MALACOLOGIA, 2025, 67(1/2): 199–213

EVOLUTION OF PUTATIVE CONE SNAIL OLFACTORY GENES AND THEIR EXPRESSION PATTERNS IN TWO CLOSELY RELATED SPECIES

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ABSTRACT

Sensory biology is a critical component of organismal fitness and selection on sensory traits can drive macroevolutionary processes like speciation. Among mollusks, chemosensation (the detection of chemical signals) is an important sensory modality. The functional basis of chemosensation is a suite of olfactory receptor (OR) genes, but despite their importance to molluscan life history, ecology, and evolution, these genes remain poorly characterized for this highly diverse phylum. Here, we explore molluscan biology through this novel lens, using transcriptomics to characterize the olfactory receptor (OR) gene repertoires of *Conus ebraeus* Linnaeus, 1758 and *C. judaeus* Bergh, 1895—a pair of closely related, co-occurring gastropod species that exhibit fine-scale niche partitioning. We identify candidate OR genes, construct a gene tree for this gene family, and compare its structure and composition between these two species. We measure and compare gene expression of candidate OR genes, test for differential expression of orthologous loci, and compare patterns of sequence evolution between paralogous and orthologous loci to determine if gene family diversification occurs within or between species. Finally, we compare rates of evolution between ortholog pairs to identify genes showing evidence of differential selective pressures. Although *C. ebraeus* and *C. judaeus* share similar OR gene family repertoires, some genes showed divergence in terms of patterns of expression and modes of selection, which suggests the adaptive evolution of olfactory abilities among species. This is the first study to characterize the genetic basis of olfaction in *Conus* Linnaeus, 1758 and provides a genetic foundation for understanding how sensory biology can contribute to the rapid diversification of this genus.

Key words: chemosensation, olfaction, transcriptome, *Conus*, evolution, gastropod, phylogenetics, expression, sensory.

INTRODUCTION

The ability to sense and interpret exogenous information is central to organismal fitness: foraging, mating success, habitat selection, and predator avoidance all depend on it. Of the various sensory modalities, chemosensation—the detection of chemical signals—can involve the most complex information landscape. Organisms encounter myriad chemicals, the relevance of which depends on identity, concentration, co-occurring compounds, and any number of other contextual cues (Bargmann, 2006). Because things like conspecific recognition (Rafferty & Boughman, 2006; Walderon et al., 2011), niche partitioning (Proffit et al., 2007; Tait et al., 2016), and adaptation to novel environments (Hayden et al., 2010; Khan et al., 2015) frequently involve chemical signals, chemosensation often drives ecological and evolutionary processes like population and community structuring and speciation (Hay, 2009; Haswell et al., 2018).

Conidae—"cone snails"—is a hyperdiverse family of predatory marine gastropods with > 1,200 species (MolluscaBase, 2024). The family has a largely circumtropical distribution with a center of exceptionally high diversity in the Indo-West Pacific. Approximately three-quarters (or 76.9% based on MolluscaBase, 2024) of species are in the genus *Conus* Linnaeus, 1758 (MolluscaBase, 2024), which has a rate of diversification double that of most marine gastropod genera (Kohn, 1990, 2014). Cone snails often occur with high species richness and in sympatry with close relatives (Kohn,

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2001; Vallejo, 2005), likely due to fine-scale niche partitioning and especially of prey resources (Kohn, 1959; Kohn & Nybakken, 1975; Duda et al., 2009a). In Okinawa, sister species *Conus ebraeus* Linnaeus, 1758, and *C. judaeus* Bergh, 1895, occupy identical marine bench habitat, but adult dietary compositions are markedly distinct, with *C. ebraeus* preying almost exclusively on errant polychaetes in the family Eunicidae and *C. judaeus* on sedentary polychaetes in the family Capitellidae (Duda et al., 2009b). Studies of this ecological specialization have mostly focused on the highly divergent and diverse venoms used by different cone-snail species to subdue prey. However, to partition niche space, organisms must first be able to sense the biotic and abiotic elements that shape it. Given the important role of olfaction in cone-snail ecology—particularly predatory behavior (Kohn, 1956, 1959; Stewart & Gilly, 2005)—it is likely key to their ecological specialization.

Gastropod mating behavior (Croll, 1983; Clifford et al., 2003; Painter et al., 2004) and habitat choice (Hadfield & Paul, 2001; Hadfield, 2011; Cahill & Koury, 2016) depend heavily on chemical cues, and chemosensation is the primary sensory modality for marine invertebrates to detect food (Kohn, 1961; Audesirk, 1975; Kamio & Derby, 2017). Subclassifying chemosensation (e.g., into olfaction, gustation) can be controversial (Derby & Caprio, 2024), but it is important to note that we are concerned with chemical cues detected from inhaled water (i.e., olfaction) as distinct from those detected by direct physical contact (i.e., gustation) because cone snails likely use different anatomical structures for these purposes. To maintain this distinction going forward, we use the term olfaction/olfactory. Cone snails typically spend daylight hours buried under substrata with only their siphon protruding, emerging to hunt only at night (Kohn, 1959). In captivity, they initiate predatory behaviors when exposed to potential prey (including, e.g., when water from a tank containing prey is added to the snail's aquarium) (Kohn, 1956; Stewart & Gilly, 2005). Moreover, prey choice trials demonstrate that snails can distinguish among potential prey items and do so with high taxonomic resolution (Kohn, 1959). Like most gastropods, cone-snail olfaction relies on a highly enervated bipectinate structure called the osphradium (Copeland, 1918; Kohn, 1961; Croll, 1983). First described by Spengel (1881), early studies of the osphradium in whelks showed that cauterizing the organ prevented individuals from sensing prey cues introduced into the siphon opening (Copeland, 1918). The cone-snail osphradium is located in the mantle cavity at the base of the siphon, adjacent to the ctenidium (gill) and directly in the path of the inhalant water currents (Spengler & Kohn, 1995). The osphradium exhibits interspecific morphological variation correlating with habitat and dietary differences of *Conus* spp. (Spengler & Kohn, 1995). Macroanatomical structures like the osphradium have been key to understanding chemosensory function and evolution (Bertmar, 1969; Smith et al., 2007), but the functional units of chemosensation that receive and transduce chemical signals are direct gene products (i.e., receptor proteins). A holistic understanding of olfactory phenotypes must therefore include olfactory receptors (OR) and the genes that encode them, but no studies to date have characterized OR genes in cone snails.

Buck & Axel (1991) first characterized the OR gene superfamily in rats, and subsequent studies quickly revealed the scale and complexity of this superfamily. ORs share basic similarities across the Tree of Life, almost always featuring 7-transmembrane domain G-protein coupled receptor (GPCR) proteins that initiate signal cascades when odorant molecules bind to extracellular domains (Hildebrand & Shepherd, 1997; Ache & Young, 2005). OR gene families show signatures of adaptive evolution, including rapid gene turnover, birth-and-death evolutionary dynamics, and elevated rates of nonsynonymous substitutions (Gilad et al., 2003; Gardiner et al., 2008; Dong et al., 2009; Hussain et al., 2009; Ramasamy et al., 2016). Invertebrate OR gene families arose independently from those of vertebrates (e.g., Robertson et al., 2003; Hansson & Stensmyr, 2011) but share many of the same structural and functional characteristics (Bargmann, 2006). With ~120,000 described species (MolluscaBase, 2024), Mollusca is the second most diverse phylum of animals (Rosenberg, 2014) but lacks strong representation among model organisms so the genetic basis of olfaction in this group is poorly characterized. Cummins et al. (2009) identified 90 putative OR genes in the gastropod *Aplysia californica* J. G. Cooper, 1863 that are organized into three distinct subfamilies, but the generality of these findings cannot be evaluated without a wider taxonomic lens.

Characterizing cone-snail OR genes in a comparative phylogenetic context can yield novel insights about their diversification and ecology. If olfactory adaptation facilitates niche partitioning, we expect interspecific divergence in overall OR gene family composition and expression as well as heterogeneous rates of evolution for individual genes. A single nonsynonymous substitution or shifts in gene expression could substantially alter olfactory function, with potentially dramatic fitness consequences: previously undetected prey might be exploited, compatible mates might become invisible, or larval settlement cues could be missed. Cone-snail venom, another adaptive predatory trait, is highly distinct among even close relatives (e.g., Olivera et al.,1999). Predator-prey interactions are likely a source of strong selection driving venom evolution, and numerous relationships have been documented between shifts in diet and conotoxin evolution (Duda & Palumbi, 2004; Duda & Lee, 2009; Duda et al., 2009a; Dutertre et al., 2014). These patterns of evolution are mirrored by changes in conotoxin gene expression among species and populations that encounter distinct prey assemblages (Chang & Duda, 2016; Weese & Duda, 2019). If olfaction plays a complementary role to venom in ecological specialization, we could observe similar patterns of gene family composition, molecular evolution, and gene expression of OR genes.

In this study, we used a transcriptomic approach to examine the OR gene repertoires of *Conus ebraeus* and *C. judaeus*—a pair of closely related species that exhibit highly distinct dietary preferences. For the first time, we identified candidate OR genes, reconstructed the evolutionary history of this gene family in *Conus*, and compared its structure and composition among two species. To determine how gene expression contributes to differences in OR repertoires of *C. ebraeus* and *C. judaeus*, we measured and compared gene expression of candidate OR genes and tested for differential expression of orthologous gene copies in the two species. Finally, we compared patterns of sequence evolution between paralogous and orthologous loci to determine whether gene family diversification occurs within species or is driven by divergence between species. We also compared rates of evolution between pairs of orthologous loci to identify genes that might be experiencing different selective pressure in the two species.

METHODS

Taxon Sampling, RNA Extraction, and Sequencing

We collected *Conus ebraeus* and *C. judaeus* individuals on an expedition to Okinawa, Japan during the summer of 2015. Upon collection, snails were brought back to Sesoko Station marine laboratory (Tropical Biosphere Research Center, University of the Ryukyus), placed in separate plastic vessels with 200 mL of seawater for up to 48 hr, and then sacrificed and dissected. Osphradium tissue was placed in cryovials with RNALater (Invitrogen, Carlsbad, California, U.S.A.) and stored temporarily at 4°C in the field prior to transport and permanent storage at −80°C at the University of Michigan. Snails and their associated tissues were deposited in the collections of the University of Michigan Museum of Zoology Mollusk Division (Table 1). We selected osphradia from five adult individuals of each species to sequence for this study. We pestle-homogenized osphradial tissue, extracted total RNA with Trizol (Invitrogen, Carlsbad, California, U.S.A.) according to the manufacturer's protocol, and submitted the RNA samples to the University of Michigan DNA Sequencing Core for quality assessment with a Bioanalyzer 2100, followed by library preparation and indexing (Illumina Tru-Seq kit, San Diego, California, U.S.A.). Libraries were sequenced with a larger batch of samples across two flowcells on an Illumina HiSeq4000. Raw sequence data is available on the NCBI Sequence Read Archive under the BioProject accession number PRJNA1154654.

Read Processing, Transcriptome Assembly, and Transcript Filtering

We filtered raw data using a read processing and transcript filtering pipeline developed by Yang & Smith (2014) and processed reads separately for each individual. We first used Rcorrector (Song & Florea, 2015) to correct suspected sequencing errors and remove uncorrectable reads, and then ran Trimmomatic v0.36 (Bolger et al., 2014) with default parameters to remove Illumina sequencing adapters and low-quality reads. We binned reads as mitochondrial or nuclear DNA by mapping them to a custom database of nine complete *Conus* mitochondrial genomes with Bowtie2 v2.3.4.3 (Langmead & Salzberg, 2012). We used FastQC v0.10.1 (Andrews, 2010) to

TABLE 1. Individuals of *Conus judaeus* and *Conus ebraeus* sequenced for this study.

Individual	UMMZ Acc. No.	Sex	Collection Locale	Collection Date
jud_1	304700	♀	Cape Bise, Okinawa, Japan	2015-07-24
jud_2	304704	♀	Cape Bise, Okinawa, Japan	2015-07-24
jud_3	304718	♀	Cape Bise, Okinawa, Japan	2015-07-24
jud_4	304721	♀	Cape Bise, Okinawa, Japan	2015-07-24
jud_5	304720	♂	Cape Bise, Okinawa, Japan	2015-07-24
ebr_1	304690	♀	Sesoko Station, Okinawa, Japan	2015-07-24
ebr_2	304692	♀	Sesoko Station, Okinawa, Japan	2015-07-24
ebr_3	304701	♀	Cape Bise, Okinawa, Japan	2015-07-24
ebr_4	304699	♂	Cape Bise, Okinawa, Japan	2015-07-24
ebr_5	304691	♂	Sesoko Station, Okinawa, Japan	2015-07-24

assess read-quality and read representation, and to cull overrepresented sequences. We then pooled the filtered reads of individuals from each species and assembled separate de novo transcriptomes for *Conus ebraeus* and *C. judaeus* with Trinity v2.4.0 (Grabherr et al., 2011).

We filtered the de novo transcriptome to remove low-quality transcripts and transcripts lacking open reading frames (ORF). First, we ran Transrate v1.0.3 (Smith-Unna et al., 2016) with default settings to determine assembly quality and remove poor-quality transcripts, and then applied custom scripts from the Yang & Smith (2014) pipeline to remove chimeric transcripts. We produced clusters of transcripts belonging to the same putative gene using Corset v1.07 (Davidson & Oshlack, 2014), selecting the longest transcript from each cluster to represent that gene. We predicted ORFs using TransDecoder v5.0.1 (Haas et al., 2013), setting minimum ORF length to 100 amino acids, and included homology searches to increase sensitivity for ORFs with functional significance. We utilized two homology searches: a BLASTp search (Altschul et al., 1990) against a custom reference database comprised of all *Conus*, *Lottia gigantea* G. B. Sowerby I, 1834, and *Alpysia californica* protein sequences from the NCBI nonredundant database, and a search of the PFAM database (Finn et al., 2014) to identify homology with common protein motifs, including transmembrane domains. TransDecoder combined the results of these searches with ORF predictions to determine which transcripts should be retained. Finally, we reduced transcript redundancy for predicted coding sequences with CD-HIT-EST and a sequence identity cutoff of 0.99, and for predicted peptide sequences with CD-HIT and a sequence identity cutoff of 0.98 (Li & Godzik, 2006; Fu et al., 2012).

Candidate OR Gene Prediction

We applied the same criteria used by Cummins et al. (2009) to extract candidate OR genes from filtered transcriptomes of each species. These included: (1) limited predicted peptide sequence similarity to previously characterized molluscan chemosensory receptor genes, (2) at least six predicted transmembrane domains, and (3) a full-length coding sequence. We used the filtered transcriptomes of each species as queries in a BLASTx (Gish & States, 1993) search (e-4) against a custom database of 1,830 predicted molluscan OR protein sequences gathered from the GenBank RefSeq database (O'Leary et al., 2016). We then predicted the number and location of helical transmembrane (TM) domains in the predicted peptide sequences of BLASTx hits using TMHMM v2.0 (Sonnhammer et al., 1998), keeping those with six or more predicted TM domains. Finally, we extracted those loci containing both an initiating methionine and terminating stop codon.

Phylogenetic and Ortholog Inference

To compare the overall OR gene diversity of *Conus ebraeus* and *C. judaeus* in a phylogenetic context, we combined predicted peptide sequences of candidate OR genes from each species with sequences of three *Aplysia californica* OR genes identified by Cummins et al. (2009) and aligned them using T-COFFEE in PSI/TM mode (Notredame et al., 2000; Floden et al., 2016). This program uses a reference database of TM proteins to inform the alignment of proteins with predicted TM domains. We used ModelFinder (Kalyaanamoorthy et al., 2017) to identify the best-fit model of substitution and inferred a gene tree of all OR loci using IQ-TREE (Nguyen et al., 2015) with 1,000 bootstrap replicates. We distinguished orthologous and paralogous loci based on relative positions in the tree. In particular, we deemed sequences of each of the two species that uniquely clustered together in the gene tree (i.e., "sister loci") to be orthologous counterparts, whereas we inferred sister loci from the same species to represent paralogous loci.

Alignments of some candidate ORs and *Aplysia* outgroup genes produced numerous gaps, necessitating the removal of many potentially phylogenetically informative sites, and thereby limiting our ability to resolve relationships within clades of more recently diverged OR genes. We therefore extracted sequences of genes comprising each of the two main clades in the full tree, realigned these subsets separately, and inferred trees for each clade using the same methodology as described above. This combined method allowed us to maximize the number of informative sites while retaining the larger scale topological features inferred from the comprehensive alignment.

Because highly divergent sequences can hamper successful estimation of evolutionary rates, we used a transcript clustering approach to remove candidate OR genes lacking a clear paralog or ortholog for subsequent analyses. We first concatenated candidate OR genes from both species into a single FASTA file and performed an all-by-all BLASTn (Altschul et al., 1990) search with an e-value of e-10. Then, we applied the Markov clustering algorithm to cluster sequences into groups based on BLASTn results (Enright et al., 2002; van Dongen & Abreu-Goodger, 2012). We used the remaining loci to produce three datasets: (1) loci belonging to subfamily one, (2) loci belonging to subfamily two, and (3) putative orthologous loci represented by both species. By separating loci into their respective subfamilies, we were able to produce alignments with fewer gaps, increasing the number of informative sites available for downstream analyses of evolutionary rates within each subfamily. For each of these three datasets, we used the same procedure described above to align and infer gene trees.

Expression Analysis

To evaluate gene expression patterns of candidate OR genes and identify loci that are differentially expressed between *Conus ebraeus* and *C. judaeus*, we estimated transcript abundance for each putative ortholog for both species and performed differential expression analysis using scripts packaged with Trinity (Haas et al., 2013). We mapped the processed-read fastq files from each individual to the filtered transcriptome of their respective species using the Perl script align_and_estimate_abundance.pl with *--est_method* set to RSEM (Li & Dewey, 2011) and *--aln_method* set to Bowtie2 (Langmead & Salzberg, 2012). We then used the script abundance_estimates_to_matrix.pl to produce a cross-sample normalized abundance matrix for each species and extracted expression values for transcripts representing putative orthologs. We then used the R function heatmap to produce heatmaps illustrating patterns of gene expression alongside a phylogeny of putative orthologs.

To detect orthologs differentially expressed between *Conus ebraeus* and *C. judaeus*, we extracted the raw RNAseq fragment counts of putative orthologous transcripts and assigned individuals of each species as biological replicates (i.e., five each). We then performed differential expression analyses using the Perl script run_DE_analysis.pl (also packaged with Trinity) with the edgeR method (Robinson et al., 2010; McCarthy et al., 2012), and a p-value cutoff of 0.05 to establish significance.

Testing Rates of Evolution

To assess rates of evolution of paralogous and orthologous OR genes, we used the codeml program in the PAML v4.9 software package (Yang, 2007) to calculate rates of nonsynonymous substitutions per nonsynonymous site (d_N) and synonymous substitutions per synonymous site (d_S) and their ratio (ω) across branches of the tree. Because tests of selection

require nucleotide alignments that preserve codon position, we reverse-translated our peptide alignments using MACSE v2.03 (Ranwez et al., 2011) and the original nucleotide transcript sequences. We implemented several different models (Table 2) and compared their log-likelihood values using likelihood ratio tests (LRT) to determine which were more likely given the data. We performed all analyses separately for the two OR gene subfamilies to utilize more complete alignments for each. Our first model fixes ω at a value of one for all branches of the tree (Model A). Next, we estimated a single ω value for all branches of the tree (Model B). Comparing these two models allowed us to determine whether OR genes (both paralogs and orthologs) are generally evolving under purifying or positive selection. Our third model (Model C) estimates three separate ω values: one for branches connecting paralogous loci, one for branches connecting putative orthologs, and one for the remainder of the tree. Comparing these models allowed us to determine if paralogs and orthologs are evolving at different rates. The fourth model (Model D) estimates one ω for branches connecting paralogs, fixes the ω for branches connecting orthologs to one, and estimates a third omega for the remaining branches. Comparison of models C and D allowed us to determine if orthologs are evolving under purifying or positive selection. Model E estimates a separate ω for each branch on the tree, which allowed us to compare the lineage-specific branches leading to each ortholog for each species and identify those that could be evolving at different rates in *Conus ebraeus* and *C. judaeus*.

RESULTS

Read Processing, Transcriptome Assembly/ Filtering, and OR Prediction

After passing through our bioinformatic pipeline, our raw reads (76,752,610 *Conus ebraeus* and 75,022,298 *C. judaeus*) yielded 40,400,587 (*C. ebraeus*) and 43,104,341 (*C. judaeus*) cleaned reads, 429,975 (*C. ebraeus*) and 370,330 (*C. judaeus*) unfiltered transcripts, 35,143 (*C. ebraeus*) and 37,944 (*C. judaeus*) filtered transcripts, and 88 (*C. ebraeus*) and 118 (*C. judaeus*) candidate OR genes.

Phylogenetics and Ortholog Inference

The tree topology is characterized by two major clades: subfamily one comprising the majority of candidate loci, and subfamily two with the remainder. *Conus ebraeus* and *C. judaeus* are represented roughly equally in subfamily one with 52 and 54 loci, respectively, whereas *C. judaeus* has nearly double the number of subfamily two loci (64) compared to *C. ebraeus* (36) (Fig. 1). Within each subfamily, loci are intermixed, and there are no distinct large clades composed entirely of one species. The concentration of loci and short branch lengths

TABLE 2. Descriptions of models used to test evolution of evolution for orthologous and paralogous OR loci in *Conus ebraeus* and *C. judaeus*. Model likelihoods.

	Subfamily one			Subfamily two		
Model description	-lnL	ω_{para}	ω_{ortho}	-lnL	ω_{para}	ω_{ortho}
Model A: Fix ω = 1, all branches	36175.3	[1]	[1]	14804.9	[1]	[1]
Model B: Estimate single ω for all branches	34862.0	0.25	0.25	14548.5	0.26	0.26
Model C: Estimate separate $\omega_{paralog}$ and $\omega_{ortholog}$	34858.0	0.24	0.23	14544.7	0.22	0.31
Model D: Estimate $\omega_{paralog}$ and fix $\omega_{ortholog}$ = 1	35208.6	0.23	[1]	14574.1	0.21	[1]
Model E: Estimate separate ω for each branch	34725.0	-	-	14489.3	-	-

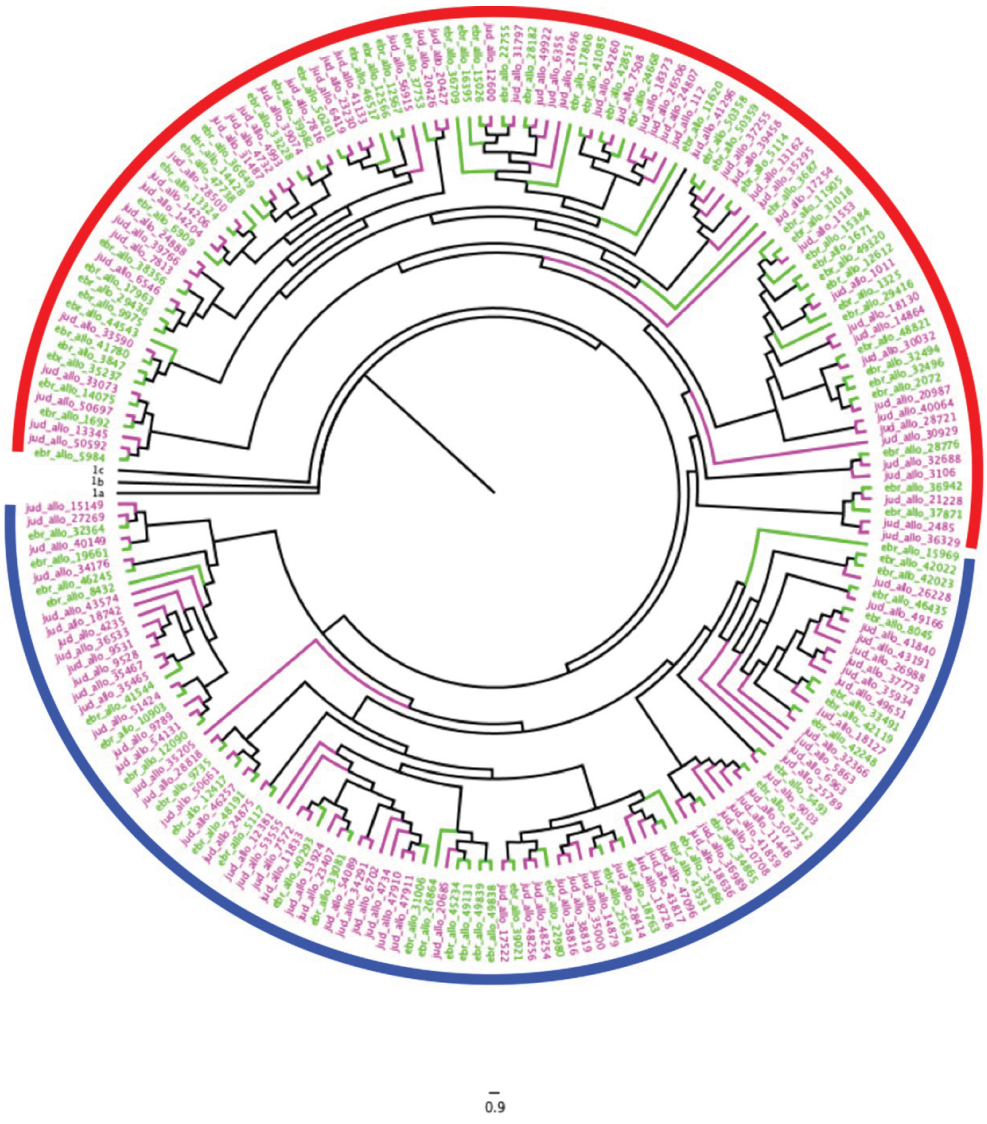

FIG. 1. Circle cladogram of all candidate OR genes identified from *Conus ebraeus* and *C. judaeus*. Maximum likelihood tree inferred using IQ-TREE and rooted to *Aplysia californica* OR gene sequences. Branches and tip labels are colored according to species (green = *C. ebraeus*, magenta = *C. judaeus*). Subfamilies are indicated by colored arcs around the tree circumference (red = subfamily one, blue = subfamily two).

observed in subfamily one suggests rapid diversification relative to subfamily two, which is sparser and has longer branch lengths. Most nodes exhibit high bootstrap support, with one exception being the first node of subfamily one.

After filtering sequences lacking obvious paralogs or orthologs, we were left with 49 loci from *Conus ebraeus* and 43 loci from *C. judaeus* for a total of 92 loci. Subfamily one includes 68 genes with 25 putative ortholog pairs, and subfamily two contains 24 genes with eight putative ortholog pairs (Fig. 2). The subfamilies are roughly even in representation of the two species with 37 *C. ebraeus* and 31

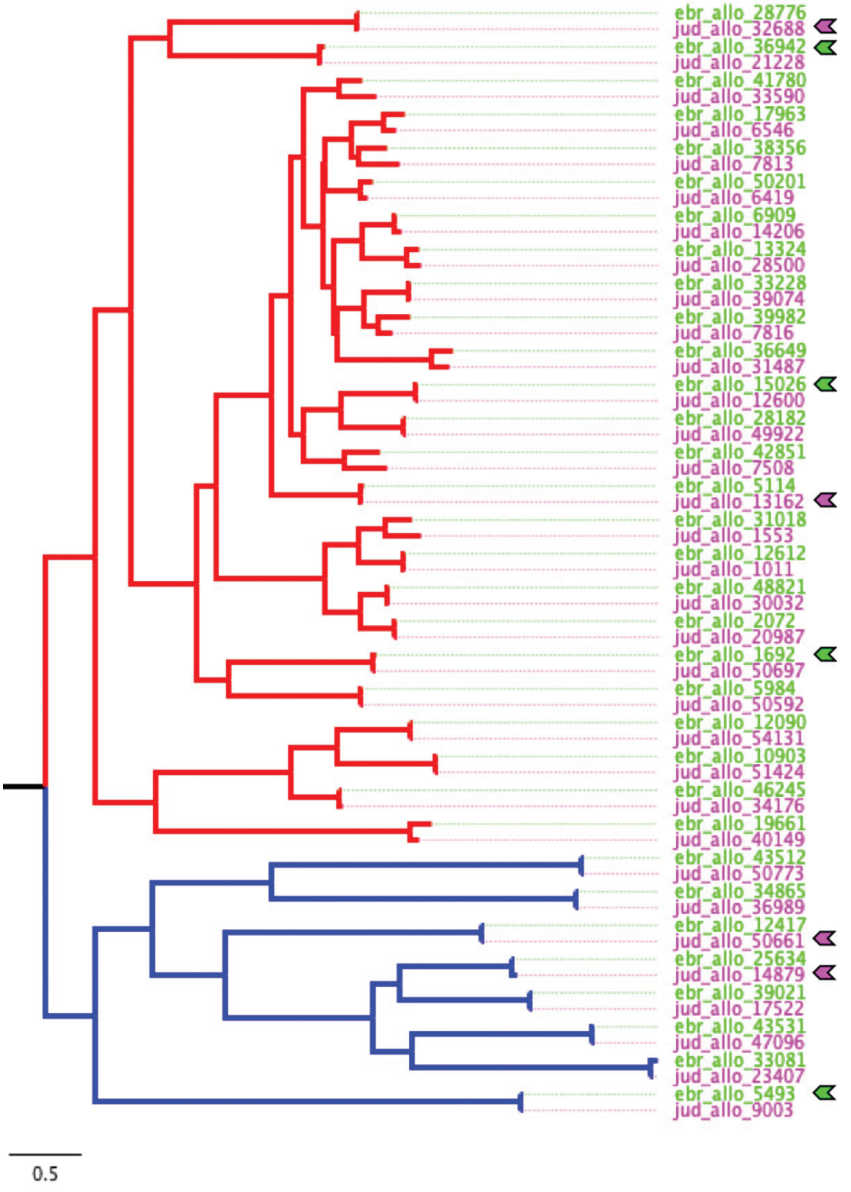

FIG. 2. IQ-TREE maximum likelihood phylogeny of putative OR orthologs. Branches are colored according to subfamily (red = one, blue = two). Tips labels colored according to species (green = *Conus ebraeus*, magenta = *C. judaeus*). Branches exhibiting ω > 1 indicated by arrows on the right.

C. judaeus genes in subfamily one, and 12 *C. ebraeus* and 12 *C. judaeus* genes in subfamily two. The richness and shorter branch lengths observed in subfamily one indicates more recent and rapid divergence relative to subfamily two.

Expression Analysis

We characterized the expression patterns of 33 candidate OR genes that we determined to be orthologous in *Conus ebraeus* and *C. judaeus*. We produced expression profiles for

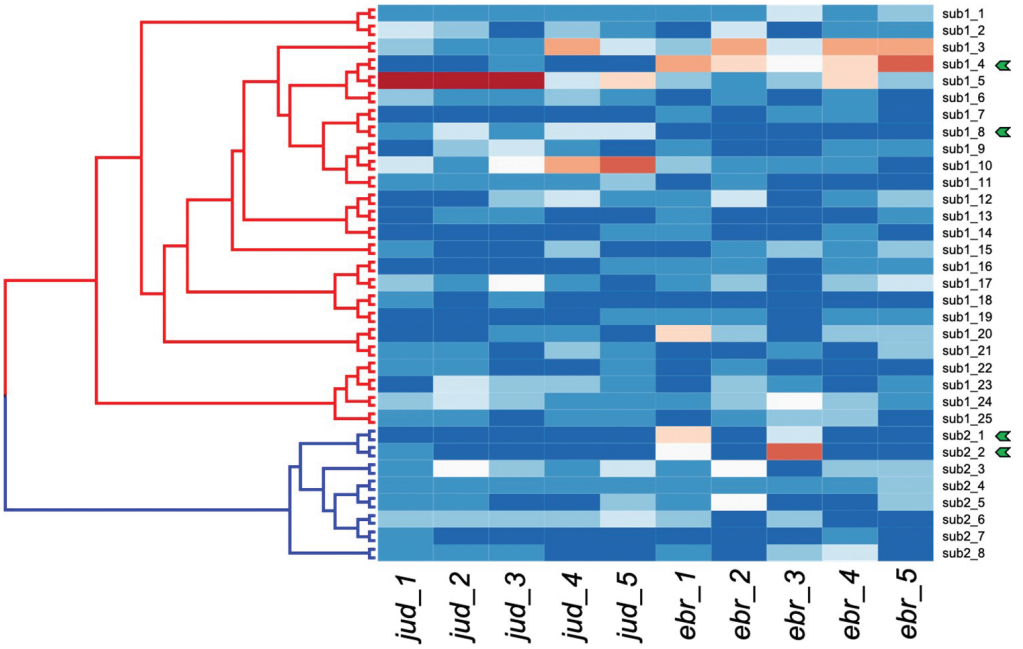

FIG. 3. Expression heatmap of 33 orthologous loci. Cells are colored according to raw RNA fragment counts, with dark blue representing the lowest and dark red the highest expression levels. Each row of cells represents a single ortholog pair and is aligned to its respective tree position. Branches of the tree are colored by subfamily (one = red, blue = two). Ortholog identifiers are provided to the right, and green arrows indicate differentially expressed orthologs.

each individual and generated a heatmap illustrating the relative abundance of each gene, which we organized phylogenetically (Fig. 3). We generally saw heterogeneous expression across the tree, with more genes that are highly expressed clustering in subfamily one, particularly within its largest subclade, and fewer highly expressed genes in subfamily 2 (Fig. 3). Overall expression patterns were broadly similar between *C. ebraeus* and *C. judaeus*, with more highly expressed genes clustering in subfamily one, but when formally tested for loci exhibiting differential expression, we recovered four orthologs that were significantly differentially expressed between the two species, two from each subfamily (Fig. 3). Both loci from subfamily two were characterized by extremely short branch lengths in both species. The differentially expressed orthologs in subfamily had longer branches leading to *C. ebraeus* than to *C. judaeus*. None of these four putative orthologs showed evidence of positive selection, nor is there a consistent pattern of larger ω values in one versus the other species.

Rates of Evolution

Our tree-wide codeml analyses suggested that both OR orthologs and paralogs are evolving under purifying selection (Tables 2, 3). Our second model estimated a single ω value of 0.25 and 0.26 in subfamily one and two, respectively, and is significantly better than model A in both cases, suggesting that loci are evolving under purifying selection. Our third model, which estimated separate ω values for branches connecting orthologs and those connecting paralogs, explains the data slightly better than model B, suggesting different rates of evolution for these classes of loci, although the estimated ω values were very similar (Table 1). The lineage-specific d_N and d_S values calculated separately for each branch in model E showed that eight of 33

TABLE 3. Hypothesis testing and LRT model comparisons.

		Subfamily one		Subfamily two	
Hypothesis	Model comparison	LRT	*p-value*	LRT	*p-value*
$\omega_{paralog} = \omega_{ortholog} = 1$	A & B	2626.6	< 0.001	512.8	< 0.001
$\omega_{paralog} \neq \omega_{ortholog}$	B & C	1.48	< 0.05	7.6	< 0.05
$\omega_{paralog} \neq \omega_{ortholog} = 1$	C & D	701.28	< 0.001	58.8	< 0.001

orthologous loci (four in *Conus ebraeus* and four in *C. judaeus*) exhibited an estimated ω greater than one (Table 4). Five of these were in subfamily one and three in subfamily two.

DISCUSSION

This study is the first to describe olfactory receptor genes in cone snails and place them in a comparative phylogenetic context. We detailed similarities and differences in gene family composition between the sister species *Conus ebraeus* and *C. judaeus* and investigated mechanisms that could contribute to these patterns. Our results added a novel perspective to adaptive trait evolution in Conidae, contributed to a better understanding of their rapid diversification, and opened new avenues for future work in this area.

The OR gene families that we identified from *Conus ebraeus* and *C. judaeus* osphradia were broadly similar, both in terms of their broad structural organization and overall numbers of candidate genes. No large subclades were unique to one species, and ~32% of all candidate OR genes belonged to a putative ortholog pair. The most glaring difference was the asymmetric representation of species in subfamily two in which we recovered nearly twice as many genes from *C. judaeus* than *C. ebraeus*. This suggests an elevated diversification of subfamily two in *C. judaeus*, potentially associated with aspects of organismal ecology that are divergent from *C. ebraeus* (e.g., prey preference). Smaller differences than this (e.g., single gene gain or loss) can still have significant impacts on ecological specialization as has been demonstrated by other adaptive traits, e.g., venom. For example, the presence or absence of a small venom gene cassette in Mojave rattlesnakes results in radically different hemorrhagic or neurotoxic venom types associated with distinct prey and could represent the early stages of ecological speciation (Strickland et al., 2018). To link differences in OR gene family composition with ecological differences

TABLE 4. Lineage specific estimates of dN and dS for orthologous loci with ω > 1.

Gene_ID	Ortholog_ID	Subfamily	dN	dS
jud_allo@32688	sub1_1	one	0.005448	0.003684
jud_allo@13162	sub1_15	one	0.007424	0.000091
ebr_allo@15026	sub1_12	one	0.001605	0.000008
ebr_allo@1692	sub1_20	one	0.005019	0.000008
ebr_allo@36942	sub1_2	one	0.013254	0.000013
ebr_allo@5493	sub2_8	two	0.003016	0.000003
jud_allo@14879	sub2_4	two	0.012092	0.000012
jud_allo@50661	sub2_3	two	0.001399	0.000001

requires additional work and an understanding of OR protein function in *Conus*. It is also important to note that limitations of transcriptomic data could contribute to observed differences because unexpressed loci and low-expression transcripts are easily missed. This is where whole genome data would be highly useful to confirm genomic differences and infer gene gain and loss.

OR gene family diversification can be driven by several processes that operate in isolation and in concert, e.g., strong positive selection and rapid birth and death dynamics (Hansson & Stensmyr, 2011; Bear et al., 2016). By estimating rates of synonymous and nonsynonymous substitutions along branches leading to both paralogous and orthologous loci, we showed that paralogous and orthologous loci of *Conus ebraeus* and *C. judaeus* exhibit slightly different rates of evolution, although both generally evolved under purifying selection (Tables 2, 3). The model that estimated separate ω values for orthologous and paralogous branches (Model C) did a significantly better job explaining the data than the model estimating a single ω value for all branches (Model B) for both subfamily one and subfamily two. Estimated ω values were very similar in subfamily one, however, at 0.24 and 0.23 for paralogs and orthologs, respectively. The values estimated for subfamily two were more divergent with $\omega_{paralog} = 0.22$ and $\omega_{ortholog} = 0.31$, which suggests different patterns of evolution of orthologs than paralogs in subfamily two and possibly a higher rate of nonsynonymous divergence. These results comport with studies of conotoxins that found no significant difference in rates of evolution of orthologs and paralogs. In a comparison of conotoxin evolution between *C. miliaris* Hwass, 1792, and *C. abbreviatus* Reeve, 1843 (close relatives of *C. ebraeus* and *C. judaeus*), Duda (2008) found that both classes of loci experience strong positive selection. Despite our finding of purifying selection across the OR gene family, it is possible that our analyses were not calibrated to detect signals of positive selection that might exist. First, estimates of d_N and d_S for long branches and deep nodes can lose accuracy, and even when subdividing candidate OR genes according to subfamilies and removing genes without obvious orthologs or paralogs, our final alignments still included some highly divergent sequences. Second, the candidate OR proteins that we recovered are much larger (~300 amino acids) than conotoxin peptides (~10–30 amino acids) (Myers et al., 1993) and could experience strong selection only in particular regions (e.g., extracellular domains) or only a few sites (e.g., active sites) (Spielman & Wilke, 2013). This has been documented for an OR gene family of teleost fishes in which purifying selection that is experienced by most of the gene swamped signals of positive selection from a small number of sites scattered throughout the sequence (Hussain et al., 2009).

We also estimated lineage-specific ω values for each of the 33 pairs of putative orthologs that we inferred. Although the majority of these loci showed evidence of purifying selection, eight branches showed ω > 1: four leading to *Conus ebraeus* and four to *C. judaeus*, none of them shared—indicating positive selection (Table 4). Because positive selection was inferred for these eight orthologs in one species but not the other, they are promising candidates to examine for a role in ecological specialization. Differential rates of evolution in orthologs are frequently linked to ecological adaptation and divergence (McBride, 2007; Brand & Ramírez, 2017).

Finally, differences in gene expression can identify candidate genes that play a more important role in one species than another. Such differences can be the result of phenotypic plasticity, but in many cases, they are the product of divergent evolution and are an important aspect of adaptive diversification (Brawand et al., 2011). We estimated the abundance of all 33 orthologs for both *Conus ebraeus* and *C. judaeus* and found a pattern of heterogeneous expression across the tree (Fig. 3) with a larger number of highly expressed orthologs found in subfamily one for both species. We also performed differential expression analysis and identified four orthologs that were differentially expressed (Fig. 3, green arrows). One ortholog was more highly expressed in *C. judaeus* and three were more highly expressed in *C. ebraeus*. None of these orthologs were among those that we inferred to experience positive selection. These genes represent ideal candidates for future studies to determine what role they play in *Conus* ecology. Shifts in conotoxin gene expression have been linked to changes in prey assemblage in *C. miliaris*, whose Easter Island population encounters a distinct prey assemblage from populations in other parts of the Indo West-Pacific (Weese & Duda, 2019). A parallel study of population-level differences of OR gene expression could reveal a similar linkage with diet. Although we were able to

identify four differentially expressed loci with our dataset, future studies comparing a larger number of individuals from each species could achieve higher resolution and capture more subtle variation. Differences in gene expression can stem from dosage effects of multiple gene copies or from upstream regulatory variation, other features that could be detected with a whole-genome analysis.

From this study, we can conclude that the OR gene repertoire of *Conus* is diverse, and the gene family organized. Furthermore, although OR gene family composition is broadly similar between *C. judaeus* and *C. ebraeus*, our results are consistent with adaptive evolution of OR genes, including the differential expression and lineage specific positive selection of orthologous genes. Taken together, our findings offer a novel lens through which to view ecological specialization in *Conus* and form a foundation for future studies of sensory evolution and its contribution to diversification in mollusks more broadly.

ACKNOWLEDGMENTS

We thank the University of Michigan Rackham Graduate Program, which provided funding for field work. We would like to acknowledge the help of Dr. Taehwan Lee with retrieving specimens and collection data from the UMMZ collections. Dr. Raquel Marchán-Rivadeneira gave valuable guidance and support for the wet-lab work carried out for this project. Dr. Joe Walker provided essential help with the bioinformatic analyses, particularly the aspects involving Phyx. We also thank an anonymous referee for comments on an earlier draft of this manuscript.

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Revised ms accepted September 13, 2024