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GPCR annotation, G proteins, and transcriptomics of fire ant (*Solenopsis invicta*) queen and worker brain: An improved view of signaling in an invasive superorganism

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ABSTRACT

Knowledge of G protein-coupled receptors (GPCRs) and their signaling modalities is crucial to advancing insect endocrinology, specifically in highly successful invasive social insects, such as the red imported fire ant, *Solenopsis invicta* Buren. In the first published draft genome of *S. invicta*, emphasis was placed on the annotation of olfactory receptors, and only the number of predicted GPCR genes was reported. Without an organized and curated resource for GPCRs, it will be difficult to test hypotheses on the endocrine role of neuropeptide hormones, or the function of neurotransmitters and neuromodulators. Therefore, we mined the *S. invicta* genome for GPCRs and found 324 predicted transcripts encoded by 125 predicted loci and improved the annotation of 55 of these loci. Among them are sixteen GPCRs that are currently annotated as "uncharacterized proteins". Further, the phylogenetic analysis of class A neuropeptide receptors presented here and the comparative listing of GPCRs in the hymenopterans *S. invicta, Apis mellifera* (both eusocial), *Nasonia vitripennis* (solitary), and the solitary model dipteran *Drosophila melanogaster* will facilitate comparative endocrinological studies related to social insect evolution and diversity. We compiled the 24 G protein transcripts predicted (15 α, 7 β, and 2 γ) from 12 G protein genes (5 α, 5 β, and 2 γ). Reproductive division of labor is extreme in this ant species, therefore, we compared GPCR and G protein gene expression among worker, mated queen and alate virgin queen ant brain transcriptomes. Transcripts for ten GPCRs and two G proteins were differentially expressed between queen and worker brains. The differentially expressed GPCRs are candidate receptors to explore hypotheses on division of labor in this species.

1. Introduction

G protein-coupled receptors (GPCRs) are integral cell surface membrane signaling proteins that as a superfamily are characterized by seven transmembrane (TM) regions; also known as 7TM receptors for this reason. GPCRs in insects are activated by a number of types of ligands (e.g. biogenic amines, neuropeptides, glutamate, and GABA), as well as light in rhodopsin. As their name indicates, GPCRs couple with trimeric G proteins. Specifically, upon receptor activation, the Gα subunit (GTP-binding and GTPase) separates from the Gβ and Gγ subunits, which remain bound to one another. G proteins (Gα and Gβγ) amplify the receptor signals through distinct intracellular cascades.

GPCR signaling is integral to insect physiology in neuronal signaling and modulation, paracrine and endocrine functions, receiving visual stimuli, ecdysis, regulation of feeding behavior, pheromone synthesis, reproduction, learning and memory, and other metabolic processes. Much has been learned on GPCR annotation and characterization from the dipterans *Drosophila melanogaster* (Hanlon and Andrew, 2015; Hauser et al., 2006) and mosquitoes (Hill et al., 2002); from other blood-feeding arthropod vectors such as the assassin bug, *Rhodnius prolixus* (hemipteran), and the tick, *Ixodes scapularis* (Gulia-Nuss et al., 2016). Among social insects, the updated honey bee (*Apis mellifera*) genome allowed discovery of "missing" genes, including GPCRs (Elsik et al., 2014).

The red imported fire ant, *Solenopsis invicta*, is an invasive and aggressive species native to South America that was introduced to the US early in the 20th century and since has spread to other temperate regions of the world. The genetic origins and expansion of their worldwide invasion have been documented (Ascunce et al., 2011). Most research on fire ants has focused on the genetics and regulation of the two social forms, monogyne and polygyne, and in understanding the significance of the "social chromosome" present in the polygyne form

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(Pracana et al., 2017; Wang et al., 2013). Further understanding of the roles of GPCRs in this superorganism could add knowledge on colony regulation and growth, and on neuro-endocrine links between feeding and reproduction (Johnson and Linksvayer, 2010). For example, in early studies on the short neuropeptide F receptor (sNPFR) in fire ants, we discovered that the sNPF receptor transcript expression is sensitive to starvation (Chen and Pietrantonio, 2006). This led to a better understanding of honey bee sNPFR signaling (Ament et al., 2011), and progressed into linking nutrition with reproduction (Bai and Palli, 2016; Lu and Pietrantonio, 2011). Systematically determining the functional role of fire ant GPCRs in queens and workers is essential to investigate social organization, as GPCRs are expected to play key roles (Castillo and Pietrantonio, 2013; Lu and Pietrantonio, 2011).

As part of a recent transcriptomic study, we identified GPCR transcripts expressed in the fire ant queen brain (Calkins et al., 2018). Mining the fire ant genome available in NCBI we found that many GPCRs were annotated with names of mammalian receptors for ligands that are not found in insects, and/or their automatic annotation was suboptimal. Yet, some GPCRs remain annotated as uncharacterized proteins. Moreover, little is known about the G proteins of fire ants, which represents another gap in GPCR signaling research. Here, to improve the current annotation of GPCRs in the *S. invicta* genome these sequences were curated for insect-specific signaling molecules and processes. Reannotations mainly focused on Class A GPCRs for biogenic amines and neuropeptides. While similar compilations exist for neuropeptides in the ant *Camponotus floridanus* (Schmitt et al., 2015), GPCRs for neurohormones in the honey bee (Hauser et al., 2006), and for biogenic amine GPCRs in *S. invicta* (Qi et al., 2018), no curated list of GPCRs exists for any of these hymenopterans. This curation is timely because the honey bee genome has been improved (official gene set (OGSv3.2); assembly Amel_4.5) from its original release (Elsik et al., 2014) and, while this paper was under review the genome of *S. invicta* was updated on NCBI (September 2018, assembly GCA_000188075.2 Si_gnH). To further understand GPCR signaling in fire ants, phylogenetic analyses of selected GPCRs, and of G alpha subunits were performed. Additionally, brain transcriptomes of queens and workers were compared for GPCRs and G proteins expression. This study represents a new, needed fundamental resource to advance the study of G proteins and GPCRs in fire ants and other hymenopterans.

2. Methods

2.1. Data mining and bioinfomatic analyses for reannotation

Preliminary mining and compilation of *S. invicta* GPCR genes were described previously using the assembly GCA_000188075.1 Si_gnG (Calkins et al., 2018). In the latter study GPCRs were identified by Blast analyses using insect GPCR sequences from other species as queries. Herein, for this improved annotation, the newest *S. invicta* assembly GCA_000188075.2 Si_gnH released on August 1st, 2018 was used. Sequences identified by Blast analyses as candidate GPCRs contained signatures of GPCRs, and the transmembrane regions were verified using the tool TMPred (Hofmann and Stoffel, 1993). Blast searches of *S. invicta* predicted protein sequences were performed with Blastp and tBlastn against the genomes of *Nasonia vitripennis, A. mellifera* and *D. melanogaster*. These three species were chosen because they have sequenced genomes, and functional analyses have been carried out for many GPCRs from the fruit fly and the honey bee, aiding the *S. invicta* GPCR reannotation. Reciprocal searches were performed to confirm orthologues and the SmartBlast tool was also used to verify the most similar receptor in *D. melanogaster*. Reannotation of GPCRs was aided by amino acid sequence alignments [tools were Cobalt at NCBI, BioEdit, and Mafft (mafft.cbrc.jp/)] and literature review focusing on amino acid signature motifs, especially for GPCRs of neuropeptide families. Phylogenetic analyses of GPCRs supporting reannotations (ours and those submitted to NCBI by Univ. of Lausanne in August 2018 corresponding to the newest assembly GCA_000188075.2 Si_gnH) were performed (see section below). For corazonin, CNMamide, and periviscerokinin receptors for which two loci were found, the identifiers 1 or 2 were added to the proposed annotations. G protein genes were mined using the Blast tool for similarity and reciprocal searches at NCBI with the filter "*Solenopsis invicta*".

2.2. Phylogenetic tree construction

Phylogenies for *S. invicta* Class A neuropeptide GPCRs, GPCRs important for juvenile hormone regulation (allatostatins), GPCRs involved in aggression (dopamine), and G alpha proteins were reconstructed using Bayesian methods as in Hjelmen and Johnston (2017). For this, proteins similar to those of *S. invicta* sequences were identified as described above (based on Blast analyses). The neuropeptide GPCR phylogenetic analysis utilized sequences from NCBI GenBank of *D. melanogaster, A. mellifera, S. invicta*, and only one sequence from *T. castaneum* (inotocin receptor). For the allatostatin and dopamine receptors phylogenetic analyses, sequences were from *A. mellifera,* the red flour beetle *(Tribolium castaneum),* the Argentine ant *(Linepithema humile), D. melanogaster,* and *N. vitripennis.* Due to the known high divergence of GPCRs in the N- and C- termini, for Bayesian analyses GPCR sequences were first trimmed at both termini to retain sequences encompassing the predicted TM1 to TM7 (Šimo et al., 2014). G protein sequences used for phylogenetic analyses were from *S. invicta*, *N. vitripennis*, *L. humile*, *A. mellifera, T. castaneum,* and *D. melanogaster* (Hanlon and Andrew, 2015) and two *Homo sapiens* sequences, $G\alpha_{12/13}$. G protein sequences were not trimmed.

For all phylogenetic analyses sequences were aligned using MAFFT version 7.402 (http://mafft.cbrc.jp/) (Katoh and Standley, 2013) with default settings. Using Mesquite version 3.5 (build 888) (Maddison and Maddison, 2018), alignments were visually inspected and terminal gaps were converted to missing data. Phylogenies were reconstructed using MrBayes 3.2.6 executable (Ronquist et al., 2012) for Windows 64-bit with four chains and four runs in the mixed amino acid model for 1,000,000 generations. Parameter outputs were visualized in Tracer 1.6 (Rambaut et al., 2014) to assure the four runs reached convergence. The consensus trees were generated in MrBayes using 10% burnins and visualized in FigTree version 1.4.2 (Raumbaut, 2012).

2.3. Fire ant colony maintenance

Polygyne *S. invicta* colonies were collected in Brazos County, TX, from May to July 2015. Colonies were maintained in a laboratory at Texas A&M University in plastic trays (27 × 40 × 9 cm) with the walls of the containers coated with Insect-a-slip (Fluon®, BioQuip products, CA, USA). Each colony was provided a 14 cm-diameter Petri dish half-filled with damp Castone (Dentsply International Inc., York, PA, USA) as a nest area. The colonies were maintained at 27 ± 2 °C in a 12:12 h light:dark photoperiod. The colonies were fed daily with a 20% honey water solution and crickets, *Acheta domestica*. Water was given *ad libitum.* Colonies contained dealate mated queens, alate queens, males, brood (eggs, larvae and pupae) and workers; details as in Chen et al. (2004).

2.4. Worker brain transcriptome

Brains from worker ants were dissected and RNA extracted as previously described (Calkins et al., 2018). Briefly, worker brains were dissected on PBS kept on ice (~3°C) and placed immediately in 100 µL of TRIzol Reagent (Thermo Fisher Scientific, Carlsbad, CA) maintained on dry ice. Once brains (approximately 150–200 per tube constituting one sample) were dissected, RNA purification was performed following the manufacturer's instructions. Samples were further purified using Direct-zol™ RNA MicroPrep kit (Zymo Research, Irvine, CA) columns, including optional DNase I treatment. Any remaining DNA was

removed using TURBO DNA-free™ Kit (Life Technologies, Carlsbad, CA). For transcriptome sequencing, four total RNA samples from worker brains were submitted to the Texas A&M AgriLife Genomic and Bioinformatic Center. The libraries were prepared with Illumina TruSeq Stranded Total RNA library preparation kit and sequenced with the HiSeq 2500 System (Illumina) 125SE (single end). The sequence raw reads were cleaned using cutadapt 1.0 to remove barcode tags and adaptors. Individual samples were processed with FastQC, and the QC reports were checked as final confirmation of sequence quality. All bioinformatic analyses were performed in the Discovery Environment web interface and platform at CyVerse (Goff et al., 2011). The RNA-seq reads that passed the quality filters (FASTQC tools) were mapped to the *S. invicta* genome (RefSeq GCA_000188075.2 Si_gnH) using TopHat 2.1.1 (Trapnell et al., 2009). Fire ant queen brain transcriptomes in NCBI GEO under accession number GSE108063 (Fire ant alate virgin and de-alate mated queen brain transcriptomes) (Calkins et al., 2018) were uploaded to the Discovery Environment at CyVerse for comparisons to worker brain transcriptomes. Differentially expressed genes were identified using Cuffdiff 2.2.1 (Trapnell et al., 2012) with a false discovery rate (FDR < 0.05).

3. Results and discussion

3.1. Gpcrs of Solenopsis invicta

In the genome of *S. invicta*, many GPCRs were annotated automatically by adjudicating them the name of the closest mammalian or vertebrate orthologue, therefore, some of these annotations are uninformative for insects. This suboptimal annotation creates confusion in interpreting the roles of these GPCRs in insects, especially for non-experts. Automatic annotations are particularly confusing for neuropeptide receptors and cognate peptide ligands which are ancestrally related to mammalian counterparts but have different functions in insects. As such, we have compiled GPCRs of *S. invicta,* and after analyses we now propose curated and insect relevant annotations (Tables 1 and 2). The list of GPCR loci presented here (Tables 2 and S1) is likely not complete as additional partial GPCR sequences and/or unannotated genes may remain to be identified. However, this report constitutes a valuable resource because this curated compilation does not yet exist for *S. invicta* and complements the only such previous effort on *S. invicta* biogenic amine receptors (Qi et al., 2018).

Previously, 83 GPCR loci were compiled from the genome of *S. invicta*, corresponding to a total of 129 predicted transcripts encoding full length or partial receptors (Calkins et al., 2018). Herein, incorporating the most recent update to the *S. invicta* genome, 125 GPCR loci corresponding to 324 transcripts are compiled (Table S1). In total we identified loci for 70 Class A GPCRs, 48 Class B, and 7 Class C (4 for Glutamate and 3 for GABA).

Table 1 lists all proposed GPCR reannotations, including our identification of GPCRs for sixteen loci currently annotated as “uncharacterized proteins”. Four of these likely encode orphan neuropeptide GPCRs, nine correspond to Methuselah-like receptors, one corresponds to a gamma-aminobutyric acid receptor, one is a metabotropic glutamate receptor-like receptor, and finally one is an adhesion GPCR. These reannotations represent an improvement over the current genome annotation, but functional analyses should be used to verify them. Herein, the annotation is improved for 55 of the 125 loci (~44%) and 164 of the 324 transcripts (~51%) mined (Table 1). Many reannotations in Table 1 were chosen for uniformity among insects (e.g. to point to the closest orthologues in other insect genomes), even when some of these annotations are still suspect (i.e., see discussion below for LOC105197486). Seven of those reannotated GPCR genes were receptors for insect neurohomones within the core set (CS) identified by Hauser et al. (2010). In Table 1, references that support the reannotation with notes on the most likely receptor function to be investigated are listed when available. For Class A GPCRs, variations of the canonical DRY/ERY motif at the cytoplasmic extension of TM3 in the *S. invicta* were noted as this motif has functional significance, as described below.

Table 2 summarizes the proposed annotations for all GPCRs currently identified, those that were correctly predicted by the automatic annotation in NCBI and those reannotations listed in Table 1. In addition, the phylogeny of Class A neuropeptide GPCRs of *S. invicta, D. melanogaster,* and *A. mellifera* are presented in Fig. 1. For comparison, another invasive ant, the Argentine ant *Linepithema humile*, has 59 identified GPCR genes (Smith et al., 2011). In *S. invicta,* all corresponding GPCRs of the core set of neurohormones present in insects (Hauser et al., 2010), except for RYamide peptides, were identified (Tables 1, 2 and S1). The recent genome update includes a gene, LOC105194591, annotated as ‘RYamide receptor-like’. However, our analysis of LOC105194591 (subsequently discussed) indicates it is an RFamide-like orphan receptor. This is expected, as neither the ligands (RYamides) or receptors for RYamides have been identified in ants (Friedman and Gordon, 2016; Nygaard et al., 2011), but do exist in the wasp *N. vitripennis* (Hauser et al., 2010). Within the variable set (VS) of hormone receptors (Hauser et al., 2010), we did not find receptors for allatostatin B (Chang et al., 2018), as expected for Hymenoptera, nor for insect kinins (Nygaard et al., 2011).

Table S1 lists all identified *S. invicta* GPCR genes and transcripts, their improved annotation, and the percentage of identity to the most closely related proteins in *D. melanogaster*, *A. mellifera,* and *N. vitripennis*. Table S1, also provides the amino acid residue length of the predicted GPCRs, as well as the number of predicted TM regions encoded. To facilitate cross referencing of loci, our preliminary reannotation efforts based on the now outdated genome (GCA_000188075.1.34) are documented in Table S2.

3.2. Biogenic amine receptors

Biogenic amines are critical to numerous physiological processes and in hymenopterans they play roles in division of labor, kin recognition, and aggression (Blenau and Thamm, 2011; Bloch et al., 2000; Boulay et al., 2001; Cuvillier-Hot and Lenoir, 2006; Farkhary et al., 2017; Grohmann et al., 2003; Kamhi et al., 2015; Muscedere et al., 2016; Nouvian et al., 2018; Wagener-Hulme et al., 1999). Specifically in *S. invicta*, queen primer pheromone regulates brain octopamine levels, which in turn regulate kin recognition and intraspecific aggression levels (Vander Meer et al., 2008).

A study by Qi et al. (2018) identified 18 biogenic amine receptors: five for serotonin (5-HT), three for dopamine, one for dopamine/ecdysteroid, six for octopamine, two for tyramine, and one orphan receptor (XP_011155898.1). This “orphan” receptor is currently annotated in NCBI as “trace amine-associated receptor 9” (Tables 2 and S1). Of these 18 receptors, we identified all but the potential 5-HT_{2b} receptor SINV21469 listed by Qi et al. (2018) which was predicted to encode only TM6-7. Notably, this predicted annotation has been removed from the current genome. Qi et al. (2018) improved the annotation of the 5-HT receptors, noted in Tables 1 and S1, which has not yet been updated in the current *S. invicta* assembly. We did however identify an additional 5-HT_{2B} receptor not found in the Qi et al. (2018) study, LOC113004247, currently annotated as “alpha-2B adrenergic receptor” featuring two predicted isoforms: “alpha-2B adrenergic receptor isoform X1” (XP_025992824.1) and “uncharacterized protein LOC113004247 isoform X2” (XP_025992825.1). We have reannotated both transcripts of LOC113004247 to ‘5-hydroxytryptamine receptor 2B’ due to the high similarity in the transmembrane regions and the presence of signatures of 5-HT_{2B}-type receptors. As such, the fire ant now has two 5-HT_2 (A and B) receptor loci in the current genome.

Herein, reannotated loci encoding dopamine receptors are LOC105206526, LOC105205353, LOC105194927, and LOC105194339 (Table 1, Fig. 2). LOC105206526, annotated as dopamine receptor 1, is herein reannotated as D1 dopamine receptor for consistency with the

Table 1
Proposed reannotation of *S. invicta* GPCRs: The current NCBI predicted protein length is expressed as number of amino acid residues for only one transcript per locus. A complete list of predicted proteins (including those from loci with multiple predicted transcripts) is in Table S1. Under "Proposed new annotation", the motif in the cytoplasmic extension of the TM3 is in parenthesis. The classification of constant set (CS) or variable set (VS) of hormone neuropeptides is as proposed by Hauser et al. (2010). Literature citations support reannotations. Orphan receptors are indicated.

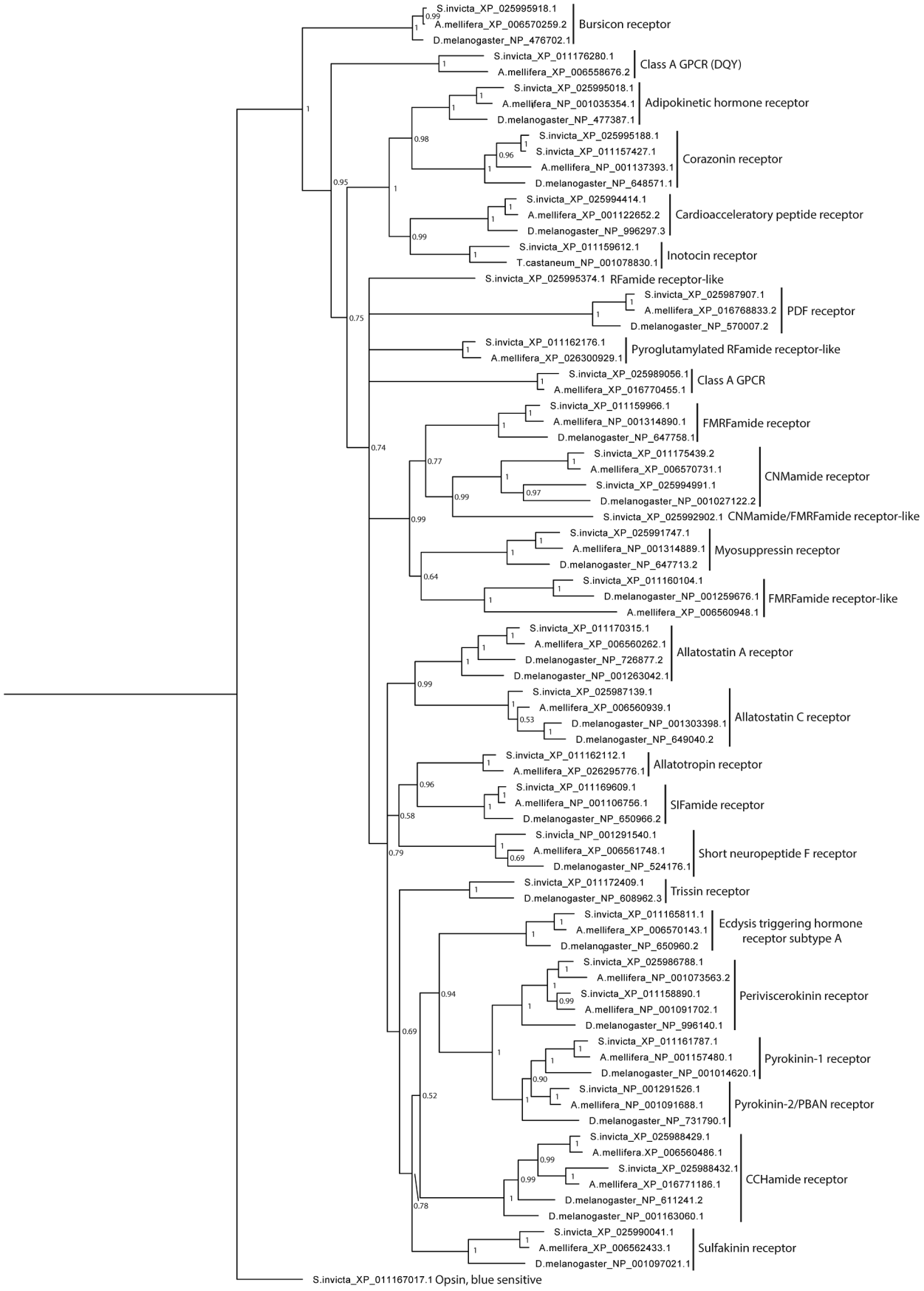
LOC	Length	Current annotation	Proposed new annotation (TM3 cytoplasmic motif)*	Notes
LOC105199174	404	5-hydroxytryptamine receptor 2A-like	5-hydroxytryptamine receptor 1 (DRY)	(Qi et al., 2018)
LOC105199572	644	5-hydroxytryptamine receptor 2B	5-hydroxytryptamine receptor 2A (DRY)	(Qi et al., 2018)
LOC113004247	780	alpha-2B adrenergic receptor	5-hydroxytryptamine receptor 2B (DRY)	
LOC105202925	570	5-hydroxytryptamine receptor 1	5-hydroxytryptamine receptor 7 (DRY)	(Qi et al., 2018)
LOC105206526	418	dopamine receptor 1	D1 dopamine receptor (DRY)	(Šimo et al., 2014)
LOC105205353	459	dopamine receptor 2	invertebrate specific D1-like dopamine receptor (InvD1L) (DRY)	(Šimo et al., 2014)/Dop2 as per (Qi et al., 2018)
LOC105194927	690	dopamine D2-like receptor	D2 dopamine receptor (DRY)	Dop3 as per (Qi et al., 2018)
LOC105194339	313	probable G-protein coupled receptor 52	dopamine/ecdysteroid receptor (DRY)	
LOC105199739	545	octopamine receptor Oamb	alpha-adrenergic-like octopamine receptor (DRY)	
LOC105198818	779	alpha-2A adrenergic receptor	alpha2-adrenergic-like octopamine receptor (DRF)	(Qi et al., 2017)
LOC105194469	406	tyramine receptor 1	tyramine receptor 2 (DRY)	(Qi et al., 2018; Reim et al., 2017)
LOC105193663	394	histamine H2 receptor	amine receptor-like (DRY)	
LOC105203747	408	somatostatin receptor type 2	allatostatin C receptor (DRY)	CS (Urlacher et al., 2016)
LOC105197452	440	orexin receptor type 1	allatotropin receptor (DRW)	VS
LOC105205389	388	gonadotropin-releasing hormone II receptor	adipokinetic hormone receptor (DRY)	CS
LOC105193776	799	lutropin-choriogonadotropic hormone receptor	bursicon receptor (ERN)	CS [*Rickets* orthologue (DLGR2)]
LOC105200112	434	growth hormone secretagogue receptor type 1	ecdysis triggering hormone receptor subtype A (ERY)	CS
LOC105194591	497	RYamide receptor-like	RFamide receptor-like (DRY)	orphan
LOC105198435	416	sex peptide receptor	myosuppressin receptor (WRY)	CS
LOC105207590	474	pyrokinin-1 receptor	CNMamide receptor 2 (ERF)	
LOC105205554	381	thyrotropin-releasing hormone receptor	CNMamide receptor 1 (ERY)	
LOC105203942	436	gonadotropin-releasing hormone receptor	corazonin receptor 2 (DRF)	VS
LOC105194275	410	gonadotropin-releasing hormone receptor	corazonin receptor 1 (DRF)	VS
LOC105195745	375	vasopressin V1a receptor-like	inotocin receptor (DRY)	VS
LOC105195234	475	neuropeptides capa receptor	periviscerokinin receptor 1 (ERY)	VS
LOC105195236	466	neuropeptides capa receptor-like	periviscerokinin receptor 2 (ERY)	VS
LOC105200002	605	neuromedin-U receptor 2-like	pyrokinin-2 receptor/PBAN receptor (ERY)	(Choi and Vander Meer, 2012)
LOC105197486	423	gastrin/cholecystokinin type B receptor	pyroglutamylated RFamide receptor-like (ERY)	
LOC105192838	547	cholecystokinin receptor	sulfakinin receptor (ERY)	VS
LOC105195500	387	prolactin-releasing peptide receptor-like	short neuropeptide F receptor (DRF)	CS (Bajracharya et al., 2014)
LOC105197902	508	calcitonin receptor	diuretic hormone 31 receptor-like	CS (Kwon et al., 2012)
LOC105194460	474	diuretic hormone receptor	CRF diuretic hormone receptor 44 – dih2-like	CS (Jagge and Pietrantonio, 2008)
LOC105196189	471Ψ	uncharacterized protein LOC105196189	methuselah-like 3 receptor	
LOC105200318	478Ψ	uncharacterized protein LOC105200318	methuselah-like B3 receptor	
LOC105207541	1901	uncharacterized protein LOC105207541	gamma-aminobutyric acid type B receptor subunit 2-like	
LOC105204438	685	uncharacterized protein LOC105204438	class A GPCR (DRY)	orphan
LOC105203919	441	uncharacterized protein LOC105203919	CNMamide/FMRFamide receptor-like	orphan
LOC105195005	752	relaxin receptor 1	leucine rich repeat-containing GPCR	LRR3 (orphan)
LOC105196073	386	probable G-protein coupled receptor B0563.6	FMRFamide receptor-like (ERY)	orphan
LOC105201914	371	histamine H2 receptor-like	class A GPCR (ENY)	orphan
LOC105208194	840	uncharacterized protein LOC105208194	class A GPCR (DQY)*	orphan
LOC105201824	465	adrenocorticotropic hormone receptor-like	class A GPCR	orphan
LOC105195799	1136	uncharacterized protein LOC105195799	class A GPCR	orphan
LOC105204554	579	secretin receptor	class B GPCR	orphan
LOC105200605	1336	probable G protein coupled receptor 158	probable G protein coupled receptor 158-like	orphan
LOC105203641	217	probable G protein coupled receptor B0563.6	probable G protein coupled receptor B0563.6-like	orphan
LOC105195815	800	probable G protein coupled receptor CG31760	probable G protein coupled receptor CG31760-like	orphan
LOC105201654	1470	uncharacterized protein LOC105201654	adhesion GPCR	
LOC105202571	486Ψ	uncharacterized protein LOC105202571	class C GPCR	orphan; contains a pfam00003: 7tm_3 domain
LOC105198935	542Ψ	uncharacterized protein LOC105198935	methuselah-like receptor	
LOC105205937	621Ψ	uncharacterized protein LOC105205937	methuselah-like receptor	
LOC105204218	455Ψ	uncharacterized protein LOC105204218	methuselah-like receptor	
LOC105201066	698	uncharacterized protein LOC105201066	methuselah-like receptor	
LOC113004514	443Ψ	uncharacterized protein LOC113004514	methuselah-like receptor	
LOC113005085	430Ψ	uncharacterized protein LOC113005085	methuselah-like receptor	

* XP_011176281.1. = This is an unusually large GPCR with an unusual TM3 "DQY" motif. CS = constant set; VS = variable set (see text). Ψ These proteins have less than 7 predicted transmembrane regions.

nomenclature in Šimo et al., (2014). LOC105205353 (XP_011173009.1) encodes the invertebrate specific D1-like dopamine receptor (Šimo et al., 2014). While analyzing the *S. invicta* genome available before August 2018, we predicted that LOC105194900 (XP_011158340.1) and LOC105194927 (XP_011158366.1) together likely encoded the D2 dopamine receptor (Table S2). LOC105194900 was previously annotated in NCBI as "serotonin (5-HT) receptor". We predicted this locus encoded TM1-2 while the second locus (LOC105194927) encoded TM3-7 of a single receptor. This was based on the analysis of the previous genomic scaffold (Si_gnG Si_gnG.scaffold02648) sequence

Table 2
Compilation of mined GPCRs from the *Solenopsis invicta* genome released in August 2018. GPCR annotation includes those proposed herein (marked with "a") and NCBI current annotations that remain unchanged. When more than one protein accession number (XP) is available for a given locus (LOC) only one is shown here.

GPCR annotation	LOC	XP
5-hydroxytryptamine receptor 1[a]	LOC105199174	XP_011164446.1
5-hydroxytryptamine receptor 2A[a]	LOC105199572	XP_011165026.1
5-hydroxytryptamine receptor 2B[a]	LOC113004247	XP_025992824.1
5-hydroxytryptamine receptor 7[a]	LOC105202925	XP_025995734.1
adenosine receptor A2b	LOC105201199	XP_011167438.1
adenosine receptor A2b-like	LOC105201947	XP_011168550.1
adhesion G protein-coupled receptor A3	LOC105201434	XP_011167742.1
adhesion GPCR[a]	LOC105201654	XP_011168055.1
adipokinetic hormone receptor[a]	LOC105205389	XP_025995016.1
allatostatin A receptor	LOC105203239	XP_011170315.1
allatostatin C receptor[a]	LOC105203747	XP_025987139.1
allatotropin receptor[a]	LOC105197452	XP_011162112.1
alpha-adrenergic-like octopamine receptor[a]	LOC105199739	XP_025990748.1
alpha2-adrenergic-like octopamine receptor[a]	LOC105198818	XP_025992087.1
bursicon receptor[a]	LOC105193776	XP_025995918.1
cardioacceleratory peptide receptor	LOC105207336	XP_025994414.1
CNMamide receptor 1[a]	LOC105205554	XP_025994991.1
CNMamide receptor 2[a]	LOC105207590	XP_011175439.2
corazonin receptor 1[a]	LOC105194275	XP_011157427.1
corazonin receptor 2[a]	LOC105203942	XP_025995188.1
CRF diuretic hormone receptor 44 – dih2-like[a]	LOC105194460	XP_025992420.1
D1 dopamine receptor[a]	LOC105206526	XP_011174320.1
D2 dopamine receptor[a]	LOC105194927	XP_025992165.1
dopamine/ecdysteroid receptor[a]	LOC105194339	XP_011157518.1
invertebrate specific D1-like dopamine receptor (InvD1L)[a]	LOC105205353	XP_011173009.1
diuretic hormone 31 receptor-like[a]	LOC105197902	XP_025987488.1
ecdysis triggering hormone receptor subtype A[a]	LOC105200112	XP_011165811.1
FMRFamide receptor	LOC105195979	XP_011159966.1
gamma-aminobutyric acid type B receptor subunit 1	LOC105202097	XP_011168784.1
gamma-aminobutyric acid type B receptor subunit 2	LOC105198865	XP_025994475.1
gamma-aminobutyric acid type B receptor subunit 2-like[a]	LOC105207541	XP_025992090.1
glucagon receptor-like	LOC105206029	XP_011173862.1
inotocin receptor[a]	LOC105195745	XP_011159612.1
latrophilin Cirl	LOC105193213	XP_025989117.1
metabotropic glutamate receptor	LOC105202369	XP_011169146.1
metabotropic glutamate receptor 2	LOC105200285	XP_011166063.2
metabotropic glutamate receptor 8	LOC105197884	XP_025987394.1
G protein coupled receptor Mth	LOC105203498	XP_011170599.1
G protein coupled receptor Mth	LOC105201065	XP_025996921.1
G protein coupled receptor Mth-like 3	LOC105201068	XP_025986430.1
G protein coupled receptor Mth2	LOC105207507	XP_011175316.1
G protein coupled receptor Mth2	LOC105199776	XP_025995885.1
G protein coupled receptor Mth2	LOC105193060	XP_025995097.1
G protein coupled receptor Mth2	LOC105204371	XP_025994309.1
G protein coupled receptor Mth2	LOC105202569	XP_011169446.1
G protein coupled receptor Mth2	LOC105207504	XP_025995892.1
G protein coupled receptor Mth2	LOC105196180	XP_025992556.1
G protein coupled receptor Mth2-like	LOC105204531	XP_011171921.1
G protein coupled receptor Mth2-like	LOC105199774	XP_011165299.1
G protein coupled receptor Mth2-like	LOC105207506	XP_011175314.1
G protein coupled receptor Mth2-like	LOC105204876	XP_011172431.1
G protein coupled receptor Mth2-like	LOC105195599	XP_011159387.1
G protein coupled receptor Mth2-like	LOC105196109	XP_011160163.1
G protein coupled receptor Mth2-like	LOC105196494	XP_025987813.1
G protein coupled receptor Mth2-like	LOC105199507	XP_025996851.1
G protein coupled receptor Mth2-like	LOC113005491	XP_025996919.1
G protein coupled receptor Mth2-like	LOC113005481	XP_025996891.1
G protein coupled receptor Mth2-like	LOC113005222	XP_025996274.1
G protein coupled receptor Mth2-like	LOC113002672	XP_025996892.1
G protein coupled receptor Mth2-like	LOC105197050	XP_025995880.1
G protein coupled receptor Mth2-like	LOC105203499	XP_025996436.1
methuselah-like 3 receptor[a]	LOC105196189	XP_025992552.1
methuselah-like B3 receptor[a]	LOC105200318	XP_025992110.1
methuselah-like receptor[a]	LOC105198935	XP_011164108.1
methuselah-like receptor[a]	LOC105205937	XP_025995886.1
methuselah-like receptor[a]	LOC105204218	XP_025996439.1
methuselah-like receptor[a]	LOC105201066	XP_025996927.1
methuselah-like receptor[a]	LOC113004514	XP_025993825.1
methuselah-like receptor[a]	LOC113005085	XP_025995889.1
probable G protein coupled receptor Mth-like 1	LOC105203758	XP_025987189.1
probable G protein coupled receptor Mth-like 1	LOC105195397	XP_025993830.1
probable G protein coupled receptor Mth-like 2	LOC105200319	XP_011166100.2
probable G protein coupled receptor Mth-like 3	LOC105202828	XP_011169824.1
probable G protein coupled receptor Mth-like 3	LOC105201067	XP_025996920.1
probable G protein coupled receptor Mth-like 3	LOC105204875	XP_025993073.1
probable G protein coupled receptor Mth-like 4	LOC105203497	XP_025996440.1
probable G protein coupled receptor Mth-like 5	LOC105195600	XP_011159388.1
G protein coupled receptor moody	LOC105195543	XP_025990528.1
G protein coupled receptor moody	LOC105195542	XP_011159290.1
muscarinic acetylcholine receptor DM1	LOC105199400	XP_011164802.1
probable muscarinic acetylcholine receptor gar-1	LOC105205262	XP_011172906.1
myosuppressin receptor[a]	LOC105198435	XP_025991747.1
neuropeptide CCHamide-1 receptor	LOC105200046	XP_025988429.1
neuropeptide CCHamide-2 receptor-like	LOC105200038	XP_025988432.1
neuropeptide SIFamide receptor	LOC105202687	XP_011169609.1
octopamine receptor beta-1R	LOC105202476	XP_025992228.1
octopamine receptor beta-2R	LOC105200419	XP_011166273.1
octopamine receptor beta-3R	LOC105197287	XP_025992265.1
green-sensitive opsin-like	LOC105208023	XP_011176051.1
opsin, blue-sensitive	LOC105200910	XP_011167017.1
opsin, ultraviolet-sensitive	LOC105199537	XP_025996224.1
rhodopsin	LOC105199190	XP_011164467.1
rhodopsin	LOC105199191	XP_025993904.1
PDF receptor	LOC105204568	XP_025987907.1
periviscerokinin receptor 1[a]	LOC105195234	XP_025986788.1
periviscerokinin receptor 2[a]	LOC105195236	XP_011158890.1
prostaglandin E2 receptor EP2 subtype	LOC105193552	XP_025991254.1
protein trapped in endoderm-1	LOC105208166	XP_011176251.1
protocadherin-like wing polarity protein stan	LOC105193015	XP_025992997.1
pyroglutamylated RFamide receptor-like[a]	LOC105197486	XP_011162176.1
pyrokinin-2 receptor/PBAN receptor[a]	LOC105200002	NP_001291526.1
pyrokinin-1 receptor	LOC105197213	XP_011161787.1
short neuropeptide F receptor[a]	LOC105195500	NP_001291540.1
sulfakinin receptor[a]	LOC105192838	XP_025990041.1
tachykinin-like peptides receptor 86C	LOC105193922	XP_025986434.1
tachykinin-like peptides receptor 99D	LOC105203085	XP_025989494.1
trace amine-associated receptor 9	LOC105193226	XP_011155898.1
trissin receptor	LOC105204861	XP_011172409.1
tyramine receptor 1	LOC105193512	XP_025991529.1
tyramine receptor 2[a]	LOC105194469	XP_011157692.1
Orphan receptors		
amine receptor-like	LOC105193663	XP_011156507.1
FMRFamide receptor-like[a]	LOC105196073	XP_011160104.1
class A GPCR[a]	LOC105201824	XP_025989008.1
class A GPCR[a]	LOC105201914	XP_025989056.1
class A GPCR[a]	LOC105208194	XP_011176280.1
class A GPCR[a]	LOC105195799	XP_011159708.1
class A GPCR[a]	LOC105204438	XP_011171810.1
class B GPCR[a]	LOC105204554	XP_025987851.1
class C GPCR[a]	LOC105202571	XP_011169449.1
CNMamide/FMRFamide receptor-like[a]	LOC105203919	XP_025992902.1
leucine rich repeat-containing GPCR[a]	LOC105195005	XP_011158500.1
probable G protein coupled receptor 158-like[a]	LOC105200605	XP_025995202.1
probable G protein coupled receptor B0563.6-like[a]	LOC105203641	XP_025989946.1
probable G protein coupled receptor CG31760-like[a]	LOC105195815	XP_025996802.1
RFamide receptor-like[a]	LOC105194591	XP_025995374.1



(caption on next page)

Fig. 1. Phylogeny of Class A neuropeptide GPCRs in *S. invicta, A. mellifera,* and *D. melanogaster*. The phylogeny was reconstructed in MrBayes with four chains and four runs of the mixed amino acid model for 1,000,000 generations with a 10% burnin. Node values are Bayesian posterior probability rounded to two significant figures. Scale bar indicates branch length. *S. invicta* protein accession numbers are cross referenced with LOC numbers in Tables 2 and S1. One protein from *T. castaneum* (NP_001078830.1) was included as an equivalent inotocin receptor which was not present in genomes from *D. melanogaster* or *A. mellifera.*

NW_011796802.1 (NCBI) in which these two predicted loci were incomplete but immediately adjacent. Further, several gaps were present in the scaffold, which likely contributed to the previous mispredictions. Our conclusion was further supported by the alignments performed for the Bayesian analysis and the resulting protein clustering (Fig. S1). In the current genome, these loci have been merged in LOC105194927, in agreement with our analysis and as such, LOC105194927 encodes the D2 dopamine receptor. Additionally, there is confusion in the arthropod literature on the nomenclature used to classify dopamine receptors. Previously, Qi et al. (2018), designated LOC105194927 (previously XP_011158366.1, currently XP_025992165.1) as Dop3, based on the similarity to the functionally characterized *A. mellifera AmDop3* (AAX62923.1 or XP_006561568.1; orthologous to *D. melanogaster* D2 receptor; Fig. 2, Table S1) (Beggs et al., 2005). We followed the clarification of nomenclature presented by Šimo et al., (2014) to rename this gene as D2 dopamine receptor. The orthologous *D. melanogaster* and *A. mellifera* proteins that were functionally characterized, decrease intracellular cAMP upon activation (Beggs et al., 2005; Hearn et al., 2002).

LOC105194339, annotated as a "probable G-protein coupled receptor 52" (Table 1), likely encodes the dopamine/ecdysteroid receptor XP_011157518.1, which was used for the tree presented in Fig. 2. In summary, *S. invicta* has one putative receptor protein for each of the four invertebrate dopamine receptor types (Fig. 2).

Improved annotations are proposed for octopamine receptors LOC105199739 and LOC105198818 currently annotated as "octopamine receptor Oamb" and "alpha-2A adrenergic receptor", respectively. LOC105199739 has specific octopamine receptor conserved domains. In contrast, the receptor encoded by LOC105198818, is specifically an alpha-2A adrenergic receptor-like octopamine receptor (Qi et al., 2017). This receptor is particular in that it possesses a DRF motif following TM3, while most biogenic amine receptors including adrenergic octopamine receptors, exhibit a DRY motif. The DRF motif has been associated with agonist-independent activity in biogenic amine receptors from insects including the honey bee (Schlenstedt et al., 2006) and other arthropods (Clark et al., 2004; Meyer et al., 2011). Thus, this proposed octopamine receptor may exhibit agonist-independent activity; it would also be interesting to test if it perhaps functions as an octopamine/tyramine receptor (Gross et al., 2015). Furthermore, LOC105198818 has the FxxxWxPFF motif in TM6, present in biogenic

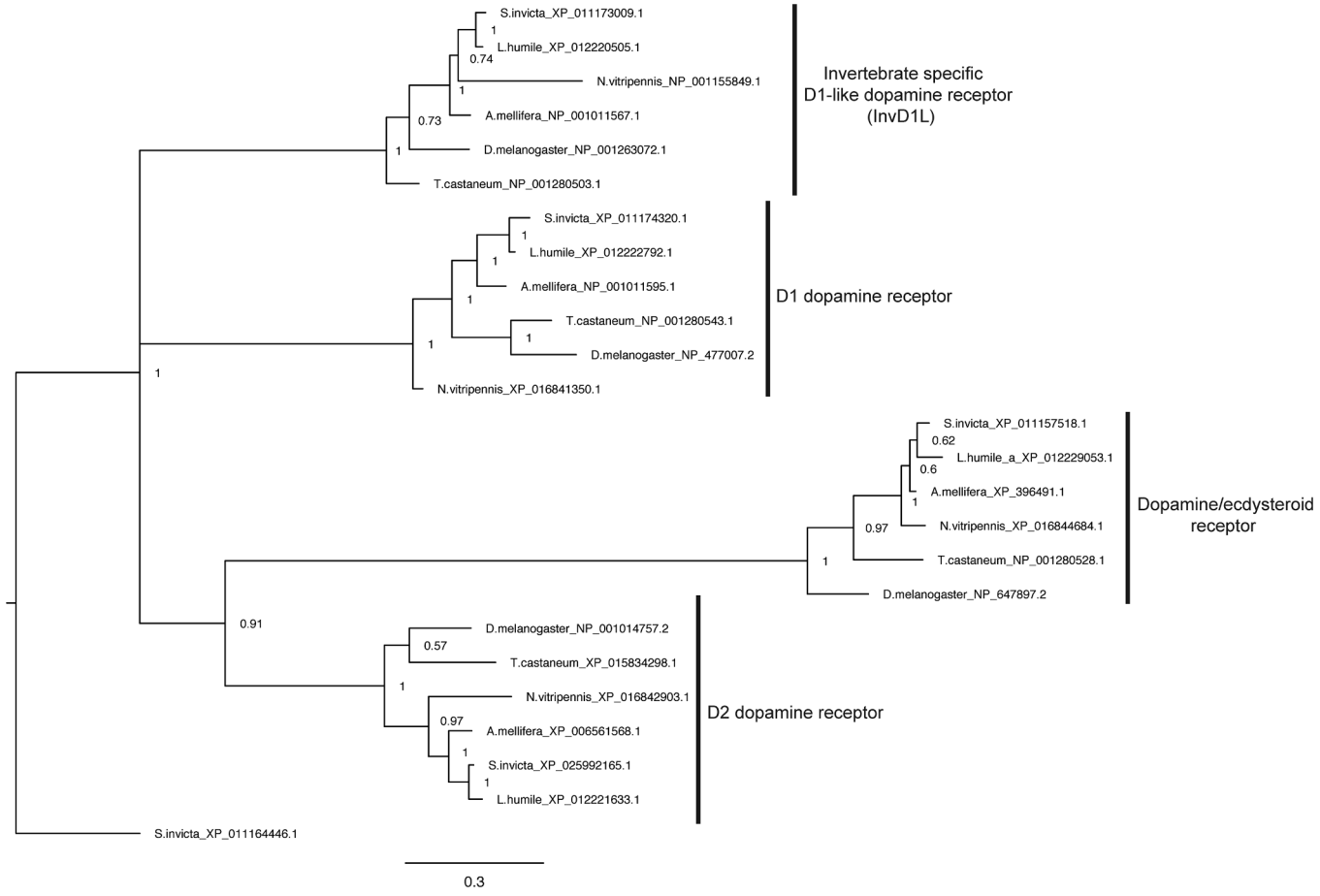


Fig. 2. Phylogeny of dopamine receptors in *S. invicta, A. mellifera, T. castaneum, L. humile, D. melanogaster, and N. vitripennis*. The phylogeny was reconstructed in MrBayes with four chains and four runs of the mixed amino acid model for 1,000,000 generations with a 10% burnin. Node values are Bayesian posterior probability rounded to two significant figures. Scale bar indicates branch length. Clades are labeled following nomenclature as in Šimo et al. (2014). *S. invicta* has one representative member in each of the dopamine clades, invertebrate specific D1-like dopamine receptor: XP_011173009.1, D1 dopamine receptor: XP_011174320.1, dopamine/ecdysteroid receptor: XP_011157518.1, and D2 dopamine receptor: XP_025992165.1. A predicted *S. invicta* 5-HT receptor (XP_011164446.1) identified by Qi et al. (2018) and listed in Table S1 as 5-HT$_1$, was used as the outgroup.

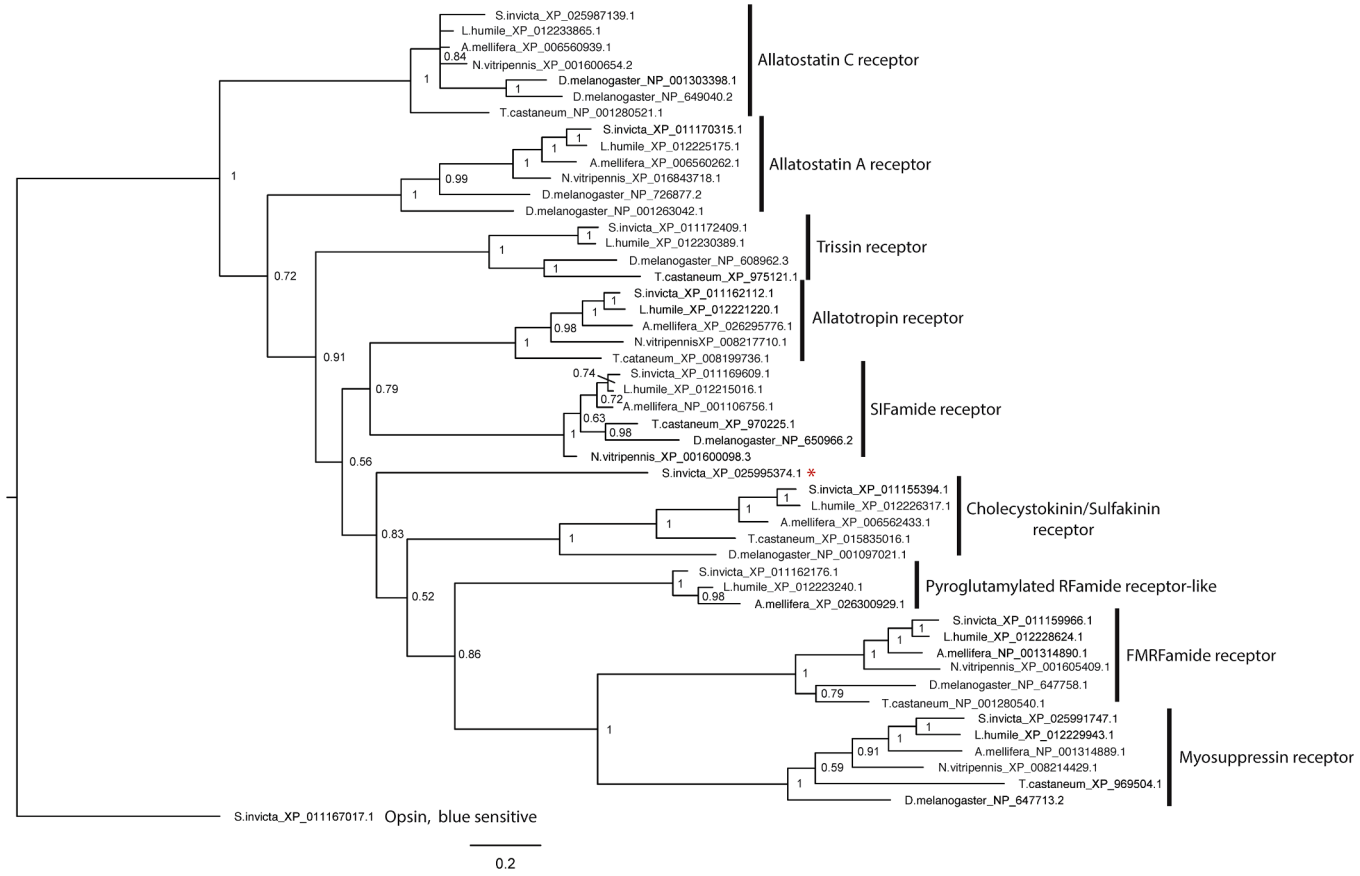


Fig. 3. Phylogeny of select neuropeptide receptors in *S. invicta, A. mellifera, T. castaneum, L. humile, D. melanogaster, and N. vitripennis*. The phylogeny was reconstructed in MrBayes with four chains and four runs of the mixed amino acid model for 1,000,000 generations with a 10% burnin. Node values are Bayesian posterior probability rounded to two significant figures. Scale bar indicates branch length. Clades are labeled based on *D. melanogaster* nomenclature. *S. invicta* protein accession numbers are cross-referenced in Tables 2 and S1. This tree was reconstructed to annotate the XP_025995374.1 orphan receptor indicated by the red asterisk, using allatostatin A, trissin, and RFamide-like receptors. The analyses show that this protein clusters with the RFamide-like receptors and was annotated as RFamide receptor-like (Tables 1 and 2). *S. invicta* "opsin, blue sensitive" acts as the outgroup.

amine receptors, however, it has the NPFIY motif in TM7 instead of NP(L/I)IY motif of adrenergic octopamine receptors (Maqueira et al., 2005).

Recent published analyses (Qi et al., 2018; Reim et al., 2017) support our reannotation of LOC105194469 (which encodes two putative transcripts) as tyramine receptor 2 (Table 1).

3.3. Neuropeptide receptors

Within neuropeptide Class A GPCRs, one locus was correctly annotated in the genome as encoding an "allatostatin-A receptor" (XP_011170315.1; LOC105203239) (Fig. 3, Tables 2 and S1), possessing the key DRY motif present in allatoregulatory peptide receptors; other motifs in these type of receptors are QRY, DRW, or DRF (Felix et al., 2015; Verlinden et al., 2015). Previously an additional locus, LOC105204861, encoding the predicted XP_011172409.1, had been annotated as "allatostatin-A receptor" (Table S2), however, this is currently annotated as a trissin receptor (Table 1, Fig. 3). This protein lacks the two asparagine residues (N) within the TM7 motif, NSxxNPxxY, conserved for allatostatin A receptors but instead features the TSxxDPxxY motif; additionally it has the ERY motif instead of the DRY motif on the cytoplasmic extension of TM3 (Felix et al., 2015) (Fig. S1). Our analysis agrees with the reannotation of LOC105204861 as a trissin neuropeptide receptor, because of high similarity to trissin receptors and the presence of the ERY motif (Fig. 3, Fig. S1), which is also present in the functionally characterized *D. melanogaster* trissin receptor (NP_608962.3) (Ida et al., 2011). Bayesian phylogentic analyses further support this reannotation (Fig. 3).

The currently annotated "somatostatin receptor type 2", LOC105203747, was reannotated as "allatostatin C receptor" (XP_025987139.1) (Fig. 3). In support of this reannotation, the orthologue receptor in *A. mellifera* functionally responds to both peptides allatostatin C and allatostatin CC (Urlacher et al., 2016).

LOC105194591 was previously annotated as "allatostatin-A receptor-like" (Table S2) and is now annotated as "RYamide receptor-like" (XP_025995374.1) (Table 1). Our alignments of allatostatin receptors (Fig. S2) and the phylogenetic analyses (Figs. 1 and 3) clearly show this sequence is not an allatostatin receptor. Blast analyses of this sequence shows signatures of cholecystokinin-like mammalian receptors, which include four types of receptors for RFamide ligands: NPFF, pyroglutaminated RFamide peptides, cholecystokinin (sulfakinin-like in insects) and orexins (insect allatotropins). The sequences of the insect allatotropins (although considered orexin orthologues) end in GF-NH_2, GY-NH_2 or SF-NH_2 and not RF-NH_2. The receptor sequence XP_025995374.1 lacks the conserved E/DRWYAI present in allatotropin and orexin receptors at the cytoplasmic extension of TM3 (Alzugaray and Ronderos, 2018), therefore, it is unlikely it encodes an allatotropin receptor (Veenstra et al., 2012). With the current information available this receptor is a candidate for one of the other above-mentioned receptor types (cholecystokinin-like) and has been reannotated as RFamide neuropeptide receptor-like rather than RYamide receptor-like (Tables 1 and 2).

LOC105197452 is currently annotated as "orexin receptor type 1" but herein was reannotated as allatotropin receptor (Tables 1 and 2,

Fig. 3) (Alzugaray and Ronderos, 2018). The insect allatotropin receptor is most similar to the orexin receptor of vertebrates (Horodyski et al., 2011). However, when functionally tested, the orexin peptide does not activate insect allatotropin receptors, whereas allatotropin does (Horodyski et al., 2011; Verlinden et al., 2013; Vuerinckx et al., 2011). In invertebrates, allatotropin signaling stimulates feeding (Mizoguchi, 2016), and additionally in insects, juvenile hormone synthesis. In *S. invicta* the neuropeptide gene LOC105203405 is annotated as "allatotropin-like" and shares substantial similarity to *A. mellifera* and *N. vitripennis* allatotropin neuropeptide. Moreover, LOC105203405 has higher identity to the predicted allatotropin neuropeptide genes from the ants *Monomorium pharaonius* and *Trachymyrmex zeteki*. In *S. invicta* the allatotropin neuropeptide and allatotropin receptor are encoded by LOC105203405 and LOC105197452, respectively.

The adipokinetic hormone (AKH) receptor in *S. invicta* is annotated as a "gonadotropin-releasing hormone II receptor" (LOC105205389) because of similarity to and common evolutionary origin of the AKH and ACP-type receptors (Adipokinetic hormone/Corazonin related Peptide) from ancestral GnH-type receptors (Gonadotropin-releasing Hormone) (Zandawala et al., 2017). This receptor in *S. invicta* closely matches the annotated AKH receptors in *A. mellifera*, *N. vitripennis*, and *D. melanogaster* (Table S1), therefore, LOC105205389 encodes the fire ant AKH receptor (Table 1).

LOC105193776 is currently annotated as "lutropin-choriogonadotropic hormone receptor" encodes the bursicon receptor (Table 1). In *D. melanogaster*, the *rickets* gene, a leucine-rich motif containing GPCR, shows similarity to a subfamily of the lutropin-choriogonadotropin mammalian hormone receptors (Ascoli et al., 2002). The product of the gene *rickets* (CG8930) in *D. melanogaster* is the bursicon receptor (Baker and Truman, 2002; Luo et al., 2005; Mendive et al., 2005). The genes in *S. invicta* (LOC105193776), *N. vitripennis* (LOC100122233), and *A. mellifera* (LOC411738) bear the closest similarity to *rickets*, thus, these likely encode the bursicon receptor (Tables 1 and S1). The hormone "bursicon" is involved in cuticular tanning and wing expansion in newly ecdysed insects; in *D. melanogaster* the active hormone is a heterodimer of two peptides, bursicon and partner of bursicon (Luo et al., 2005; Mendive et al., 2005). The cognate receptor was not correctly annotated in the *S. invicta* genome, although the genes for its ligands, the bursicon peptide (LOC105203304) and partner of bursicon (LOC105192964) were so annotated in the draft genome. In NCBI these loci appear now combined into LOC105203304, currently annotated as an uncharacterized protein. The first half of the new protein XP_025993019.1 encoded by LOC105203304 shares high identity (75–95%) with bursicon peptide while the second half shares high identity with partner of bursicon (57–68%) in *D. melanogaster* and *N. vitripennis*. Moreover, XP_025993019.1 shares 82% identity with the "single-chain bursicon precursor" previously predicted in *A. mellifera* but later corrected, because the bursicon and partner of bursicon propeptides were experimentally determined to be two genes in *A. mellifera* (Van Loy et al., 2007). As such, LOC105203304 in the fire ant should be further analyzed to determine if indeed represents an incorrect fusion of two separate genes.

Ecdysis triggering hormone (ETH) initiates the insect ecdysis sequence. A potential receptor, ETHR in *S. invicta,* is encoded by LOC105200112, currently annotated as "growth hormone secretagogue receptor type 1". Previously, an additional ETHR candidate, LOC105200117, was annotated as "thyrotropin-releasing hormone receptor-like" (Table S2) but has now been removed in the new assembly.

Currently annotated as "Sex peptide receptor", LOC105198435, likely encodes the myosuppressin receptor. Myosuppressins inhibit insect muscle contraction. In *S. invicta*, the receptor encoded by this gene exhibits the WRY motif in the intracellular loop following TM3. This motif is also present in the *A. mellifera* (NP_001314889.1) and *N. vitripennis* (XP_008214429.1) predicted orthologous receptors, as well as in the *D. melanogaster* receptor, which has been functionally characterized and is correctly annotated as "Myosuppressin receptor 1" (NP_647713.2) (Table S1) (Egerod et al., 2003). Thus, although annotated as "sex peptide receptors" (Table S1), the above mentioned receptors of *S. invicta*, *A. mellifera,* and *N. vitripennis* most likely encode myosuppressin receptors. While myosuppressin receptors are similar to sex peptide receptors from *D. melanogaster Bombyx mori*, and *T. castaneum*, the sex peptide receptor motif is QRY (Poels et al., 2010). The sex peptide receptor in *D. melanogaster* functionally responds to myoinhibitory peptides (MIPs) (Kim et al., 2010; Verlinden et al., 2015). Therefore, we proposed LOC105198435 in *S. invicta* encodes the myosuppressin receptor. This is in agreement with previous reports that sex peptide ligand genes are not present in ant genomes (Nygaard et al., 2011).

Currently two loci LOC105203942 and LOC105194275 are annotated as "gonadotropin-releasing hormone receptor" (Table 1). Blast analyses revealed high similarity to corazonin receptors (Table S1), thus, they were so reannotated supported by phylogenetics (Fig. 1). Previously there was a third locus annotated as "gonadotropin-releasing hormone receptor-like", LOC105192960, encoding TM1-4 (202 amino acid residues) that has now been merged with LOC105203942, previously encoding only TM5-7 (208 amino acid residues) of the corazonin receptor. Genome analyses revealed these two previous loci were in separate scaffolds, SI_gnG.scaffold01499 (~2208 bp) and SI_gnG.scaffold7973 (~200,000 bp), which likely contributed to the previous assignment of two different loci numbers.

LOC105200002, "neuromedin-U receptor 2-like", encodes the pheromone biosynthesis-activating neuropeptide (PBAN)-like receptor (Accession JX657040.1), as it was functionally demonstrated in *S. invicta,* where it activates synthesis of trail pheromone (Choi and Vander Meer, 2012).

The "gastrin/cholecystokinin type B receptor-like", LOC105197486, was herein reannotated as "pyroglutamylated RFamide peptide receptor-like" based on very high similarity to the orthologues proteins in other hymenopterans so named, including the bumble bee, *Bombus terrestris*. However, we speculate that in fire ant the two transcripts may correspond to two forms of the elevenin receptor (Table 1). This is based on reciprocal Blast analyses using the coleopteran *T. castaneum* EFA11608.1 (TC014211) and the *B. terrestris* (AIV98045.1; Mathiassen and coworkers, direct NCBI submission) elevenin receptor sequences. These sequences are listed as such by Uchiyama et al., who deorphanized the orthologous receptor in the hemipteran *Nilaparvata lugens* (Uchiyama et al., 2018). It is noteworthy, however, that the peptide tested in *N. lugens* by the latter authors, of sequence KVNCRKFVYAPVCRGVAA, is not an RFamide peptide and is not amidated (Yoshiaki Tanaka, *personal comm.*). Moreover, the elevenin receptor is lost in *B. mori* and *D. melanogaster* (Uchiyama et al., 2018), justifying the low similarity of the *S. invicta* sequence to the closest similar in *Drosophila*, the ETH receptor (Table S1). Further research is needed to clarify the role of this receptor, which is present (with very high sequence similarity) in all hymenopterans examined.

We previously cloned (Chen and Pietrantonio, 2006) and deorphanized the *S. invicta* sNPF receptor (AAY88918.1), which was unresponsive to sNPF peptides of canonical C-terminal sequence LRLRFa. This receptor is however activated by novel sNPY ligand(s) (Bajracharya et al., 2014). Despite this published work, both receptor (LOC105195500) and cognate peptide (LOC105194759) genes are currently misannotated in NCBI for *S. invicta.* Published accession numbers, however, lead to the correct annotated receptor and propeptide, respectively. There are six transcripts predicted for the *S. invicta* sNPF receptor gene that encode identical proteins; the transcripts' differences are in untranslated regions (Table S1). These differences in transcript length may reflect differences in mRNA stability related to the observed variation in the number and location of sNPF receptor immunoreactive cells detected in worker and queen brains (Bajracharya et al., 2014; Castillo and Pietrantonio, 2013). The honey bee ligand is annotated as "short neuropeptide F-like" (XP_003250155.1) (Ament

et al., 2011) and its receptor is still misannotated as "prolactin-releasing receptor-like" (Table S1).

In the current *S. invicta* genome two loci for tachykinin receptors are present, tachykinin-like peptides receptor 99D (LOC105203085) and tachykinin-like peptides receptor 86C (LOC105193922), each encode a single full GPCR. In the previous assembly, two loci annotated as "tachykinin-like peptides receptor 99D" LOC105208274 and LOC105203085 encoded the N-terminal (TM1-3) and C-terminal (TM4-7) regions, respectively (Table S2). The predicted XP_011176389.1 "tachykinin-like peptides receptor 99D" (TM 1–3) was on a smaller scaffold (~3,500 bp) than XP_011170144.1 "tachykinin-like peptides receptor 99D" (TM4-7), and upstream of the latter there were predicted gaps. This may explain the two previous LOC numbers attributed to these protein fragments. The protein encoded by joining these two predicted fragments is the orthologue of *B. mori* receptor A24 that functionally responds to a number of tachykinin-related peptides (He et al., 2014) and to ion transport-like peptide (AY950503), but with a higher EC_{50} for the latter (Nagai-Okatani et al., 2016; Nagai et al., 2014). These two *S. invicta* loci were merged in the current assembly under LOC105203085. Similarly, for tachykinin-like peptides receptor 86C (Jiang et al., 2013; Poels et al., 2009), LOC105205294 and LOC105193922 encoded the N-terminal (TM1-4) and C-terminal (TM5-7) regions, respectively, of the receptor, which is the orthologue of *B. mori* A33 receptor (Nagai et al., 2014). In this case both predicted fragments were at the ends of two different scaffolds; these loci were merged in the new assembly under LOC105193922. Our analyses support the merging of these previously split loci.

3.4. Unclassified receptors, orphan receptors and orphan ligands

We identified at 15 orphan GPCRs, although some were assigned likely ligands (Table 1). The receptor encoded by LOC105204438 (Table 1) currently annotated as "uncharacterized protein" has the DRY motif and exhibits a NPWIR motif in TM7 similar but not identical to the NP(L/I)IY present in adrenergic-like octopamine receptors (Maqueira et al., 2005). The unique TM6 motif FxxxWxP of aminergic receptors is absent. This putative receptor has the signature of Class A GPCRs but no other specific motifs are present to place it within the class. For this reason the reannotation is "class A GPCR."

Blast searches with the currently annotated *S. invicta* "Histamine H2 receptor-like" (LOC105201914) (Table S1) against the *A. mellifera* genome identified a GPCR annotated as RYamide receptor-like (LOC100578739). Blast searches against all hymenopterans revealed a variety of annotations for very similar predicted receptors such as histamine H2-receptor like, melatonin receptor, NPY receptor, and RYamide receptor. Functional analyses are needed to correctly annotate this gene. In Table 1 we reannotated this gene as "class A GPCR" and noted it as orphan because phylogenetic analysis did not aid in further classifying this receptor (XP_025989056.1 in Fig. 1).

Another orphan GPCR is encoded by LOC105208194, with a predicted 840 amino acid residue protein, XP_011176280.1, with very high similarity among hymenopterans. This unusual receptor is predicted as a Class A GPCR and has a DQY motif at the cytoplasmic end of TM3 (Fig. 4). The gene encodes a complete protein and the three predicted transcripts only differ in the untranslated regions. In Table 1, this predicted receptor was reannotated as "class A GPCR" because there is no similarity to any other protein outside orthologous receptors in insects, and it was noted as orphan. The phylogenetic analysis shows a unique placement in the tree (Fig. 1).

Despite the fact that orcokinin peptides have been identified in all insects species analyzed, including the ants *Camponotus floridanus* (Schmitt et al., 2015), and that the orcokinin gene is present in *S. invicta* (LOC105208194, annotated as 'uncharacterized LOC105208194'), the receptor remains unidentified in insects (Jiang et al., 2015).

3.5. Differentially expressed GPCRs in worker and queen brain

Transcriptomic analyses comparing the brains of workers to those of mated queens and alate virgin queens (Table 3) identified ten differentially expressed GPCR genes. Two biogenic amine receptors were more highly expressed in workers than in queens. One predicted non-coding tyramine receptor transcript was more highly expressed in worker brains. Expression of the newly annotated Invertebrate specific D1-like dopamine receptor (LOC105205353) transcript XM_011174707.2 (Table 1) was significantly higher in worker than in mated queen brains. Higher dopamine levels have been correlated specifically with subordinate workers in the queenless ant, *Stebllognathus peetersi* (Cuvillier-Hot and Lenoir, 2006), and perhaps has a similar role in workers of *S. invicta*. However, higher dopamine levels in reproductive *S. invicta* virgin queens are associated with higher fertility (Boulay et al., 2001), suggesting the roles of dopamine in *S. invicta* could be caste-dependent. Transcripts for two opsins, ultraviolet-sensitive (LOC105199537) and blue-sensitive (LOC105200910), had significantly higher expression in queens than workers. This difference in visual receptors between the queens and workers parallels the brain morphological differences between the two, with queens having much larger optic lobes than workers. Further, transcripts for one orphan GPCR and four Methuselah 2 receptors were more highly expressed in the worker brain.

3.6. G proteins

In *S. invicta* twelve G protein genes (five Gα, five Gβ, and two Gγ) are annotated, with twenty-four total transcripts predicted (fifteen Gα, seven Gβ, and two Gγ). For comparison, in the model insect, *D. melanogaster*, thirteen G proteins are identified, which include nine α (one of them annotated as a pseudogene), three β, and two γ (Hanlon and Andrew, 2015).

The phylogenetic relationship of the five predicted Gα subunits was investigated (Fig. 5). To better place the *S. invicta* predicted "guanine nucleotide-binding protein subunit alpha homolog" (LOC105200292), the human $G\alpha_{12}$ (Q03113.4) and $G\alpha_{13}$ (Q14344.2) were included in the analysis (Fig. 5). The percentage of similarity of the *S. invicta* Gα proteins and an outgroup is shown in Table S3. Results from our analyses determined *S. invicta* has one gene for each of the typical Gα subunits ($G\alpha_s$, $G\alpha_o$, $G\alpha_i$, $G\alpha_q$) but lacks the atypical $G\alpha_f$ subunit present in *D. melanogaster* and *T. castaneum*. The 'guanine nucleotide-binding protein subunit alpha homolog', XP_025994567.1 (LOC105200292) most likely encodes a $G\alpha_{12/13}$ protein, as it groups in the clade containing the human $G\alpha_{12/13}$ proteins (Fig. 5).

The current annotation of *S. invicta* G proteins, along with their brain gene expression in workers (NCBI GEO Series GSE117432), alate virgin queens, and mated queens (NCBI GEO Series GSE108063), are shown in Table 4. Brain transcriptomic analyses of *S. invicta* queens and workers revealed that of the 24 G protein predicted transcripts NM_001304588.1 (LOC105193280), encoding a putative β subunit, was the most highly expressed in the brain transcriptomes (Table 4). Among α subunits (Fig. 5), transcript XM_026131214.1 (LOC105197512) was the most highly expressed in the brain transcriptomes in both castes (Table 4). Two G proteins were differentially expressed among worker and queen brains, one Gα (LOC105205688, XM_011175158.2) and one Gβ (LOC105201581, XM_011169638.1). In the genome, LOC105197512 is predicted to encode a $G\alpha_q$ protein with ten transcripts including one non-coding transcript: XR_003268273.1.

In summary, the GPCR compilation and improved reannotation (Tables 1, 2 and S1), the phylogenetic analyses of GPCRs (Figs. 2 and 3) and Gα proteins (Fig. 5), and comparative transcriptomics of GPCRs (Table 3) and G proteins (Table 4) in queen and worker brains, constitute a fundamental resource for research in fire ant signaling. This information will support testing hypotheses on the function of GPCRs in this species, other ants, and Hymenoptera broadly, encouraging

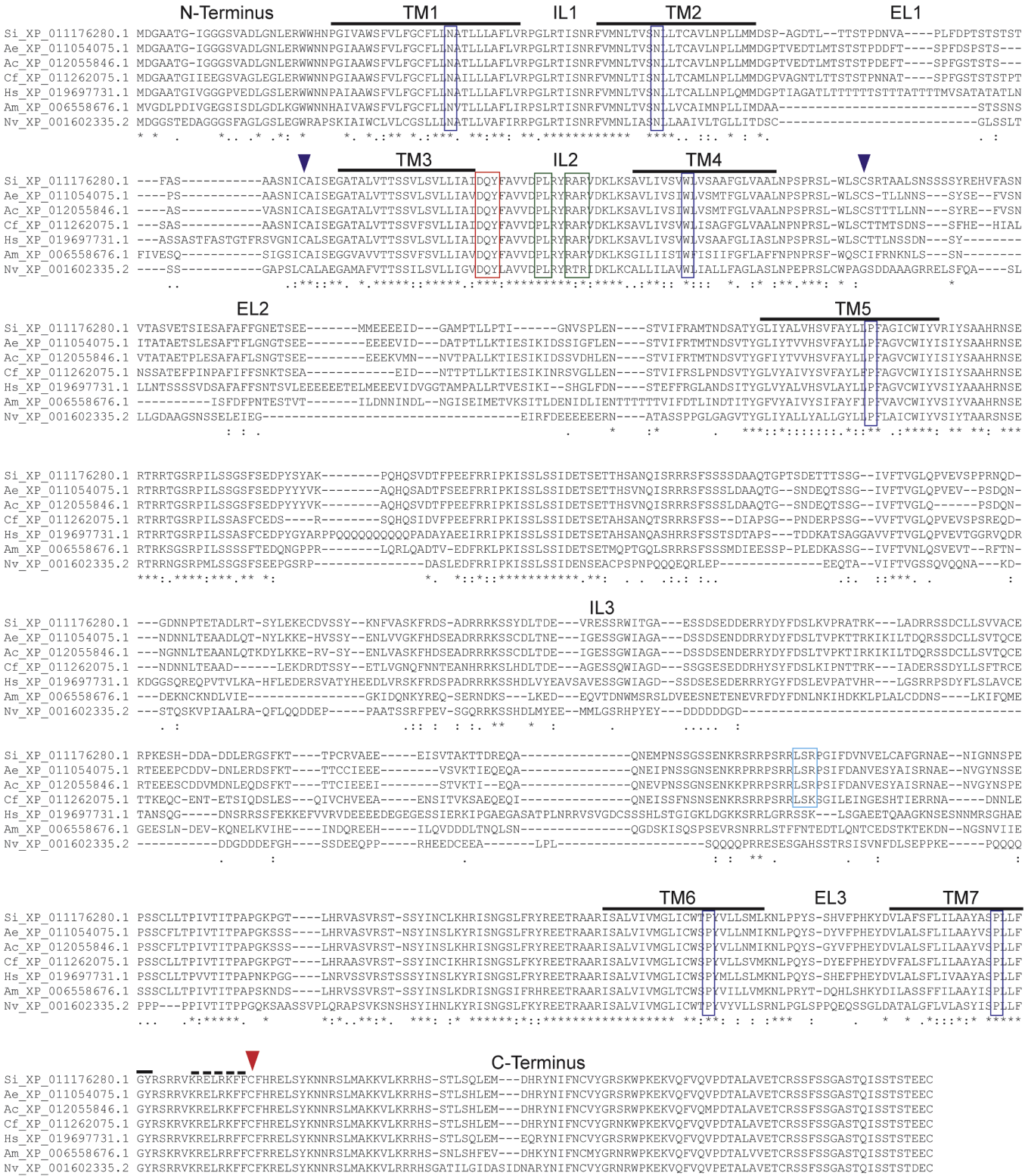


Fig. 4. Alignment of rhodopsin-like, atypical "DQY"-motif containing GPCRs mined from the genomes of hymenopterans. Ants (Si = *Solenopsis invicta*, Ae = *Acromyrmex echinatior*, Ac = *Atta cephalotes*, Cf = *Camponotus floridanus*, Hs = *Harpegnathos saltator*), *Apis mellifera* (Am), and *N. vitripennis* (Nv). Predicted transmembrane regions (TM) are indicated by a solid black line; helix 8 is marked by a dashed black line; the order of intracellular (IL) and extracellular loops (EL) is shown. Cysteines that may form disulfide bonds as in Class A GPCRs are indicated by purple arrowheads. The atypical 'DQY' motif, normally (D/E)RY, at the cytoplasmic extension of TM3 is boxed in red; and the red arrowhead indicates a probable C-terminal palmitoylation site. Indicated below the alignment are: conserved amino acid residues '*'; conservative replacements ':'; and semi-conservative replacements '.'. In dark blue boxes are conserved residues in Rhodopsin-like, Class A GPCRs (Millar and Newton, 2010). Missing residues highly conserved in Class A GPCRs include D in TM2, and a complete conserved NPxxY motif in TM7 (Katritch et al., 2012). Potential motifs for $G\alpha_{q/11}$ coupling (PL and RAR) are boxed in green and those for $G\alpha_s$ are in cyan blue (LSR) (Millar and Newton, 2010). Clustal multiple sequence alignment was performed with the MAFFT tool from the Computational Biology Research Consortium (CBRC) found at mafft.cbrc.jp, with the default settings.

Table 3
Differentially expressed GPCRs among brain transcriptomes of workers, virgin alate queens and dealate mated queens. Letters (a and b) indicate significant differences among genes. NT indicates 'no test'.

LOC	XM	Current annotation	Proposed new annotation	Worker RPKM	AlateRPKM	Mated RPKM
LOC105193512	XR_003268793.1	tyramine receptor 1		19.57[a]	5.24[b]	5.74[b]
LOC105205353	XM_011174707.2	dopamine receptor 2	invertebrate specific D1-like dopamine receptor (InvD1L) (DRY)	31.81[a]	21.25[ab]	18.68[b]
LOC105199400	XM_011166501.2	muscarinic acetylcholine receptor DM1 isoform X1		43.94[a]	22.20[b]	20.74[b]
LOC105199537	XM_026140439.1	opsin, ultraviolet-sensitive isoform X1		0.42[a]	25.25[b]	6.70[ab]
LOC105200910	XM_011168715.2	opsin, blue-sensitive		0.10[a]	13.81[b]	8.53[b]
LOC105203641	XM_026134161.1	probable G-protein coupled receptor B0563.6	probable G protein coupled receptor B0563.6-like	179.59[a]	86.08[b]	136.90[ab]
LOC113005481	XM_026141106.1	G-protein coupled receptor Mth2-like		0.81[a]	0[b]	0.14[ab]
LOC105196494	XM_026132029.1	G-protein coupled receptor Mth2-like		1.51[a]	0.06[ab]	0.08[b]
LOC105202569	XM_011171144.2	G-protein coupled receptor Mth2		1.73[a]	4.23[ab]	5.19[b]
LOC105199776	XM_026140100.1	G-protein coupled receptor Mth2		0.87[a]	0[b]	0[NT]

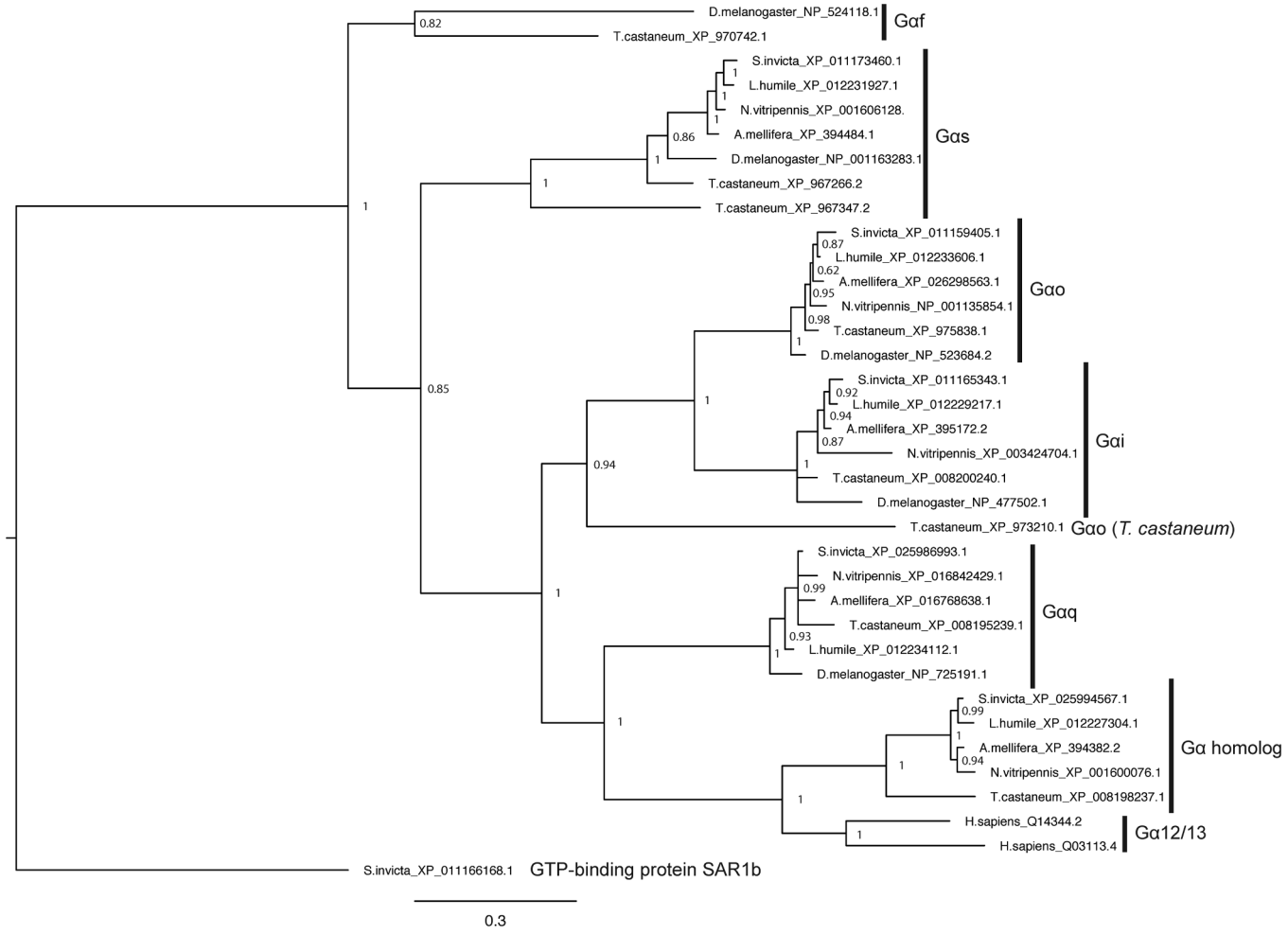


Fig. 5. Phylogeny of Gα proteins in *S. invicta, A. mellifera, T. castaneum, L. humile, D. melanogaster, and N. vitripennis*. Human $G\alpha_{12}$ and $G\alpha_{13}$ were included in the analysis as they were 63% identical to the *S. invicta* "Guanine nucleotide-binding protein subunit alpha homolog" (Gα homolog in the tree). The phylogeny was reconstructed in MrBayes with four chains and four runs of the mixed amino acid model for 1,000,000 generations with a 10% burnin. Node values are Bayesian posterior probability rounded to two significant figures. Scale bar indicates branch length. Clades are labeled based on *D. melanogaster* or *Homo sapiens* ($G\alpha_{12/13}$). *S. invicta* has one gene for each of the typical Gα subunits ($G\alpha_s$: XP_011173460.1, $G\alpha_o$: XP_011159405.1, $G\alpha_i$: XP_011165343.1, $G\alpha_q$: XP_011162230.1) and one Gα homolog (XP_025994567.1) that likely encodes a $G\alpha_{12/13}$. *S. invicta* lacks the atypical $G\alpha_f$ subunit present in *D. melanogaster* and *T. castaneum*. The outgroup protein, *S. invicta* SAR1b (XP_011166168.1), is a member of the ADP-ribosylation factor (ARF) family and guanine nucleotide-binding protein that does not participate in the G protein canonical trimer ($\alpha\beta\gamma$) (Donaldson and Jackson, 2011) .

Table 4
Table of G proteins genes in the *Solenopsis invicta* genome and the expression of the predicted transcripts in the brains of workers, and alate virgin and de-alate mated queens. Different genes alternate between grey and white rows. Letters indicated difference of expression at $q < 0.05$ based on cuffdiff2 analyses. *This gene likely encodes a $G\alpha_{12/13}$ protein (Fig. 5).

Name	LOC	XM	Subunit type	mated	virgin	worker
guanine nucleotide-binding protein G(o) subunit alpha	LOC105195608	XM_011161103.2	α	60.11	57.11	59.03
guanine nucleotide-binding protein G(i) subunit alpha	LOC105199791	XM_011167041.2	α	14.36	15.61	14.03
guanine nucleotide-binding protein G(s) subunit alpha	LOC105205688	XM_011175158.2	α	**5.44[a]**	**5.83[a]**	**13.25[b]**
guanine nucleotide-binding protein G(q) subunit alpha isoform X1	LOC105197512	XM_026131208.1	α	1.81	0.97	0
guanine nucleotide-binding protein G(q) subunit alpha isoform X2	LOC105197512	XM_026131209.1	α	0.92	2.24	0
guanine nucleotide-binding protein G(q) subunit alpha isoform X3	LOC105197512	XM_026131210.1	α	2.93	3.97	0.87
guanine nucleotide-binding protein G(q) subunit alpha isoform X4	LOC105197512	XM_026131211.1	α	5.43	5.36	0.16
guanine nucleotide-binding protein G(q) subunit alpha isoform X5	LOC105197512	XM_026131212.1	α	23.42	22.16	9.27
guanine nucleotide-binding protein G(q) subunit alpha isoform X6	LOC105197512	XM_026131213.1	α	9.27	5.71	1.88
guanine nucleotide-binding protein G(q) subunit alpha isoform X7	LOC105197512	XM_026131214.1	α	31.91	23.10	52.80
guanine nucleotide-binding protein G(q) subunit alpha isoform X8	LOC105197512	XM_026131215.1	α	0	0	0.39
guanine nucleotide-binding protein G(q) subunit alpha isoform X9	LOC105197512	XM_026131216.1	α	0.09	0	0.03
guanine nucleotide-binding protein G(q) subunit alpha	LOC105197512	XR_003268273.1	α	11.86	13.78	1.52
guanine nucleotide-binding protein subunit alpha homolog isoform X1	LOC105200292*	XM_026138782.1	α	1.90	1.65	1.89
guanine nucleotide-binding protein subunit alpha homolog isoform X2	LOC105200292*	XM_026138783.1	α	0.04	0.12	0.45
guanine nucleotide-binding protein subunit beta-like protein	LOC105193280	NM_001304588.1	β	772.96	1007.80	571.50
guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	LOC105196793	XM_011162905.2	β	13.74	13.61	20.61
guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	LOC105196793	XM_011162906.2	β	10.41	11.63	17.58
guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	LOC105196793	XM_011162907.2	β	8.53	8.10	13.80
guanine nucleotide-binding protein subunit beta-2	LOC105201581	XM_011169638.2	β	**85.37[a]**	**120.97[a]**	**10.93[b]**
guanine nucleotide-binding protein subunit beta-5	LOC105201804	XM_011170008.2	β	46.93	44.67	39.87
guanine nucleotide-binding protein subunit beta-like protein 1	LOC105202002	XM_026132429.1	β	12.88	15.30	15.30
guanine nucleotide-binding protein subunit gamma-1	LOC105195725	XM_011161278.2	γ	85.60	92.49	79.15
guanine nucleotide-binding protein subunit gamma-e	LOC105205228	XM_011174531.2	γ	195.30	245.25	294.99

comparative studies. At least 15 GPCRs are still orphan (Table 2), underscoring the need for additional functional receptor deorphanization and endocrinological studies in this species.

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Appendix A. Supplementary data

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