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Expression of odorant co-receptor Orco in tissues and development stages of *Glossina morsitans morsitans, Glossina fuscipies fuscipies* and *Glossina pallidipies*

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ABSTRACT

Tsetse flies (*Glossina*) depend on their olfactory system to identify host, larviposition sites and mates. Previous studies have characterized olfactory organs and evaluated the odorant receptor (OR) expression to host-derived chemicals. However, no studies thus far have investigated the odorant co-receptor (Orco) associated with these olfaction processes in tissues and developmental stages in tsetse flies. In this study, quantitative polymerase chain reaction was done with *Glossina morsitans morsitans, Glossina pallidipies* and *Glossina fuscipes fuscipes* tissues (antennae and legs) and developmental stage (larvae and pupae) to quantify Orco expression levels and *G. m. morsitans* OR genes in *G. m. morsitans* larvae and pupae. Our findings indicate that expression of Orco were elevated in *G. f. fuscipes* male antennae and legs relative to *G. f. fuscipes* female antennae and relative to male antennae in *G. m. morsitans* and *G. pallidipies.* However, in female *G. m. morsitans* antennae, expression of Orco was higher than in female *G. pallidipies* and *G. f. fuscipes*. Orco levels were also significantly higher in pupal stages of *G. f. fuscipes* and *G. pallidipies* relative to the three larval stages and *G. m. morsitans* pupae. Two *G. m. morsitans* OR genes (GmmOR20 and GmmOR28) were highly expressed in *G. m. morsitans* larvae and pupae respectively. This study demonstrate that the Orco gene was significantly expressed in adult male and female tissues as well as in the developmental stages of *Glossina* species. The *G. m. morsitans* OR genes reported in larvae and pupae could mean co-expression of Orco and ORs in developmental stages. These findings pinpointed the olfactory differences that exists amongst *G. m. morsitans, G. pallidipies* and *G. f. fuscipes*, and provide valuable information to the probable role of olfactory genes in *Glossina* contributing further to the understanding of olfactory processes and the evolution of olfactory genes in tsetse flies.

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Introduction

Tsetse flies (*Glossina*) are vectors of trypanosomes that cause Human African Trypanosomiasis (HAT) and African Animal Trypanosomiasis (AAT) [1,8]. *Glossina morsitans morsitans* and *Glossina pallidipies* are vectors of HAT and AAT while *Glossina fuscipies fuscipies* are vectors of HAT [7]. Olfaction plays a critical role in tsetse biology including habitat seeking, selecting larviposition sites, finding mates and host specifity [46]. Olfactory proteins identified in tsetse species include soluble proteins (odorant binding proteins (OBPs), pheromone binding proteins (PBPs), chemosensory proteins (CSPs), odorant degrading enzymes (ODEs) [24,25,28], receptor proteins (odorant receptors (ORs), gustatory receptors (GRs) and ionotropic receptors (IRs) [28,36]. It is postulated that odors are bound by OBPs and transported in the sensillium to ORs located within the odorant receptor neuron (ORN) where the signal is detected and transferred to higher brain centers for processing [21,37].

Odorant binding proteins were reported to be highly transcribed in female antennae of *G. m. morsitans* and affected by the insect's nutritional status [25] while expression of CSPs in *G. m. morsitans* was linked to female host seeking behavior [24]. Obiero et al. [36] reported six ORs to be associated with mating deterrence in female *G. m. morsitans* and observed that the overal reduction in chemoreceptor repertoire could be linked to the restricted hematophagous diet of tsetse flies. Genomic comparision of chemosensory genes in five tsetse species revealed that *Glossina* genes are dispersed across distantly located scaffolds unlike in *Drosophila melanogaster* where the genes occur in clusters, devoid of sugar receptors and have expanded carbon dioxide associated receptors [28]. Expression analysis of OR genes in *G. m. morsitans* reported that GmmOR33 and GmmOR45 were highly expressed in female and male antennae, respectively, while GmmOR26 and GmmOR34 were high in female and male legs, respectively. The findings identified sex- and tissue-specific *G. m. morsitans* ORs and confirmed that OR gene expression could be conserved in function, with the antenna being the main olfactory organ [35].

Insect ORs are odorant-gated nonselective cation channels that are highly divergent across different insect species [4,32,40]. However, there exists a specialised group of ORs called odorant co-receptor (Orco) that is found co-expressed with all other ORs [19]. Orco sequence is highly conserved across phylogenetically distant insect species [13,15,22] and is probably involved in formation of functional receptor-odor complexes [12,14] and efficient localisation of the odorant-OR complex onto dendrites of the ORN to initiate signal transduction [33].

Recent studies have reported expression and conservation of Orco in olfactory and non-olfactory tissues of insects across different orders [13,39,48]. Here, we report expression of Orco in tissues (antennae and legs) and developmental stages (larvae and pupae) of *G. m. morsitans, G. pallidipies* and *G. f. fuscipies* and expression of *G. m. morsitans* OR genes in *G. m. morsitans* larvae and pupae.

Materials and methods

Bioinformatics analysis of odorant receptor co-receptor (Orco)

Glossina m. morsitans Orco (GMOY00561) identified by [36], was used to search for *G. pallidipies* and *G. f. fuscipies* orthologs in VectorBase (VB-2014-04) (https://www.vectorbase.org/) [30]. The *G. m. morsitans* Orco was also used to query OrthoMCL (v.5) (http://orthomcl.org/orthomcl/) [10] for corresponding Orco orthologs. Conserved amino acid domain in *Glossina* Orco (*G. m. morsitans, G. pallidipies* and *G. f. fuscipies*) and Orco orthologs identified in other insects were determined by blast search in NCBI CDD database (v.3.15) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) [6,29]. Multiple sequence alignment of the *Glossina* Orco and identified Orco orthologs was performed using T Coffee (http://tcoffee.crg.cat/) [9]. Transmembrane domains in the Orco orthologs was predicted using TMHMM server (v.2.0) (http://www.cbs.dtu.dk/services/TMHMM/) [17]. The graphical data was then compared to T Coffee aligned sequences to give a visual representation of the actual position of the transmembrane domains. Protein motifs were then determined by submitting *Glossina* Orco protein sequences (for *G. m. morsitans G. pallidipies* and *G. f. fuscipies*) to the motif elicitation tool MEME Suite (v.4.10.2) (http://meme-suite.org/tools/meme) [3] with parameters set as '0-order model of sequences, with minimum and maximum width of motifs being set at 5 and 10 respectively, as well as minimum and maximum sites for motif search set at 2 and 5 respectively.

Insect preparation and tissues dissection

Glossina f. fuscipies, G. m. morsitans and *G. pallidipies* were obtained from Biotechnology Research Centre (BRC) of the Kenya Agricultural and Livestock Research Organization (KALRO) - Muguga Station in March 2016. The flies were reared under standard laboratory conditions (temperature 25 ± 1 °C; relative humidity 75 ± 10% and fed on sterilized pig blood every 24 h, at 10 a.m., using an artificial membrane) in the insectary at the BRC-KALRO [31]. Twelve day old male and female adult flies were collected 10 h after blood meal. The larvae were collected upon larviposition and immediately frozen in liquid nitrogen while pupae were collected four hours after larviposition and frozen in liquid nitrogen. Adult male and female *G. f. fuscipies, G. m. morsitans* and *G. pallidipies* flies were initially immobilized by placing them at 4 °C for about a minute and then placed in a petri-plate on ice. The antennae and legs were then cut off from immobilized *Glossina* fly placed under a 10x dissecting microscope (Optika ST-30-2LF) and immediately frozen in liquid nitrogen, and stored in a 1.5 ml Eppendorf tube at −20 °C until RNA extraction. The process was performed for all the samples within 24 h after dissection.

Extraction of total RNA and cDNA synthesis

Total RNA was extracted from 100 antennae and 100 legs dissected from adult male and females tsetse flies separately, two larvae and two pupae from each *Glossina* species (*G. f. fuscipies, G. m. morsitans* and *G. pallidipies*) using TRIzol reagent (Invitrogen, Carlsbad CA, USA) according to the manufacturer's protocol. The genomic DNA was digested with RNase free-DNAse 1 (Fermentas, Life sciences, USA), and the purity and integrity of the RNA obtained checked using a NanoDrop 2000 spectrophotometer and 1.2% formaldehyde agarose gel electrophoresis. cDNA templates were prepared from purified RNA samples (20 ng/µL) of male and female antenna and legs using RevertAid First Strand cDNA synthesis kit (Fermentas, Thermoscientific, UK) according to the manufacturer's protocol. 20 ng/µl template RNA followed by 0.25 µM Oligo $(dT)_{18}$ primer were put in a micro centrifuge tube, nuclease free water added to bring the volume to 12 µl. This was mixed gently, spun down and incubated at 65 °C for 5 min. The tubes were cooled on ice for 1 min and final concentrations of the following reagents were added; 1 × reaction buffer (250 mM Tris-HCL pH 8.3, 250 mM KCl, 20 mM $MgCl_2$, 50 mM Dithiothreitol), 1 U/µl Ribolock RNAse inhibitor, 1 mM dNTP Mix and 10 U/µl RevertAid M-MULV Reverse Transcriptase. The reaction mixture was spun briefly and incubated at 42 °C for 60 min, followed by termination at 70 °C for 5 min and finally chilled on ice.

Reverse transcription PCR (RT-PCR)

Primers were designed for *Glossina* Orco (*G. m. morsitans, G. pallidipies* and *G. f. fuscipies*), *G. m. morsitans* actin sequence (GMOY001776, retrieved from VectorBase, VB-2014-04) (https://www.vectorbase.org/) and 46 *G. m. morsitans* OR genes [36] using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) [45]. The primer sequence length was set at a range of 18 to 20 bp with a temperature range of 55 °C to 58 °C. The GC content was set at 20% with the product size range of 150–200 bp. The designed primers were synthesized by Inqaba Biotec, South Africa (Supplementary Tables 1 and 2). The integrity of *G. m. morsitans, G. pallidipies* and *G. f. fuscipies* antennae, leg, larvae and pupae cDNA was validated through RT-PCR amplification with reference gene *G. m. morsitans* actin primers in an ABI GeneAMP 9700 (Applied Biosystem). The Orco primers were used to amplify *G. m. morsitans, G. pallidipies* and *G. f. fuscipies* antennae, leg, larvae and pupae cDNA while the 46 *G. m. morsitans* OR primers were used to amplify *G. m. morsitans* larvae and pupae cDNA. The RT-PCR was done in a total volume of 20 µl containing: 6 µl nuclease free water, 10 µl × 2 Dream *Taq* master mix, and 1 µl (10 mM) each of the forward and reverse primers and 2 µl (20 ng/µl) of cDNA template, with cycling conditions set at 95 °C for 5 min for initial denaturation, followed by 35 cycles of subsequent denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 1 min followed by final extension at 72 °C for 7 min. The amplified RT-PCR products were analysed on a 1.5% agarose gel.

Quantitative real time polymerase chain reaction (qPCR)

Amplified *G. m. morsitans, G. pallidipies* and *G. f. fuscipies* Orco and *G. m. morsitans* OR genes were further quantified by qPCR using a Stratagene Mx3000P qPCR system (Agilent Technologies UK Ltd, Cheshire, UK). The samples were run in triplicate with *G. m. morsitans* actin for normalization of the template cDNA. The PCR efficiency of the gene was initially validated before gene expression analysis. Reaction volumes of 10µl containing 1 × Maxima SYBR Green/ROX master mix (Fermentas, Thermoscientific, Lithuania), 2 ng/µl of cDNA template, 0.3 mM of each primer and nuclease free water were prepared. Thermocycling conditions were set at one cycle of 95 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s. Data acquisition was performed during the extension step.

Gene expression analysis

Gene expression levels were calculated by delta delta Ct (ddCt) method. The dataset for Ct for the Orco and ORs with *G. m. morsitans* actin were initially log transformed using the formula log10 (ddCt + 0.001) to normalize the variation in the amount of cDNA in each reaction [26]. Due to the absence of specific standardized statistical methods [18] to calculate the differential expression interpretation of the biological significance, the mean and variance of the expressed genes were statistically calculated. To this end, a well-reviewed DESeq2 statistical [41] analysis package for Gene Expression (GE) analysis available as part of the Bioconductor suite on the R platform [27] was used. In DESeq2, the means and variances were calculated using binomial distribution model and considered significantly expressed at P-value < 0.05.

Phylogenetic tree analysis

Phylogenetic relationships of *Glossina* Orco (*G. m. morsitans, G. pallidipies,* and *G. f. fuscipes*) with selected Orco othologs from other insects (*D. melanogaster, An. gambiae, Ae. aegypti, B. mori, A. mellifera,* L. *migratoria, S. gregaria, T. castaneum* and *O. nubilalis*) was generated by MEGA (v.6.06) using Maximum Likelihood method with bootstrap value of 1000 iterations and Jones-Taylor-Thornton (JTT) substitution model [44].

Table 1
Glossina morsitans morsitans, Glossina fuscipes fuscipes and Glossina pallidipies Orco orthologs in OrthoMCL.

Glossina Orco (Accession No)	AA Length	Exons	TMDs	Best OrthoMCL Hit	Accession No	E Value	% Identity
G. m. morsitans (GMOY005610)	591	4	7	*Drosophila melanogaster*	FBpp0112105	0.0	N/A
G. f. fuscipies (GFUI035140-PA)	463	3	7	*Drosophila melanogaster*	FBpp0112105	0.0	97.5
G. pallidipies (GPAI035133-PA)	477	3	7	*Drosophila melanogaster*	FBpp0112105	0.0	95.7

Orco orthologs identified for *G. m. morsitans, G. f. fuscipes* and *G. pallidipies* from OrthoMCL database. AA length—Amino Acid length; TMDs – Transmembrane domain; E Value—Expect value; % Identity—Percentage Identity.

Table 2
BlastX match of Orco gene from different insect orders.

Query	Hit type	PSSM-ID	From	To	E-Value	Bitscore	Accession	Short name
AmelOrco	Superfamily	251,636	67	463	2.14503e-30	119.314	cl20237	7tm_6 superfamily
BmorOrco	Superfamily	251,636	70	457	9.52449e-38	140.5	cl20237	7tm_6 superfamily
OnubOrco	Superfamily	251,636	136	405	1.14517e-13	70.0086	cl20237	7tm_6 superfamily
DmelOrco	Specific	251,636	70	472	1.19832e-50	176.324	pfam02949	7tm_6
	superfamily	251,636	70	472	1.19832e-50	176.324	cl20237	7tm_6 superfamily
AgambOrco	superfamily	251,636	68	464	7.75819e-43	154.753	cl20237	7tm_6 superfamily
AaegOrco	superfamily	251,636	68	464	8.53781e-44	157.449	cl20237	7tm_6 superfamily
LmigOrco	superfamily	251,636	63	445	5.1345e-23	97.743	cl20237	7tm_6 superfamily
SgregOrco	superfamily	251,636	64	450	4.20565e-21	92.3502	cl20237	7tm_6 superfamily
GmmOrco	superfamily	251,636	70	447	7.71465e-32	124.707	cl20237	7tm_6 superfamily
TcasOrco	superfamily	251,636	12	282	3.13709e-20	87.3426	cl20237	7tm_6 superfamily

Orco from different insect orders showing areas of conservation and belonging to the 7 transmembrane protein superfamily in NCBI CDD (conserved domain database). The abbreviations represent the different insects used and their accession numbers. i.e AmelOrco: *Apis mellifera* (AOAO87ZPQ3), BmorOrco: *Bombyx mori* (H9JU00), OnubOrco: *Ostrinia nubilalis* (D2KWR3), DmelOrco: *Drosophila melanogaster* (Q9VNB5), AgambOrco: *Anopheles gambiae* (Q7QCC7), AaegOrco: *Aedes aegypti* (Q178U6), LmigOrco: *Locusta migratoria* (H2ELT0), SgregOrco: *Schistocerca gregaria* (H2ELT1), GmmOrco: *G. m. morsitans* (GMOY005610) and TcasOrco: *Tribolium castaneum* (C0Z3Q6).

Results

Orco gene identification

Glossina m. morsitans Orco gene retrieved from VectorBase had 197 bp, 4 exons with 7 transmembrane domains. *Glossina. f. fuscipies* and *Glossina pallidipies* Orco had 154 bp and 159 bp respectively with seven transmembrane domains and 3 exons. The three *Glossina* Orco all had *Drosophila melanogaster* Orco (FBpp0112105) as the best ortholog match with high amino acid identities (Table 1). Blast search of *Glossina m. morsitans* Orco orthologs identified similar sequences from *Drosophila melanogaster, Anopheles gambiae, Aedes aegypti, Bombyx mori, Apis mellifera, Locusta migratoria, Schistocerca gregaria, Ostrinia nubilalis* and *Tribolium castaneum* that belonged to the 7 transmembrane protein superfamily (Table 2). Multiple sequence alignment of *G. m. morsitans, G. f. fuscipes* and *G. pallidipies* Orco with Orco orthologs from *Drosophila melanogaster, Anopheles gambiae, Aedes aegypti, Bombyx mori, Apis mellifera, Locusta migratoria, Schistocerca gregaria, Ostrinia nubilalis* and *Tribolium castaneum*, revealed a highly conserved amino acid in the C-terminus (Fig. 1).

Motif search and phylogenetic alignment

Protein motifs search revealed that the most abundant amino acids were Glutamine (Q), Serine (S) and Cysteine (C) (Fig. 2). An alignment of the protein motifs by MAST tool in MEME suite revealed the most abundant and frequent amino acid residues to be Glutamine (Q), Serine (S) and Cysteine (C) (Fig. 2).

The phylogenetic analysis revealed that the *Orco* gene in tsetse fly, fruit fly and mosquitoes were closely related while silk moth, honey bee, locusts, red flour beetle and European corn occurred along different nodes (Fig. 3).

Orco expression in tissues and developmental stages

Quantitative-PCR (qPCR) was used to determine Orco expression in tissues (antennae and legs) and developmental stages (larvae and pupae) of *G. m. morsitans, G. f. fuscipes* and *G. pallidipies* and *G. m. morsitans* ORs expression in *G. m. morsitans* larvae and pupae. Overall, Orco was highly expressed in the female antennae than male antennae, while in the legs, expression of Orco was high in the male than female legs. In the males, higher expression of Orco was seen in *G. f. fuscipes* antennae while expression in the *G. pallidipies* and *G. m. morsitans* antennae was similar (Fig. 4A). Among the females, higher Orco expression was observed in *G. m. morsitans* antennae while expression in *G. f. fuscipes* and *G. pallidipies* antennae was relatively low (Fig. 4A).

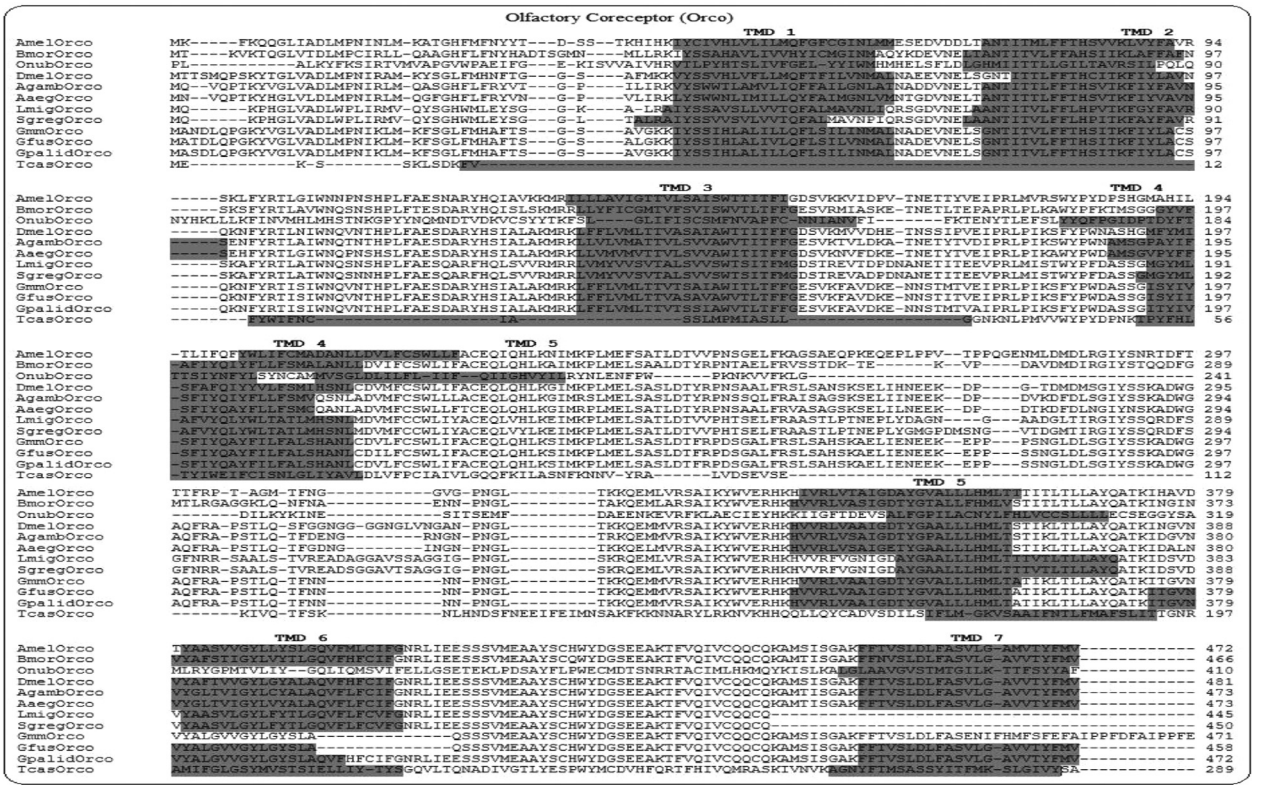

Fig. 1. Alignment of Orco protein of different insect species with transmembrane domains (TMD) indicated with shaded grey shade. The numbers to the right show the position of the last amino acid residue in the alignment. The abbreviations at the left represent the different insects used and their accession numbers. i.e Dmel: *Drosophila melanogaster* (Q9VNB5), Gmm: *G. m. morsitans* (GMOY005610), Gfus: *Glossina f. fuscipes* (GFUI035140-PA), Gpal: *Glossina pallidipies* (GPAI035133-PA), Agamb: *Anopheles gambiae* (Q7QCC7), Aaeg: *Aedes aegypti* (Q178U6), Bmor: *Bombyx mori* (H9JU00), Amel: *Apis mellifera* (AOAO87ZPQ3), Lmig: *Locusta migratoria* (H2ELT0), Sgreg: *Schistocerca gregaria* (H2ELT1), Onub: *Ostrinia nubilalis* (D2KWR3), Tcas: *Tribolium castaneum* (C0Z3Q6).

Among the males, a higher expression was reported in *G. f. fuscipes* legs and low expression observed in *G. pallidipies* and *G. m. morsitans* legs. There was no significant difference in expression of Orco among the female *G. f. fuscipes, G. pallidipies* and *G. m. morsitans* legs (Fig. 4A).

The developmental stages had a higher Orco expression in pupae than the larvae (Fig. 4B). The expression levels in the larvae of *G. m. morsitans, G. f. fuscipes* and *G. pallidipies* wasn't significantly different. However, Orco expression in *G. f. fuscipes* and *G. pallidipies* pupae was significantly enriched than in *G. m. morsitans* pupae (Fig. 4B).

Of the 46 *G. m. morsitans* OR genes, Gmm OR20 was highly amplified in *G. m. morsitans* larvae (Fig. 5A) while Gmm OR28 was highly expressed in *G. m. morsitans* pupae (Fig. 5B). This verified that the putative *G. m. morsitans* ORs were indeed differentially expressed in *G. m. morsitans* developmental stages (larvae and pupae).

Discussion

In this study we established that Orco in the three tsetse species (*G. m. morsitans, G. f. fuscipes* and *G. pallidipies*) had amino acid within the range of 463 to 591 residues and seven transmembrane domain. Similar results were reported in *Cimex lectularius* Orco gene which consisted of 451 amino acids with the predicted seven transmembrane domain in other insects [11,13,38,48]. The *G. m. morsitans* Orco had 120aa to 130aa larger than *G. f. fuscipes* and *G. pallidipies* Orco which could result in longer amino or carboxyl terminus that results in larger sizes of the intracellular/extracellular loops [14]. Our analysis indicate that *Orco* gene had different number of exons in *G. m. morsitans, G. f. fuscipes* and *G. pallidipies* Orco when compared to eight identified in *C. lectularius* Orco [13], eight and six identified in *An. gambiae* and *D. melanogaster* respectively [38]. Genomic coordinates of *Glossina* genes revealed that they are dispersed across different scaffolds while in *D. melanogaster* and other insects, these genes occur in clusters [20,28,42].

The highly similar *Glossina* Orco sequences is in agreement with previous data that insect Orco have at least 50% orthologous conservation, unlike other insect ORs which do not show sequence conservation [15,47]. Multiple sequence alignment of the different Orco orthologs revealed that sequence conservation and clustering of the transmembrane domains was biased towards the extracellular C-terminus as reported in other insects [13,22,33,48]. There is no literature explaining this

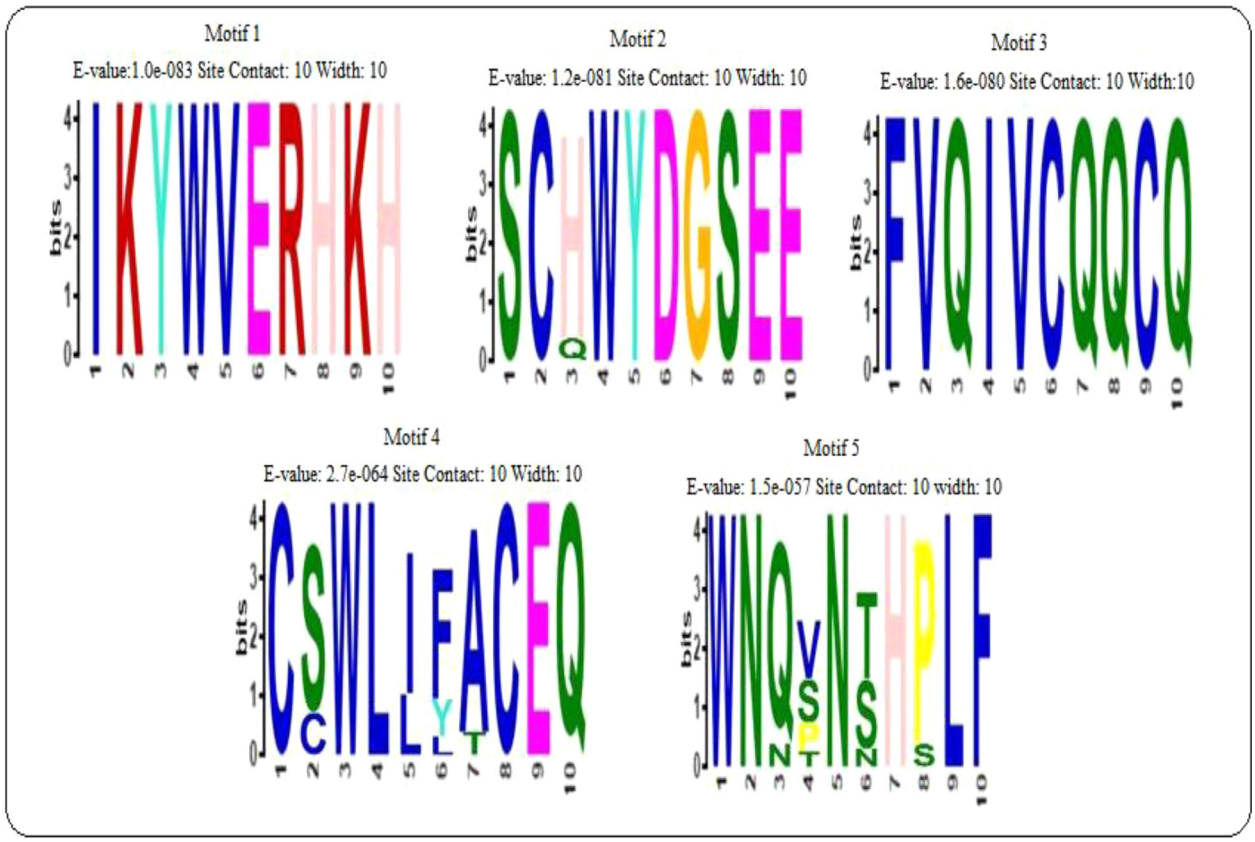

Fig. 2. Protein motif profile of *Glossina* Orco protein sequences (for *G. m. morsitans G. pallidipies* and *G. f. fuscipies*) showing the most abundant amino acid. From the four motifs (motif 1, motif 2, motif 3, motif 4 and motif 5) the most abundant and frequent amino acid residues are Glutamine (Q), Serine (S) and Cysteine (C).

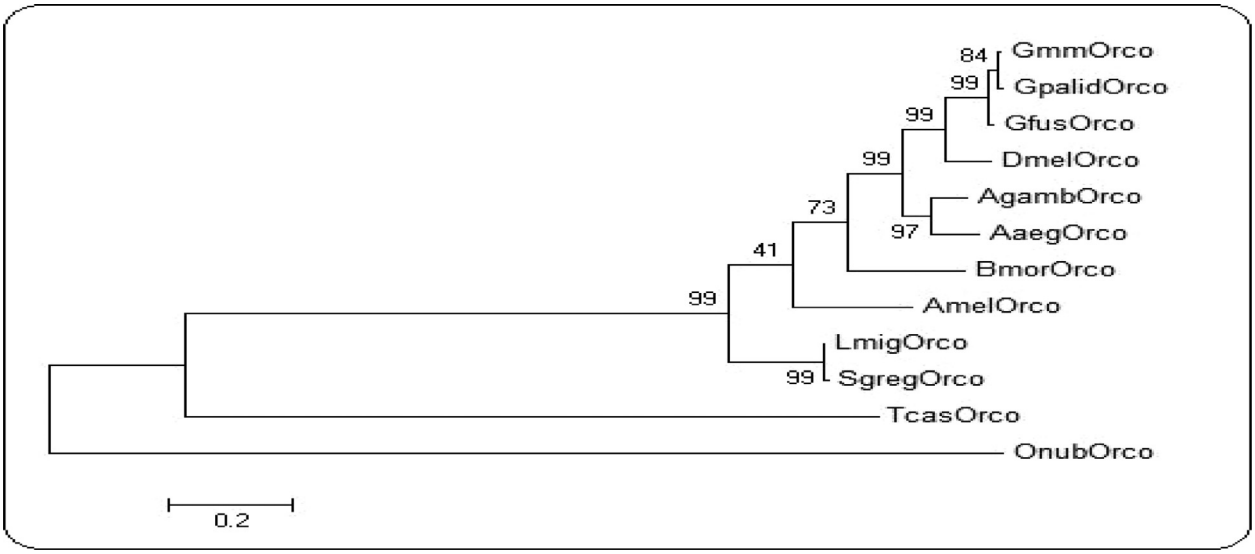

Fig. 3. Phylogenetic relationships of *Glossina* Orco with selected Orco othologs from other insects. The tree was generated by MEGA using Maximum likelihood method and Jones-Taylor-Thornton (JTT) substitution model. Reliability of the nodes was evaluated by 1000 bootstrap replicates. The codes represent: GmmOrco – *Glossina morsitans morsitans* Orco, GpalidOrco - *Glossina pallidipies* Orco, GfusOrco - *Glossina fuscipes fuscipes* Orco, DmelOrco - *Drosophila melanogaster* Orco, AgambOrco - *Anopheles gambiae* Orco, AaegOrco - *Aedes aegypti* Orco, BmorOrco - *Bombyx mori* Orco, AmelOrco - *Apis mellifera* Orco, LmigOrco - *Locusta migratoria* Orco, SgregOrco - *Schistocerca gregaria* Orco, TcasOrco - *Tribolium castaneum* Orco, OnubOrco - *Ostrinia nubilalis* Orco.

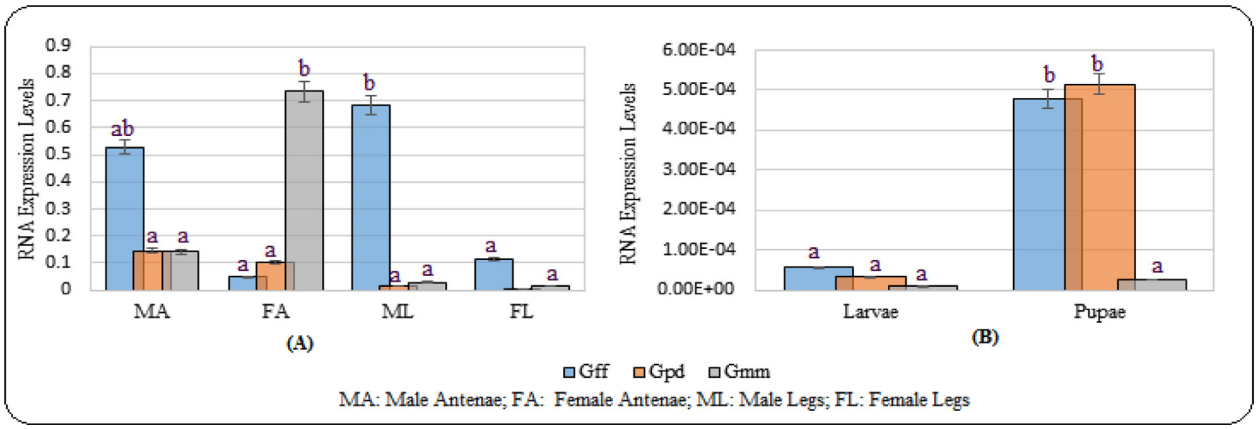

Fig. 4. The quantitative-PCR (qPCR) expression for Orco in (A) tissues (antennae and legs) and (B) developmental stage (larvae and pupae) of three *Glossina* species (Gff - *Glossina fuscipes fuscipes;* Gpd - *Glossina pallidipies* and Gmm - *Glossina morsitans morsitans*). Expression values were normalized with the control expression data from the same samples. Tukey-Kramer HSD ($p < 0.05$) was used to separate the mean. The means with similar letter are not significantly different. Letters a, b, and ab are difference in significance with reference to control where a: significantly low expression; b: significantly high expression and ab: expression significantly not different with control.

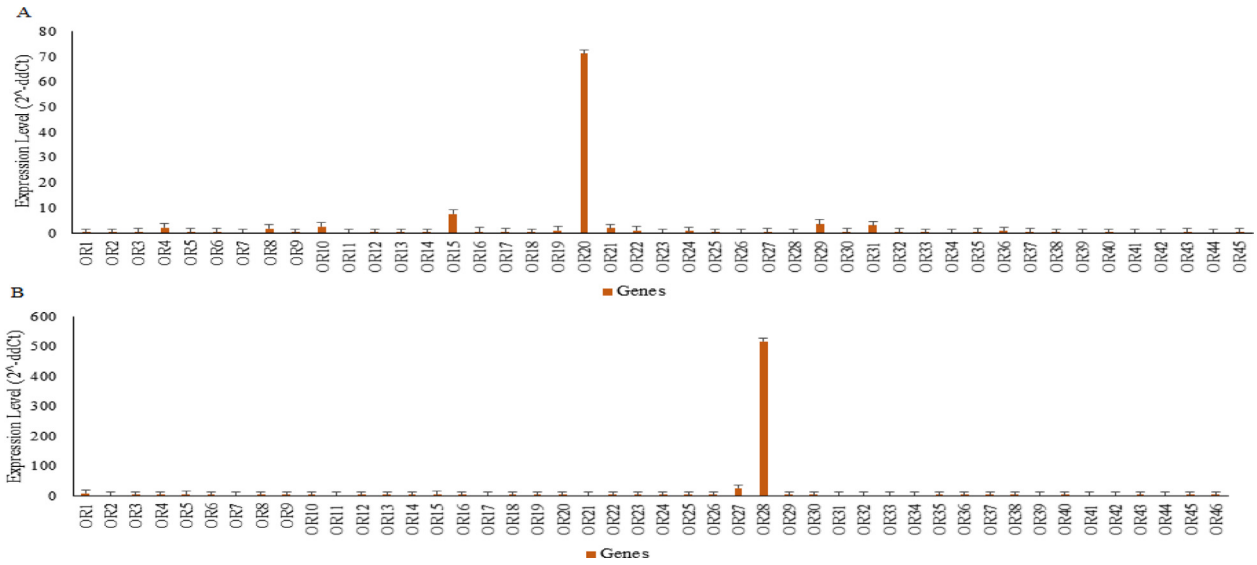

Fig. 5. Quantitative PCR (q-PCR) analysis of *Glossina morsitans morsitans* OR genes expression levels in larvae (A) and pupae (B). The expression level of *G. m. morsitans* OR gene in both larvae and pupae was calculated by ddCt method in which the discrepancy between the Ct for the Gmm OR gene and internal control was first calculated to normalize the variation in the amount of cDNA in each reaction [26].

phenomenon but we believe that this is possibly because the N-terminus is mainly used to anchor the gene to the cell membrane, while the extracellular C-terminus is involved in formation of functional receptor-odor complexes to initiate a signal trasduction process that ultimately results in a behavioral response [11,14].

The most abundant amino acids among the motifs were found to be Glutamine (Q), Serine (S) and Cysteine (C), all being polar uncharged amino acids. Similar studies in *D. melanogaster* and *B. mori* reported that the most conserved amino acid residues were Glutamine (Q), Asparagine (N) (polar uncharged amino acids) and Tyrosine (Y) which they believed were responsible for correct OR-Orco cation channel functionality [2,33].

Phylogenetic relationship of *Glossina* Orco revealed that the *D. melanogaster* were the closest relatives followed by mosquitoes. This is supported by evolutionary studies, which indicate that Diptera split into two lineages about 260 million years ago, i.e. Brachycera (which includes both *Glossina* and *Drosophila*) and Nematocera (which includes mosquitoes) [25]. The phylogenetic relationship of Orco in this study is in concurrence with insect phylogenetic relationships that separates the insects into respective orders [48,49].

The higher expression profile of Orco in antennae than in the legs, confirms that the antennae is the main olfactory organ in insects [21]. However, varied expression levels of Orco between the female and male tissues (antennae and legs) and the different *Glossina* species could provide a molecular basis for potential biological and behavioral differences in the

olfactory responses of the three tsetse species. Orco expression in locust was reported to be the same in both male and female antennae and attributed to similarity in sensilla types and numbers between the sexes [48] while in bed bugs Orco expression was low in male antennae than female antennae [13].

In this study, Orco expression in male *Glossina* legs was significantly higher than in the female legs. Orco expression in male legs is consistent with other studies that reported similar patterns in male bed bugs legs [13] and mosquitoes legs [23,42]. The ubiquitous expression of Orco transcripts have been reported in antennae, mouthparts, tarsi and wings of L. *migratoria* and *S. gregaria* [48].

The low expression of Orco in *Glossina* larvae is similar to studies that reported low expression of Orco in developmental stages and other non-olfactory organs [16,20,43]. The high expression of Orco in *Glossina* pupae could suggests development of olfactory system in the resting stage.

Expression of *G. m. morsitans* OR genes in developmental stages showed that GmmOR20 and GmmOR28 were highly expressed in larvae and pupae respectively. GmmOR20 and GmmOR28 were reported to be highly expressed in male *G. m. morstians* legs and not significantly expressed in female *G. m. morstians* antennae and legs and male *G. m. morstians* antennae [35]. The ORs detected in larvae and pupae (GmmOR20 and GmmOR28) are most likely not conserved in adult male and female *G. m. morstians* and their detection could indicate development of olfactory system in the different growing stages. In insects, Orco form heterodimeric complexes with OR protein (OrX) [5,34]. Given the previous characterization and expression of OR genes in *G. m. morsitans* antennae, legs [35], larvae and pupae (reported in this study), we speculate that Orco and ORs are expressed ubiquitously in different tsetse tissues (antennae and legs) and developmental stages (larvae and pupae). The specific functions, whether olfactory or non-olfactory need to be investigated further.

Conclusion

The olfactory system is critical for the survival of *Glossina* as olfactory cues are sensed through activation of olfactory proteins localized on the antennae. Orco plays an important role in localization of ORs within dendrite membranes of ORNs, and its expression in *Glossina* antennae, legs, larvae and pupae could imply its diverse role in tsetse survival. Orco transcripts were elevated in *G. f. fuscipies* male antennae relative to *G. f. fuscipies* female antenna. In contrast, Orco was elevated in female *G. m. morsitans* antennae relative to males antennae. Orco levels were also significantly higher in pupal stages of *G. f. fuscipies* and *G. pallidipes* relative to the larval stages in the three *Glossina* species. Comparative analysis of Orco in *G. m. morsitans, G. pallidipies* and *G. f. fuscipies* could pinpoint the conserved role of Orco and ORs in signal transduction. It is evident that alignment of *Glossina* Orco with its orthologs revealed a highly conserved amino acid sequences that were phylogenetic related.

Author contributions

Albert Ondimu Moindi, Cyrus Tare, Peter Juma Ochieng, Fred Wamunyokoli and Steven Ger Nyanjom conceived and designed the experiments, performed the experiments and analysed the data; prepared and wrote the manuscript.

Conflict of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sciaf.2018.e00011.

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