RESEARCH NOTE

REVISED Comparative genomics identifies male accessory gland proteins in five *Glossina* species [version 2; referees: 2 approved]

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Abstract

Accessory gland proteins (ACPs) are important reproductive proteins produced by the male accessory glands (MAGs) of most insect species. These proteins are essential for male insect fertility, and are transferred alongside semen to females during copulation. ACPs are poorly characterized in *Glossina* species (tsetse fly), the principal vector of the parasite that causes life-threatening Human African Trypanosomiasis and Animal trypanosomiasis in endemic regions in Africa. The tsetse fly has a peculiar reproductive cycle because of the absence of oviposition. Females mate once and store sperm in a spermathecal, and produce a single fully developed larva at a time that pupates within minutes of exiting their uterus. This slow reproductive cycle, compared to other insects, significantly restricts reproduction to only 3 to 6 larvae per female lifespan. This unique reproductive cycle is an attractive vector control strategy entry point. We exploit comparative genomics approaches to explore the diversity of ACPs in the recently available whole genome sequence data from five tsetse fly species (*Glossina morsitans, G. austeni, G. brevipalpis, G. pallidipes* and *G. fuscipes*). We used previously described ACPs in *Drosophila melanogaster* and *Anopheles gambiae* as reference sequences. We identified 36, 27, 31, 29 and 33 diverse ACP orthologous genes in *G. austeni, G. brevipalpis, G. fuscipes, G. pallidipes* and *G. morsitans* genomes respectively, which we classified into 21 functional classes. Our findings provide genetic evidence of MAG proteins in five recently sequenced *Glossina* genomes. It highlights new avenues for molecular studies that evaluate potential field control strategies of these important vectors of human and animal disease.

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REVISED **Amendments from Version 1**

We have revised the Abstract, Introduction and Discussion sections to address the over-emphasis that ACPS play a crucial role in the tsetse fly life reproductive cycle, and thus vector control.

We have revised the Discussion section to show that this is a preliminary study using the initial release of the Glossina genomes, which are publicly available, and future studies using RNASeq/transcriptome datasets of the male accessory glands and testis may identify rapidly evolving Glossina-specific ACPs, which perhaps do not bear a well-known protein domains, and could escape *in silico* investigation dependent on a comparative genomic approach alone.

We have amended the Results and Discussion sections to reflect that we identified possible signatures of selection, since a wider analysis including more closely related taxa was not performed.

We used a different approach (outlined in the Methods section with references 17 & 18) to that used by Scolari *et al.*, 2016. Our approach is able to distinguish functional redundancy (orthologs from paralogs) from BLASTp reciprocal hits; and resolve the many-to-many orthologous relationships inherent in comparisons across multiple genomes (reference 17). Only orthologs with a reciprocal BLASTP E-value cut-off 1e-5 and inflation index 2.5 (default settings) were considered for further analysis. We also provide annotated (gene name and exact genome location) sequences for each of the orthologous ACP clusters in the Supplementary materials section.

Further, we have examined transcriptome results from Scolari *et al.*, 2016 (reference 37), which uses a different approach (reciprocal BLASTp hits). This analysis only identified 4 non-annotated ACPs (GMOY000024, GMOY007757, GMOY009744, GMOY012189) orthologous to *D. melanogaster* not detected in our analysis; and no orthologs to *A. gambiae* ACPs.

See referee reports

Introduction

Accessory gland proteins (ACPs) are important reproductive proteins produced by the male accessory glands (MAGs) of most insect species. These proteins are essential for male insect fertility, and are transferred alongside semen to females during copulation[1]. ACPs trigger significant physiological and behavioral changes in females after copulation, which include: egg laying, reduced sexual receptivity and refractoriness to subsequent inseminations, induce the expression of immune peptides and reduction of female lifespan[1–5]. ACPs are only resynthesized after transfer of seminal fluid to females, but topical application of juvenile hormone on the male's cuticles stimulates *in vivo* re-synthesis to pre-mating levels[2]. Female *Anopheles gambiae* mosquitoes copulated by males with degenerate testes and MAGs fail to oviposit and readily re-mate[1]. Conversely, those copulated by males with degenerate testes but fully developed MAGs lay unfertilized eggs and do not re-mate[1]. This underscores the relevance of ACPs as an entry point for vector borne disease control.

ACPs are poorly characterized in *Glossina* (tsetse fly), compared to *Drosophila* and *Anopheles* species[1,6]. The tsetse fly is the principal vector of the parasite that causes life-threatening human (sleeping sickness) and cattle (nagana) trypanosomiasis in endemic regions in Africa[7]. Over 60 million people and 80 million cattle are at risk of contracting disease[8]. Female tsetse flies only mate once during their lifespan and store the male ejaculate in their spermathecae, which they subsequently use to self-fertilize[9]. They have a peculiar reproductive cycle because of the absence of oviposition, with females producing a single fully developed larva at a time that pupates within minutes of exiting their uterus. This slow reproductive cycle, compared to other insects, significantly restricts reproduction to only 3 to 6 larvae per female lifespan[10]. This unique reproductive cycle is an attractive vector control target. An improved understanding of tsetse fly's reproductive biology, and specifically ACPs that are crucial determinants of successful reproduction in other insect species, may provide valuable possible vector control strategy entry points.

Comparisons between ACP gene orthologs in *Drosophila simulans* and *D. melanogaster* show they are rapidly evolving, relative to non-ACP genes[11–13]. However, there is strong ACP peptide structural homology between closely related species, which decreases as species phylogenetic distances increase[14]. This rapid rate of ACP genes evolution has made it challenging to reliably identify orthologs across insect species in the absence of genomic data[15,16]. The recently available whole genome sequence data from five tsetse fly species (*Glossina morsitans*, *G. austeni*, *G. brevipalpis*, *G. pallidipes* and *G. fuscipes*) has made it possible to revisit detailed examination of ACP gene distribution and genetic diversity in tsetse flies. We exploit comparative genomics approaches to interrogate these genomes, using previously described ACPs in *D. melanogaster* and *A. gambiae* as reference sequences.

Methods

Identification of *Glossina*, *Anopheles* and *Drosophila* species ACP homologs

The proteomes of *G. austeni, G. morsitans, G. pallidipes, G. fuscipes* and *G. brevipalpis* were retrieved manually from VectorBase (www.vectorbase.org). The retrieved *Glossina* protein sequences alongside previously described ACP sequences from *A. gambiae* (n=57) and *D. melanogaster* (n=173) (Supplementary Table 1)[1], used as references, were assigned to homologous clusters using OrthoMCL with default settings (BLASTP E-value cut-off 1e-5 and inflation index 2.5)[17] (Supplementary File 1). Clusters with singletons were omitted from further processing. Mapped orthologs were subsequently processed using BMX[18].

Sequence alignment and phylogeny reconstruction

Multiple sequence alignments were performed using MUSCLE[19]. Maximum likelihood (ML) phylogenetic analysis of the multiple aligned sequences with bootstrap values of 100 replicates was performed using PHYML version 3.5[20].

Determining the direction and extent of selection pressure

The magnitude and direction of selection pressure on the ACP sequences was tested based on the ratio ($\omega = d_N/d_S$) of the average number of non-synonymous substitutions per non-synonymous site (d_N) to the average number of synonymous substitutions per synonymous site (d_S). If $\omega = 1$, amino acid substitution is assumed to be under neutral selection, $\omega > 1$ is indicative of positive selection whereas $\omega < 1$ is evidence of negative or purifying selection. Sequence alignments of each of the ACP clusters

containing *A. gambiae*, *D. melanogaster*, *G. austeni*, *G. brevipalpis*, *G. fuscipes*, *G. morsitans* and *G. pallidipes* were generated. Each alignment was then uploaded to the SNAP program[21] (www.hiv.lanl.gov), which calculates synonymous and non-synonymous substitution rates to determine the magnitude of selection pressure.

Data visualization

A list of the relative abundance of ACPs with secretory signals identified within each ortholog cluster for *Glossina, Anopheles* and *Drosophila* species was generated (Supplementary Figure 1). The 21 ACPs clusters were visually presented in a single circular ideogram using CIRCOS software[22].

Results

Identification of ACP gene orthologs in *Glossina* species

We analyzed five recently sequenced *Glossina* genomes: *Glossina morsitans*, *G. austeni*, *G. brevipalpis*, *G. pallidipes* and *G. fuscipes*, to examine the presence of orthologs to ACP genes previously identified in *Drosophila melanogaster* (n=173) and *Anopheles gambiae* (n=57)[1,6]. First, we identified 41 ACP ortholog clusters that we broadly classified into 23 groups based on the encoded protein's functional class. These ACPs have a distinct species distribution with: 12 ortholog clusters common to *Glossina, A. gambiae,* and *D. melanogaster* species; and some clusters only present in *A. gambiae* and *Glossina* species (n=7) or *D. melanogaster* and *Glossina* species (n=5) (Supplementary Table 1). The remaining 17 clusters consist of ACP orthologs exclusive to either *A. gambiae* (n=7) or *D. melanogaster* (n=10). Next, we shortlisted genes that encode proteins carrying classical secretory signals[23] to distinguish the matched testes-specific secreted male accessory gland proteins from other insect peptides. We identified 36, 27, 31, 29 and 33 ACP orthologs with secretory signals in *G. austeni*, *G. brevipalpis*, *G. fuscipes, G. pallidipes* and *Glossina morsitans* genomes respectively (Figure 1), across 21 functional class groups. α2-macroglobulins (Group 1) and heat shock proteins (Group 17) are the most abundant ACP orthologs in *Glossina* species (Figure 1). Interestingly, *Glossina* species lack orthologs to Acp70A (Group 18) and andropin (Group 19), which has antimicrobial properties and safeguards the male ejaculate, and stimulation of long-term post mating responses in females respectively[1,24,25].

Most ACP genes are under positive selection

We inferred the direction and magnitude of selection pressure on the identified ACP orthologs using dN/dS ratios. We observed possible signatures of positive selection in all genes except five are evolving under positive selection (Figure 1). We found that α2-macroglobulins, which have been shown to be important in mosquito and *Drosophila* immunity[26,27], display signatures of purifying selection suggesting they are critical for successful reproduction and all deleterious variations are purged. Our analysis was restricted to reference genes present in *A. gambiae* and *D. melanogaster*, and future studies that integrate data from more closely related taxa will highlight evolutionary changes associated with ACPs in more detail. We reconstructed the phylogeny of ACP orthologs within each cluster (Supplementary Figure 1). We failed to identify any pattern associated with the diverse ecological niche and unique reproductive style in *Glossina* species in our analysis.

ACP distribution in *Glossina* species

Distribution of ACP orthologs varies widely between species (Figure 1). *Glossina* species have a disproportionately large number of α2-macroglobublin and heat shock proteins, which are important in immunity in *A. gambiae* and *Glossina* species[26–29]. Our analysis did not detect β-defensin orthologs, which are antimicrobial peptides involved in immune responses[30], in the *G. pallidipes* and *G. brevipalpis* genomes. We also did not detect Acp29AB, Acp70A, and andropin orthologs in this comprehensive catalogue of *Glossina* genes. This raises the possibility that these genes were lost by tsetse flies after evolutionary radiation of insects into multiple taxa, and alternative species-specific proteins might compensate for the same roles.

Discussion

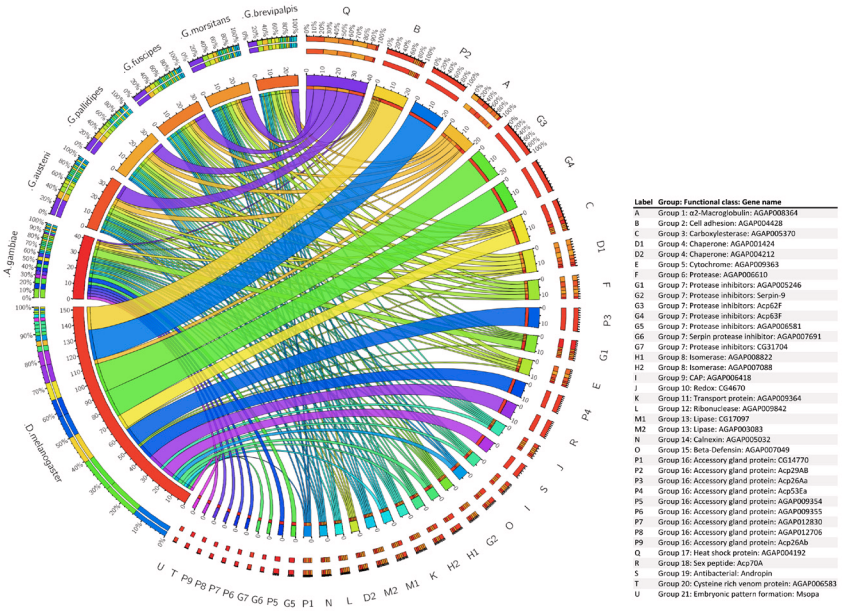
We performed comparative genomics analysis to detect the presence of male accessory gland proteins (ACPs) orthologs previously identified in *A. gambiae* and *Drosophila*[1]. The motivation here was to improve knowledge on the biology of *Glossina* species ACPs given the importance of reproductive molecules in strategic designs of vector control. We identified 21 functional classes of ACP orthologs with secretory signals in five *Glossina* species genomes. We observed genetic signatures of a high rate of ACP protein divergence, supporting similar findings on male reproduction-related genes in *Drosophila*[16]. ACPs exhibit high evolutionary changes, thus displaying between species divergence and within species polymorphism[3,31]. We restricted analysis to reference genes present in *A. gambiae* and *D. melanogaster*, and future studies integrating datasets from more closely related taxa will be useful to understand evolutionary changes associated with ACPs in more detail. α2-macroglobulins and heat shock proteins are the most abundant ACP orthologs in *Glossina* species. α2-macroglobulins are important in mosquito and *Drosophila* immunity[26,27], and display signatures of purifying selection, suggesting they are critical for successful reproduction and all deleterious variations are purged. α2-macroglobulin over-representation, and the absence of other ACP orthologs implicated in immunity in the *Glossina* genomes points to their critical role in ensuring successful tsetse fly reproduction. Heat shock protein silencing in *A. gambiae* down-regulates up to 50% of male accessory gland proteins, half of which are male reproductive tract specific and encode the homologs of 13 known *Drosophila* ACPs that include Acp70A[1]. Interestingly, *Glossina* species lack orthologs to Acp70A, andropin, Acp26Ab, Acp29AB, and Acp62F, which play critical roles in successful reproduction in *Anopheles* and *Drosophila* species[1,24,25].

Acp70A or sex peptide stimulates long-term post mating behavior, resulting in non-receptivity to mating and increased oviposition[1,25,32]. Andropin is an antimicrobial peptide transferred to the female during copulation, and defends the female reproductive tract against microbes[33]. Andropin also protects the male ejaculate from Gram-positive and Gram-negative bacterial infections[1]. Acp26Ab stimulates oviposition in *Drosophila melanogaster* females[34], and together with Acp26Ab protects the male ejaculate from microbial infections, and displacement

A **B** **C** **D**

Group	Functional class	Gene name	ds	dn	dn/ds	G. austeni	G. brevipalpis	G. fuscipes	G. morsitans	G. pallidipes	A. gambiae	D. melanogaster
Group 1	α2-Macroglobulin	AGAP008364	0.496	0.467	0.940	4	3	3	2	4	1	3
Group 2	Cell adhesion	AGAP004428	0.124	0.203	1.636	1	1	1	1	1	1	16
Group 3	Carboxylesterase	AGAP005370	0.243	0.487	2.002	1	2	1	1	1	2	10
Group 4	Chaperone	AGAP001424	0.098	0.271	2.767	2	2	3	3	3	1	1
	Chaperone	AGAP004212	0.043	0.092	2.144	1	1	1		1	1	
Group 5	Cytochrome	AGAP009363	0.180	0.238	1.319	2	3	1		2	1	1
Group 6	Protease	AGAP006610	0.320	0.315	0.984	3	2	2	3	2	1	
Group 7	Protease inhibitors	AGAP005246	0.172	0.349	2.033	1	1	1	1	1	5	1
	Protease inhibitors	Serpin-9	0.137	0.199	1.453	1	1	1	1	1	1	
	Protease inhibitors	Acp62F	NA	NA	NA							20
	Protease inhibitors	Acp63F	0.010	0.014	1.392							19
	Protease inhibitors	AGAP006581	0.107	0.118	1.097						3	
	Serpin protease inhibitor	AGAP007691	0.024	0.028	1.173						2	
	Protease inhibitors	CG31704	NA	NA	NA							2
Group 8	Isomerase	AGAP008822	0.075	0.106	1.426	1			1		1	3
	Isomerase	AGAP007088	0.104	0.079	0.755	1		1		1	1	2
Group 9	CAP	AGAP006418	0.445	0.370	0.831	1		1	1	1	1	2
Group 10	Redox	CG4670	0.235	0.260	1.109	1	1	1	1	1	1	2
Group 11	Transport protein	AGAP009364	0.054	0.160	2.978	1	1	1	1	1	1	
Group 12	Ribonuclease	AGAP009842	0.136	0.234	1.721		1	1	1	1	1	
Group 13	Lipase	CG17097	0.111	0.287	2.580	1	1	1		1		2
	Lipase	AGAP003083	0.150	0.168	1.124	1	1	1	1	1	1	
Group 14	Calnexin	AGAP005032	0.066	0.130	1.979	1		1	1	1	1	
Group 15	Beta-Defensin	AGAP007049	0.121	0.381	3.157	1		1	1		1	3
Group 16	Accessory gland protein	CG14770	0.064	0.118	1.865	1	1		1	1		1
	Accessory gland protein	Acp29AB	0.004	0.005	1.333							21
	Accessory gland protein	Acp26Aa	0.006	0.008	1.407							13
	Accessory gland protein	Acp53Ea	0.010	0.013	1.326							10
	Accessory gland protein	AGAP009354	0.011	0.024	2.259						3	
	Accessory gland protein	AGAP009355	NA	NA	NA						2	
	Accessory gland protein	AGAP012830	0.028	0.019	0.682						2	
	Accessory gland protein	AGAP012706	0.039	0.097	2.482						2	
	Accessory gland protein	Acp26Ab	NA	NA	NA						2	
Group 17	Heat shock protein	AGAP004192	0.099	0.291	2.939	10	5	8	8	8	1	
Group 18	Sex peptide	Acp70A	NA	NA	NA							10
Group 19	Antibacterial	Andropin	0.025	0.026	1.032							8
Group 20	Cysteine rich venom protein	AGAP006583	0.171	0.340	1.993						2	
Group 21	Embryonic pattern formation	Msopa	NA	NA	NA							2
		Orthologous ACPs identified				36	27	31	29	33	42	152

E



Label	Group: Functional class: Gene name
A	Group 1: α2-Macroglobulin: AGAP008364
B	Group 2: Cell adhesion: AGAP004428
C	Group 3: Carboxylesterase: AGAP005370
D1	Group 4: Chaperone: AGAP001424
D2	Group 4: Chaperone: AGAP004212
E	Group 5: Cytochrome: AGAP009363
F	Group 6: Protease: AGAP006610
G1	Group 7: Protease inhibitors: AGAP005246
G2	Group 7: Protease inhibitors: Serpin-9
G3	Group 7: Protease inhibitors: Acp62F
G4	Group 7: Protease inhibitors: Acp63F
G5	Group 7: Protease inhibitors: AGAP006581
G6	Group 7: Serpin protease inhibitor: AGAP007691
G7	Group 7: Protease inhibitors: CG31704
H1	Group 8: Isomerase: AGAP008822
H2	Group 8: Isomerase: AGAP007088
I	Group 9: CAP: AGAP006418
J	Group 10: Redox: CG4670
K	Group 11: Transport protein: AGAP009364
L	Group 12: Ribonuclease: AGAP009842
M1	Group 13: Lipase: CG17097
M2	Group 13: Lipase: AGAP003083
N	Group 14: Calnexin: AGAP005032
O	Group 15: Beta-Defensin: AGAP007049
P1	Group 16: Accessory gland protein: CG14770
P2	Group 16: Accessory gland protein: Acp29AB
P3	Group 16: Accessory gland protein: Acp26Aa
P4	Group 16: Accessory gland protein: Acp53Ea
P5	Group 16: Accessory gland protein: AGAP009354
P6	Group 16: Accessory gland protein: AGAP009355
P7	Group 16: Accessory gland protein: AGAP012830
P8	Group 16: Accessory gland protein: AGAP012706
P9	Group 16: Accessory gland protein: Acp26Ab
Q	Group 17: Heat shock protein: AGAP004192
R	Group 18: Sex peptide: Acp70A
S	Group 19: Antibacterial: Andropin
T	Group 20: Cysteine rich venom protein: AGAP006583
U	Group 21: Embryonic pattern formation: Msopa

Figure 1. Identification of male accessory gland proteins (ACPs) in five *Glossina* species. (**A**) We identified 38 clusters of ACPs, which were classified into 21 functional classes. (**B**) The identified *Glossina* species ACPs are orthologous to well-characterized ACP genes in *Anopheles gambiae* and *Drosophila melanogaster genomes*. (**C**) The magnitude and direction of selection pressure on the ACP sequences was tested based on the ratio ($\omega = d_N/d_S$) of the average number of non-synonymous substitutions per non-synonymous site (d_N) to the average number of synonymous substitutions per synonymous site (d_S). A ratio greater than 1 indicates positive selection, and a ratio less than 1 indicates purifying selection. (**D**) The relative abundance of ACPs identified per ortholog cluster. We identified 36, 27, 31, 29 and 33 ACP orthologs with secretory signals in *G. austeni, G. brevipalpis, G. fuscipes, G. pallidipes* and *Glossina morsitans* genomes, respectively. (**E**) Schematic presentation showing the relative abundance of each ACP functional class in *Glossina* species, *A. gambiae*, and *Drosophila melanogaster*. α2-macroglobulins [A] and, heat shock proteins [Q] are the most abundant ACPs in *Glossina* species.

by a second ejaculate[1]. *Drosophila* Acp29AB and Acp62F up-regulate genes for egg production and muscle development, although Acp29AB or Acp62F null males do not show a reproduction impairment phenotype[35]. Acp62F also protects sperm in the female reproductive tract from protease attack[36].

A limitation in our study was the absence of transcriptome data to measure ACP differential gene expression. Analysis of transcriptome data available to others using a different approach (reciprocal BLASTp hits) identified only 4 non-annotated ACPs (GMOY000024, GMOY007757, GMOY009744, GMOY012189) orthologous to *D. melanogaster* not detected in our analysis; and no orthologs to *A. gambiae* ACPs[37]. Future studies focused on transcriptome datasets of male accessory glands and testis may identify rapidly evolving *Glossina*-specific ACPs, which perhaps do not bear a well-known protein domains, and could escape *in silico* investigation dependent on a comparative genomic approach alone.

Our analysis detected orthologous *Glossina, Anopheles* and *Drosophila* ACPs belonging to the same functional classes, suggesting a conserved role for these proteins across all three genera. However, some ACPs may represent lineage-specific ACPs that may have evolved to perform species-specific reproductive functions. Our findings support evolutionary adaptation to different reproductive styles. Tsetse fly females produce a single fully developed larva at a time that pupates within minutes of exiting their uterus, and may have lost non-essential ACP genes after adaptation.

Conclusions

Our findings provide genetic evidence of male accessory glad proteins in five recently sequenced *Glossina* genomes. It provides new avenues for molecular studies that evaluate potential field control strategies of these important vectors of human and animal disease.

Data availability

Anopheles gambiae and *Drosophila melanogaster* ACPs were obtained from: DOI, 10.1073/pnas.0703904104[1].

Glossina species genome sequences were obtained from VectorBase.

Competing interests

No competing interests were disclosed.

Grant information

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Supplementary material

Supplementary Table 1: (A) *Anopheles gambiae* (n=57) and *Drosophila melanogaster* (n=173) ACP orthologs. (B) ACP ortholog clusters identified in *Glossina*, *A. gambiae*, and *D. melanogaster* species prior to signal-peptide filtering.

Click here to access the data.

Supplementary File 1: Amino acid sequences of the ACP ortholog clusters.

Click here to access the data.

Supplementary Figure 1: Reconstructed phylogenies of the ACP orthologs within each cluster.

Click here to access the data.

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Open Peer Review

Current Referee Status: ✔ ✔

Version 2

Referee Report 06 December 2017

doi:10.21956/wellcomeopenres.14370.r28295

✔ **Kristipati Ravi Ram**
Embryotoxicology Laboratory, Environmental Toxicology Group, Council of Scientific & Industrial Research (CSIR)- Indian Institute of Toxicology Research, Lucknow, Uttar Pradesh, India

Authors have adequately addressed the concerns raised on the previous version.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 23 October 2017

doi:10.21956/wellcomeopenres.13476.r26501

? **Kristipati Ravi Ram**
Embryotoxicology Laboratory, Environmental Toxicology Group, Council of Scientific & Industrial Research (CSIR)- Indian Institute of Toxicology Research, Lucknow, Uttar Pradesh, India

The paper by Abry *et al*., is aimed at identification of accessory gland proteins (Acps) in five *Glossina* species through bioinformatic approaches. Given the importance of reproductive molecules in strategic designs of vector control, the reported data are of significant value. However, the study is quite preliminary, and hence it feels that authors have gone overboard with the interpretation of these preliminary results. Primarily, authors are required to be clear about the rationale of their study given that the testicular/MAG contributions towards spermatophore formation through transcriptomic and proteomic approaches in *G. morsitans* has already been reported earlier (Scolari *et al.* 2016, Scientific Reports). The inclusion of data on reciprocal hits would increase the reliability of predicted/putative Acps. Further, RT-PCR based analysis of the enrichment/expression of at least a couple of identified putative Acp orthologs in male accessory glands (MAGs) from any of the Glossina species would add excellent value to the data and support to the conclusions drawn. Alternatively, authors may compare their datasets with RNAseq/proteomic data of *G. morsitans* (Scolari *et al.*, 2016) to assess if the identified genes are enriched/expressed in MAGs or form the part of male contributions to the spermatophore.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 20 Nov 2017

Benard Kulohoma,

We thank the reviewers for the comments. Our study is a preliminary *in silico* exploration of the MAG repertoire of *Glossina* species, and provides initial findings about genome annotations that identifies ACPs orthologs. Our aim was to establish whether there are male accessory gland (ACPs) orthologs across the Glossina species genomes, by comparing them to well characterised ACP orthologs from *Drosophila melanogaster* and *Anopheles gambiae*, given the importance of reproductive molecules in strategic designs of vector control.

- We have revised the manuscript to address the over-emphasis that ACPS have a play crucial role in the tsetse fly life reproductive cycle, and thus vector control. Lines 45, 73, 163, and 164.
- We have revised the manuscript to show that this is a preliminary study using the initial release of the Glossina genomes, which are publicly available, and future studies using RNASeq/transcriptome datasets of the male accessory glands and testis may identify rapidly evolving Glossina-specific ACPs, which perhaps do not bear a well-known protein domains, and could escape *in silico* investigation dependent on a comparative genomic approach alone. Lines 194 - 201.
- We have amended the manuscript to reflect that we identified possible signatures of selection, since a wider analysis including more closely related taxa was not performed. Lines 137, 140-143, and 165-166.

Competing Interests: None declared

Referee Report 04 September 2017

doi:10.21956/wellcomeopenres.13476.r25531

Emiliano Mancini
Department of Science, Roma Tre University, Rome, Italy

The paper by Abry *et al.* presents a comparative genomics analysis to identify male accessory gland proteins in *Glossina* species. The paper is an "*in silico*" exploration of the MAG repertoire of *Glossina*, and has the merit to open the road to further investigation on these important reproductive proteins and their functions. However I think that the authors should give the right relevance to their results, which are, in a sense, quite preliminary, but presented, in my opinion, with too much emphasis.

First of all the authors have to clarify the aim of the work, especially in the context of vector control. Although it is true that a deep understanding of ACPS in *Glossina* can be useful for limit fertility of *Glossina* species and thus control their spread (these proteins have important roles in female post-mating behaviour, such as single mating, storing and sustaining viability of sperms etc.), I think that ACPS do not necessarily have a realistic connection with the peculiar post-mating characteristic of the reproductive cycle of *Glossina* (e.g. absence of eggs deposition). Since this point is recurrently discussed and emphasized in the paper, I suggest the author either to better explain how the ACPS can have a role on the origin of this particular life cycle, or to eliminate this point which, otherwise, remains quite speculative.

It is important that authors stress the importance that their work should be completed in the future by RNAseq of male accessory glands and testis of *Glossina*. This because some rapidly evolving ACPS could escape *in silico* investigation, as they could be *Glossina*-specific and not corresponding to any ortholog, or simply because they do not bear a well-known protein domain. Species-specific proteins can not be obtained by a comparative genomic approach alone, so that RNA analysis is required to reveal (probably) the most important, peculiar ACPS in this important vector.

Finally, I would be very cautious in assigning positive selection to genes with dN/dS>1, since a wider analysis including more closely related taxa (and data on their intra-specific variability) should be performed. However, based on the dN/dS estimation here provided, I think that the authors should just argue about "possible signatures of positive selection" on these genes.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 20 Nov 2017

Benard Kulohoma,

We thank the reviewers for the comments. Our study is a preliminary *in silico* exploration of the MAG repertoire of *Glossina* species, and provides initial findings about genome annotations that identifies ACPs orthologs. Our aim was to establish whether there are male accessory gland (ACPs) orthologs across the Glossina species genomes, by comparing them to well characterised ACP orthologs from *Drosophila melanogaster* and *Anopheles gambiae*, given the importance of reproductive molecules in strategic designs of vector control.

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Competing Interests: None declared