hashiguchi, Y. and Nishida, M. (2007). Evolution of trace amine associated receptor (taar) gene family in vertebrates: Lineage-specific expansions and degradations of a second class of vertebrate chemosensory receptors expressed in the olfactory epithelium. *Mol Biol Evol*, 24(9):2099 – 2107.
- Hwang, J. I., Moon, M. J., Park, S., Kim, D. K., Cho, E. B., Ha, N., Son, G. H., Kim, K., Vaudry, H., and Seong, J. Y. (2013). Expansion of secretin-like G protein-coupled receptors and their peptide ligands via local duplications before and after two rounds of whole-genome duplication. *Mol Biol Evol*, 30(5):1119 – 1130.

- Ishiguro, M. (2004). Ligand-binding modes in cationic biogenic amine receptors. *ChemBioChem*, 5(9):1210 – 1219.
- Jordan, G. and Goldman, N. (2012). The effects of alignment error and alignment filtering on the sitewise detection of positive selection. *Mol Biol Evol*, 29:1125 – 1139.
- Julenius, K. and Pedersen, A. G. (2006). Protein evolution is faster outside the cell. *Mol Biol Evol*, 23:2039 – 2048.
- Kakarala, K. K. and Jamil, K. (2014). Sequence-structure based phylogeny of GPCR class A rhodopsin receptors. *Mol Phylogenet Evol*, 74(C):66 – 96.
- Katoh, K. and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. *Mol Biol Evol*, 30:772 – 780.
- Krishnan, A., Almén, M. S., Fredriksson, R., and Schioth, H. B. (2012). The origin of gpcrs: Identification of mammalian like rhodopsin, adhesion, glutamate and frizzled gpcrs in fungi. *PLoS ONE*, 7(1):e29817.
- Kristiansen, K. (2004). Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacol Therapeut*, 103(1):21 – 80.
- Lagerstrom, M. C. and Schioth, H. B. (2008). Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov*, 7:339 – 357.
- Le, S. and Gascuel, O. (2008). An improved general amino acid replacement matrix. *Mol Biol Evol*, 25:1307 – 1320.
- Lindemann, L., Ebeling, M., Kratochwil, N. A., Bunzow, J. R., Grandy, D. K., and Hoener, M. C. (2005). Trace amine-associated receptors form structurally and functionally distinct subfamilies of novel G protein-coupled receptors. *Genomics*, 85(3):372 – 385.
- Loytynoja, A. and Goldman, N. (2008). Phylogeny-aware gap placement prevents errors in sequence alignment and evolutionary analysis. *Science*, 320:1632 – 1635.
- Luan, P.-t., Ryder, O. A., Davis, H., and Zhang, Y.-p. (2013). Incorporating indels as phylogenetic characters: impact for interfamilial relationships within Arctoidea (Mammalia: Carnivora). *Mol Phylogenet Evol*, 66:748 – 756.
- Mason, J. S., Bortolato, A., Congreve, M., and Marshall, F. H. (2012). New insights from structural biology into the druggability of G protein-coupled receptors. *Trends in Pharmacol Sci*, 33(5):249 – 260.
- Morrison, D. A. (2008). A framework for phylogenetic sequence alignment. *Plant Syst Evol*, 282(3-4):127 – 149.
- Nichols, D. E. and Nichols, C. D. (2008). Serotonin receptors. *Chemical Reviews*, 108(5):1614 – 1641.
- Oberai, A., Joh, N. H., Pettit, F. K., and Bowie, J. U. (2009). Structural imperatives impose diverse evolutionary constraints on helical membrane proteins. *Proc Natl Acad Sci USA*, 106:17747 – 17750.
- Ogden, T. H. and Rosenberg, M. S. (2006). Multiple sequence alignment accuracy and phylogenetic inference. *Syst Biol*, 55(2):314 – 328.
- O'Sullivan, O., Suhre, K., Abergel, C., Higgins, D. G., and Notredame, C. (2004). 3DCoffee: Combining protein sequences and structures within multiple sequence alignments. *J Mol Biol*, 340:385 – 395.
- Pei, J., Kim, B. H., and Grishin, N. V. (2008). PROMALS3D: a tool for multiple protein sequence and structure alignments. *Nuc Acids Res*, 36(7):2295 – 2300.
- Penn, O., Privman, E., Landan, G., Graur, D., and Pupko, T. (2010). An alignment confidence score capturing robustness to guide tree uncertainty. *Mol Biol Evol*, 27:1759–1767.
- Privman, E., Penn, O., and Pupko, T. (2012). Improving the performance of positive selection inference by filtering unreliable alignment regions. *Mol Biol Evol*, 29:1–5.
- Pruitt, K. D., Brown, G. R., Hiatt, S. M., Thibaud-Nissen, F., Astashyn, A., Ermolaeva, O., Farrell, C. M., Hart, J., Landrum, M. J., McGarvey, K. M., Murphy, M. R., O'Leary, N. A., Pujar, S., Rajput, B., Rangwala, S. H., Riddick, L. D., Shkeda, A., Sun, H., Tamez, P., Tully, R. E., Wallin, C., Webb, D., Weber, J., Wu, W., DiCuccio, M., Kitts, P., Maglott, D. R., Murphy, T. D., and Ostell, J. M. (2013). RefSeq: an update on mammalian reference sequences. *Nucleic Acids Res*, 42(D1):D756–D763.
- Roeder, T. (2005). Tyramine and octopamine: Ruling behavior and metabolism. *Annual Review of Entomology*, 50(1):447 – 477.
- Schloss, P. D. (2010). The effects of alignment quality, distance calculation method, sequence filtering, and region on the analysis of 16S rRNA gene-based studies. *PLoS Comput Biol*, 6(7):e1000844.
- Schoneberg, T., Schulz, A., Biebertmann, H., Hermsdorf, T., H. R., and Sangkuhl, K. (2004). Mutant G protein-coupled receptors as a cause of human diseases. *Pharmacol Ther*, 104:173 – 206.
- Spielman, S., Dawson, E., and Wilke, C. (2014). Limited utility of residue masking for positive-selection inference. *Mol Biol Evol*, 31:2496–2500.
- Spielman, S. J. and Wilke, C. O. (2013). Membrane environment imposes unique selection pressures on transmembrane domains of G protein-coupled receptors. *J Mol Evol*, 76(3):172 – 182.
- Stamatakis, A. (2006). Phylogenetic models of rate heterogeneity: a high performance computing perspective. In *Proc. of*

- Stamatakis, A. (2014). RAxML Version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, 30(9):1312 – 1313.
- Stäubert, C., Bösel, I., Bohnkamp, J., Rompler, H., Enard, W., and Schöneberg, T. (2010). Structural and functional evolution of the trace amine-associated receptors TAAR3, TAAR4 and TAAR5 in primates. *PLoS ONE*, 5(6):e11133.
- Stäubert, C., Le Duc, D., and Schöneberg, T. (2013). Examining the dynamic evolution of G protein-coupled receptors. In *G protein-coupled receptor genetics: research and methods in the post-genomic era*, pages 23 – 43. Springer, Totowa, NJ.
- Stevens, T. J. and Arkin, I. T. (2001). Substitution rates in alpha-helical transmembrane proteins. *Prot Sci*, 10:2507 – 2517.
- Sundstrom, G., Dreborg, S., and Larhammar, D. (2010). Concomitant duplications of opioid peptide and receptor genes before the origin of jawed vertebrates. *PLoS One*, 5:e10512.
- Talavera, G. and Castresana, J. (2007). Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology*, 56(4):564–577.
- Tourasse, N. J. and Li, W.-H. (2000). Selective constraints, amino acid composition, and the rate of protein evolution. *Mol Biol Evol*, 17:656 – 664.
- Vassilatis, D. K. et al. (2003). The G protein-coupled receptor repertoires of human and mouse. *Proc Natl Acad Sci USA*, 100(8):4903 – 4908.
- Warnow, T. (2012). Standard maximum likelihood analyses of alignments with gaps can be statistically inconsistent. *PLoS Curr*, 4:RRN1308.
- Widmark, J., Sundstrom, G., Ocampo, D., and Larhammar, D. (2011). Differential evolution of voltage-gated sodium channels in tetrapods and teleost fishes. *Mol Biol Evol*, 28:859 – 871.
- Wistrand, M., Käll, L., and Sonnhammer, E. L. L. (2006). A general model of G protein-coupled receptor sequences and its application to detect remote homologs. *Prot Sci*, 15(3):509 – 521.
- Wong, K. M., Suchard, M. A., and Huelsenbeck, J. P. (2008). Alignment uncertainty and genomic analysis. *Science*, 319(5862):473 – 476.
- Wu, M., Chatterji, S., and Eisen, J. A. (2012). Accounting for alignment uncertainty in phylogenomics. *PLoS ONE*, 7(1):e30288.
- Yamamoto, K., Mirabeau, O., Bureau, C., Blin, M., Michon-Coudouel, S., Demarque, M., and Vernier, P. (2013). Evolution of dopamine receptor genes of the D1 class in vertebrates. *Mol Biol Evol*, 30(4):833 – 843.
- Yegorov, S. and Good, S. (2012). Using paleogenomics to study the evolution of gene families: Origin and duplication history of the relaxin family hormones and their receptors. *PLoS ONE*, 7(3):e32923.

FIGURES AND TABLES

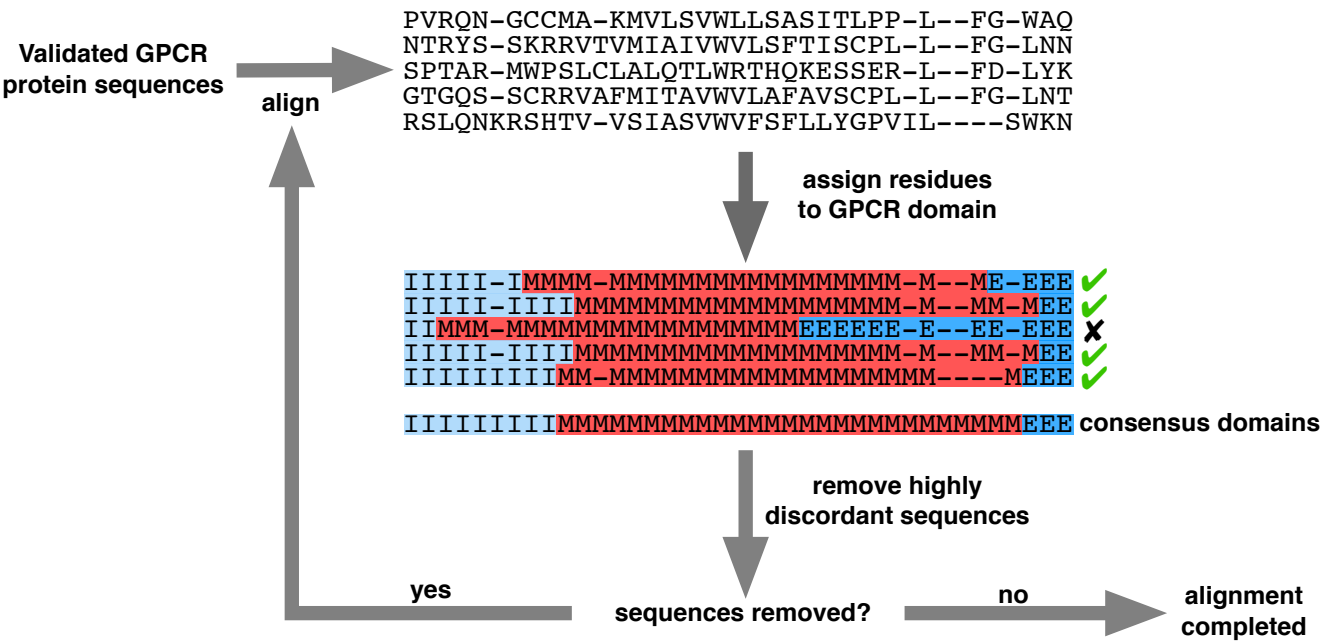


Figure 1. Iterative alignment strategy to create a structurally-curated MSA of vertebrate biogenic amine receptors. A total of 3464 sequences were initially input (“Validated GPCR protein sequences”), and the final MSA contained 3039 protein sequences. Residues marked with “I” represent intracellular residues, those marked with “M” represent transmembrane residues, and those marked with “E” represent extracellular residues. MSA gaps were treated as missing data when determining each column’s consensus structural domain. Sequences were removed (“remove highly discordant sequences”) if $\geq 5\%$ of columns belonged to a different structural domain than the respective consensus domain.

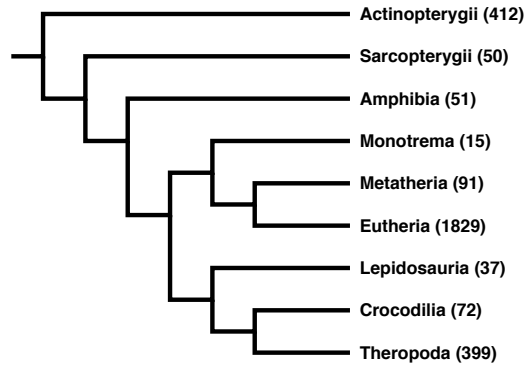


Figure 2. Cladogram of the taxonomic distribution of all sequences in the final structurally-curated MSA. All sequences belonged to the Euteleostomi clade of jawed vertebrates. Numbers in parentheses indicate the total number of sequences from the respective clade. We note that our MSA is particularly enriched for sequences from Eutherian (placental mammal) species, likely due to the stringent filters we applied to sequence collection that favored fully-sequenced genomes.

Receptor Class	Abbreviation	N
Serotonin receptors	5HTR	972
Trace amine-associated receptors	TAAR	343
Histamine receptors	HRH	286
Muscarinic cholinergic receptors	mAChR	353
Adrenergic receptors	ADR	611
Dopamine receptors	DRD	464
Unknown receptors	Unknown	10

Table 1. Biogenic amine receptor classes, and their abbreviations, considered in this study. The receptor class “Unknown” refers to the corresponding uncharacterized clade in Figure 4, and the column “N” indicates the total number of sequences for each broad receptor class in our structurally-curated MSA.

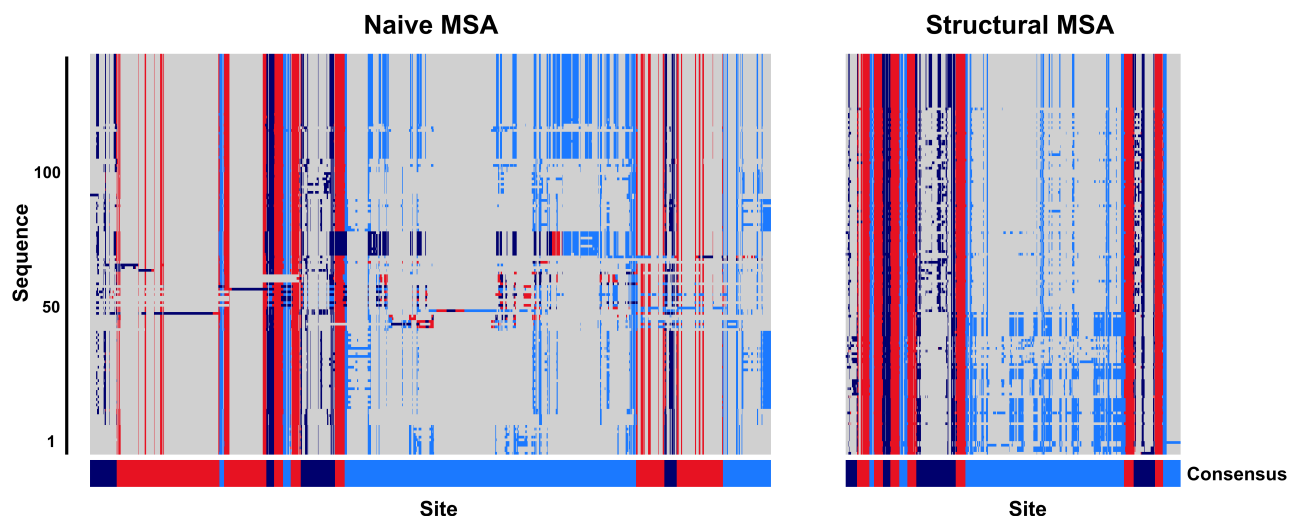


Figure 3. Graphical representation of a subset of the naive and structurally-curated biogenic amine receptor MSAs. Each image displays 130 MSA rows focused specifically on the MSA section containing the seven TM domains. Dark blue represents predicted extracellular residues, red represents predicted TM residues, lighter blue represents predicted intracellular residues, and gray represents MSA gaps. The bottom bar below each MSA figure shows the consensus domain structure for each MSA. Note that all columns which contain only gaps have been removed from this figure for visual clarity.

MSA	Partitioned	$\ln L$	k	ΔAIC
Structural Masked	Yes	-505500.8	6115	0
Structural Masked	No	-515991.7	6095	1752
Structural Unmasked	Yes	-515343.6	6115	19685
Structural Unmasked	No	-515991.7	6095	20941
Naive	No	-589703.7	6945	170047

Table 2. ΔAIC scores relative to the best performing for phylogenies. The column labeled “Partitioned” indicates whether phylogenetic inference was conducted with distinct TM (transmembrane) and EM (extramembrane) partitions. AIC is computed as $AIC = 2(k - \ln L)$, where k is the number of free parameters of the model, and $\ln L$ is the log-likelihood (Akaike, 1974; Burnham and Anderson, 2004). AIC scores are reported here relative to the phylogeny with the lowest AIC score (structural masked with partitions), representing the best-fitting phylogeny.

Protein ID	Nucleotide ID	Current Classification	Proposed Classification
XP_005797918.1	XM_005797861.1	DRD-2	DRD-3
XP_003967971.1	XM_003967922.1	DRD-2	DRD-3
NP_001266433.1	NM_001279504.1	mAChR-4	mAChR-2
XP_001520508.2	XM_001520458.3	HRH-3	HRH-4
XP_005282846.1	XM_005282789.1	HRH-4	HRH-3
XP_001920844.1	XM_001920809.1	TAAR-4-like	TAAR-12
NP_001076571.1	NM_001083102.1	TAAR-64	TAAR-13
XP_006014096.1	XM_006014034.1	TAAR-9-like	TAAR-4
XP_003201718.2	XM_003201670.2	TAAR-1-like	TAAR-10
NP_001076546.1	NM_001083077.1	TAAR-11-like	TAAR-10
NP_001083418.1	NM_001089949.1	uncharacterized	ADRB
NP_001103208.1	NM_001109738.1	uncharacterized	HRH-2
NP_001124143.1	NM_001130671.1	uncharacterized	TAAR-12
XP_001337671.1	XM_001337635.2	5HTR-4-like	TAAR*
XP_003976403.1	XM_003976354.1	5HTR-4-like	TAAR*
XP_005810466.1	XM_005810409.1	5HTR-4-like	TAAR*
XP_003454279.1	XM_003454231.1	5HTR-4-like	TAAR*
XP_004549625.1	XM_004549568.1	5HTR-4-like	TAAR*
XP_002935532.2	XM_002935486.2	5HTR-4-like	TAAR*
XP_006013317.1	XM_006013255.1	5HTR-4-like	TAAR*
XP_005510029.1	XM_005509972.1	5HTR-7-like	Unknown
XP_002187301.2	XM_002187265.2	Octopamine receptor-like	Unknown
XP_002937327.2	XM_002937281.2	Octopamine receptor-like	Unknown
XP_005045681.1	XM_005045624.1	Octopamine receptor-like	Unknown
XP_005144673.1	XM_005144616.1	Octopamine receptor-like	Unknown
XP_005229932.1	XM_005229875.1	Octopamine receptor-like	Unknown
XP_005428400.1	XM_005428343.1	Probable GPCR No9-like	Unknown
XP_005490920.1	XM_005490863.1	Probable GPCR No9-like	Unknown
XP_005518128.1	XM_005518071.1	Probable GPCR No9-like	Unknown
XP_006111669.1	XM_006111607.1	Octopamine receptor-like	Unknown
XP_420867.2	XM_420867.4	Octopamine receptor	Unknown

Table 3. Misannotated and uncharacterized sequences identified through phylogenetic analysis. Based on sequence placement in the structurally-curated phylogeny (Figure 4) we propose updated classifications for 31 biogenic amine receptor sequences. The proposed classifications “Unknown” and “TAAR*” refer to the corresponding clades in Figure 4.

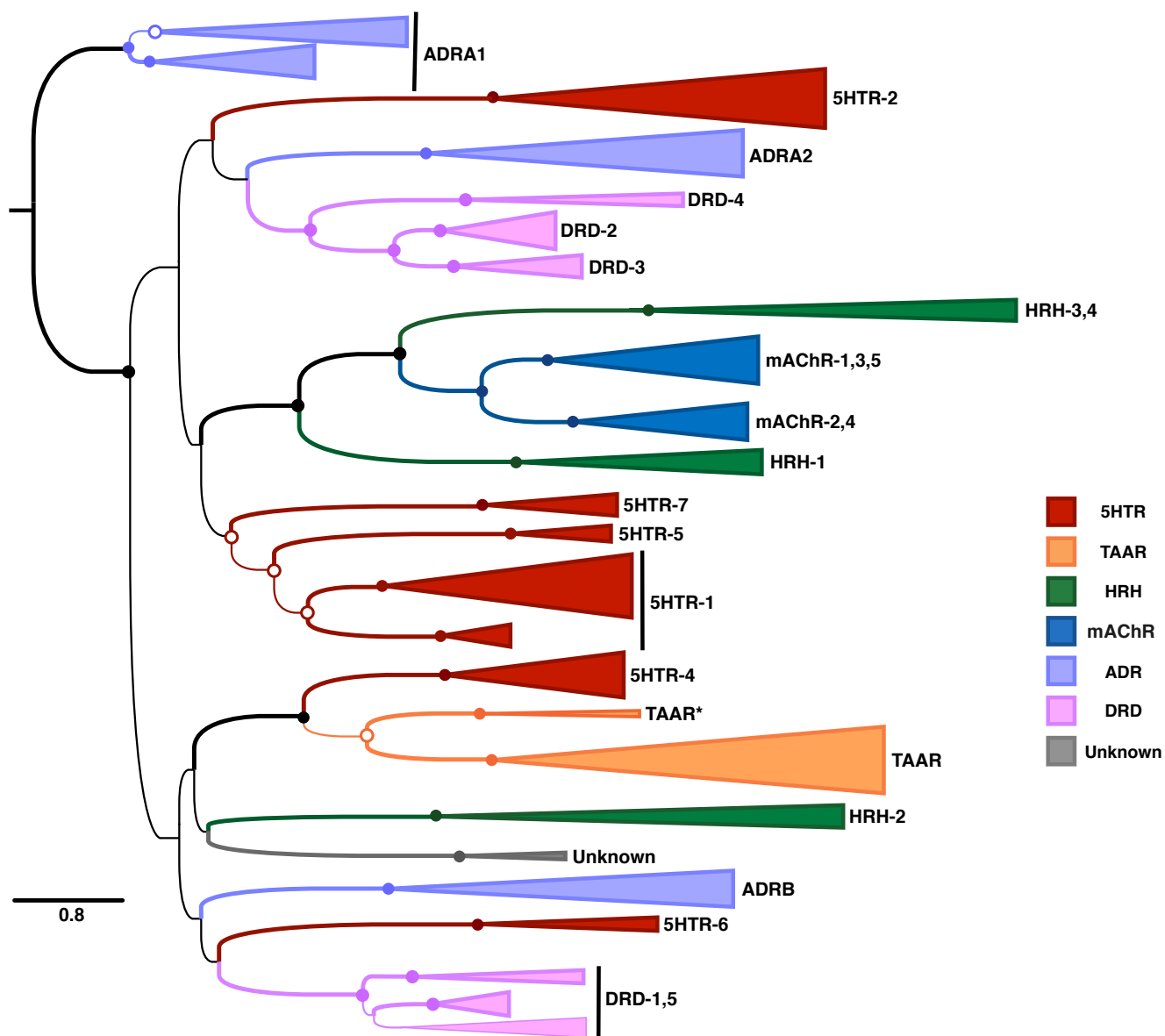


Figure 4. Maximum likelihood phylogeny of vertebrate biogenic amine receptors built using the masked structural MSA in RAXML. Nodes with open circles indicate $\geq 50\%$ bootstrap support, and nodes with closed circles and thick lines indicate $\geq 90\%$ bootstrap support. Biogenic amine receptors are abbreviated as in Table 1. The clade labeled “Unknown” could not be clearly identified as one of the major receptor types and may represent a previously unrecognized biogenic amine receptor clade.

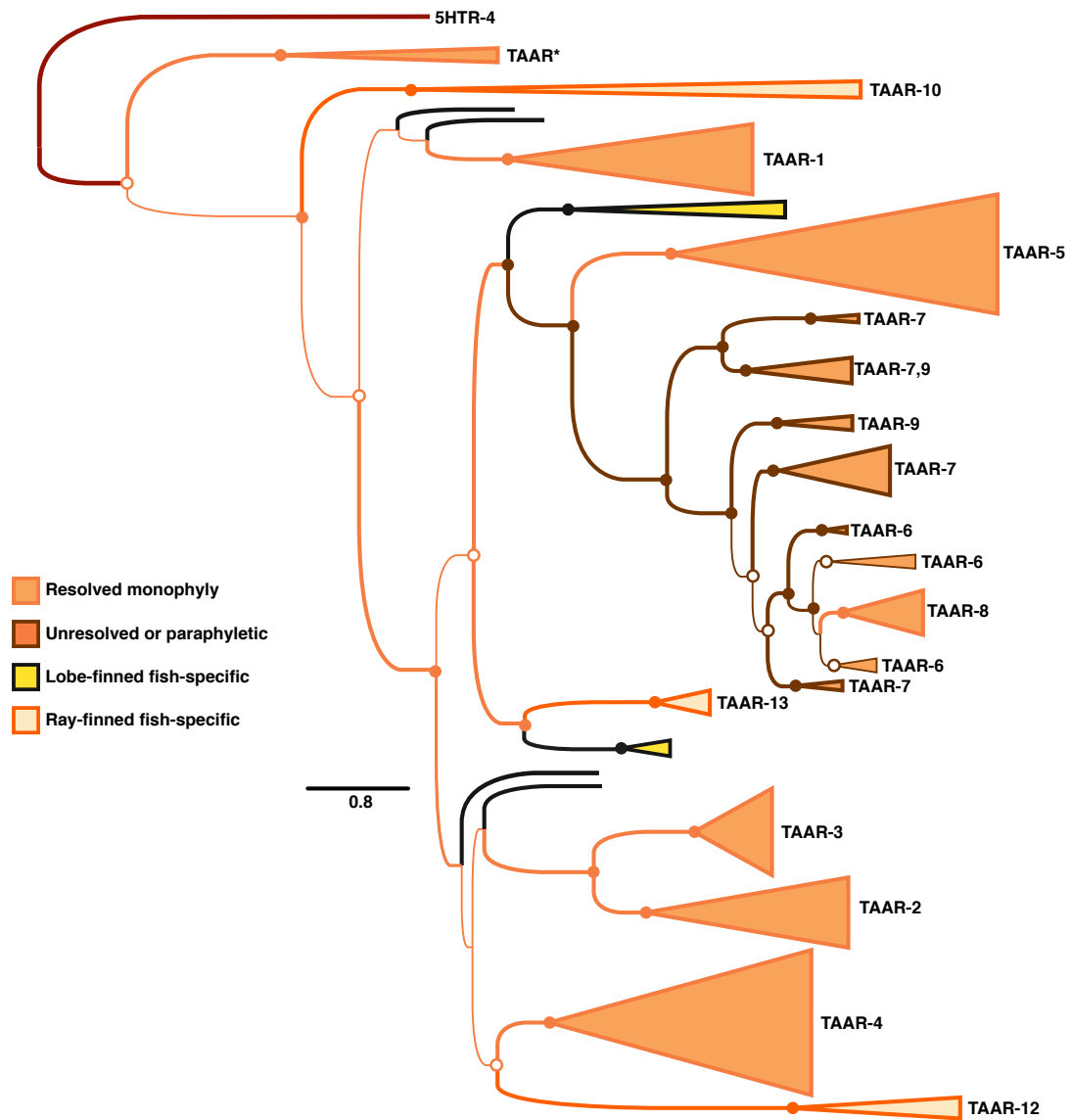


Figure 5. Subclade of the TAAR receptors within the phylogeny shown in Figure 4. Nodes with open circles indicate $\geq 50\%$ bootstrap support, and nodes with closed circles and thick lines indicate $\geq 90\%$ bootstrap support.