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RESEARCH ARTICLE

Computational identification of novel microRNAs and their targets in the malarial vector, *Anopheles stephensi*

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Abstract MicroRNAs are a ~22 nucleotide small non-coding RNAs found in animals, plants and viruses. They regulate key cellular processes by enhancing, degrading or silencing protein coding targets. Currently most of the data on miRNA is available from Drosophila. Given their important post-transcriptional role in several organisms, there is a need to understand the miRNA mediated processes in normal and abnormal conditions. Here we report four novel microRNAs ***ast-mir-2502, ast-mir-2559, ast-mir-3868*** and ***ast-mir-9891*** in *Anopheles stephensi* identified from a set of 3,052 transcriptome sequences, showing average minimum free energy of −31.8 kcal/mol of duplex formation with mRNA indicating their functional relevance. Phylogenetic study shows conservation of sequence signatures within the Class Insecta. Furthermore, 26 potential targets of these four miRNAs have been predicted that play an important role in the mosquito life-cycle. This work leads to novel leads and experimental possibilities for improved understanding of gene regulatory processes in mosquito.

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Introduction

MicroRNAs are small non-coding RNAs ~22 nucleotides long, involved in a range of gene regulation and post transcriptional modification events in animals, plants and viruses (Bartel 2004). Even though miRNAs do not encode for protein, they regulate a number of translation processes by enhancing, degrading or silencing gene expression (Bartel 2009). MicroRNAs are initially found in a premature state called primary miRNA (**pri-miRNA**) (Denli et al. 2004), which is a hairpin structure. This molecule is later processed by an RNase-III like enzyme **Drosha (Drsh-1)** to form a stem-loop structure called **pre-miRNA** (Grishok et al. 2001), transported to cytoplasm by **Exportin-5** (Kim et al. 2003) and further processed by a **Dicer (Dcr-1)** complex to form the mature single stranded **miRNA** (Kim et al. 2003; Zing et al. 2004; Zang et al. 2004). Mature miRNA sequences are incorporated into the RNA induced silencing complex, which recognizes specific targets in the mRNA sequence and induces post transcriptional gene silencing in several organisms (Khvorova et al. 2003; Schwarz et al. 2003).

The global health statistics indicate that vector-borne diseases contribute to 17 % of the world's infectious disease burden (World Health Organization Factsheet 2014), of which malaria forms a significant proportion. The protozoan parasite, Plasmodium spends major part of its life cycle in the female *Anopheline* species. The asexual sporozoites from mosquito vector are transferred into human system, when an infected Anopheles takes a human blood meal. Sporozoites are transported through blood

vessels to the liver where they invade hepatocytes and develop into the sexually active form, merozoites (Sturm et al. 2006). On rupturing of the infected hepatocytes, merozoites enter the blood stream and invade erythrocytes. The incubation period for the parasite to manifest in the human host varies from 2–3 weeks (Miller et al. 2002; Grüring et al. 2011). It would be relevant to understand parasite life cycle in Anopheles species, for effective control of malaria.

A. stephensi is a mosquito vector that is responsible for 12 % of the malarial cases spread across the Indian subcontinent (Tikar et al. 2011). It belongs to the same subgenus as *A. gambiae* and comprises of two-species namely *A. stephensi sensu stricto* and *A. stephensi mysorensis*, responsible for the spread of malaria in the Indian subcontinent. *A. stephensi* takes human blood meal largely indoors (endophilic and endophagic respectively) though during rainy season, the mosquito breeds and feeds itself outdoors (Sinka et al. 2011). Due to prevalence of stagnant water pools in the cities the urban population is always at high risk of vector borne diseases.

Several experimental studies, to determine the role of microRNAs in malarial infection, have been initiated in the past (Rathjen et al. 2006; Mead and Tu 2008; El-Assaad et al. 2011; La Monte et al. 2012). However, a lot more data are required for better understanding of the mosquito biology. Given the inherent limitation in conducting large-scale experiments, the use of computational approach holds the key for predicting novel molecules and interactions responsible for malaria infection and effectively narrow down the set of relevant experiments in future. In this study, we used bioinformatics tools, computational algorithms and statistical analysis for identification of novel microRNA molecules and their targets in the malarial vector *Anopheles stephensi.*

Materials and methods

Identifying pre-miRNA signatures in RNA transcriptome of *Anopheles stephensi*

A total of 3,052 stem-loop pre-miRNA sequences belonging to 23 organisms of the Hexapoda sub-phylum were extracted from miRBase v21 [URL—http://www.mirbase.org/] (Kozomara and Griffiths-Jones 2014). The sequences were obtained only from organisms with stable completely sequenced genomes. 61,734 RNAseq transcripts of *A. stephensi* (Hittinger et al. 2010) submitted to Vectorbase [URL—https://www.vectorbase.org/] (Megy et al. 2012) were extracted. Redundant data were removed. Figure 1 outlines the methodology used in the present work.

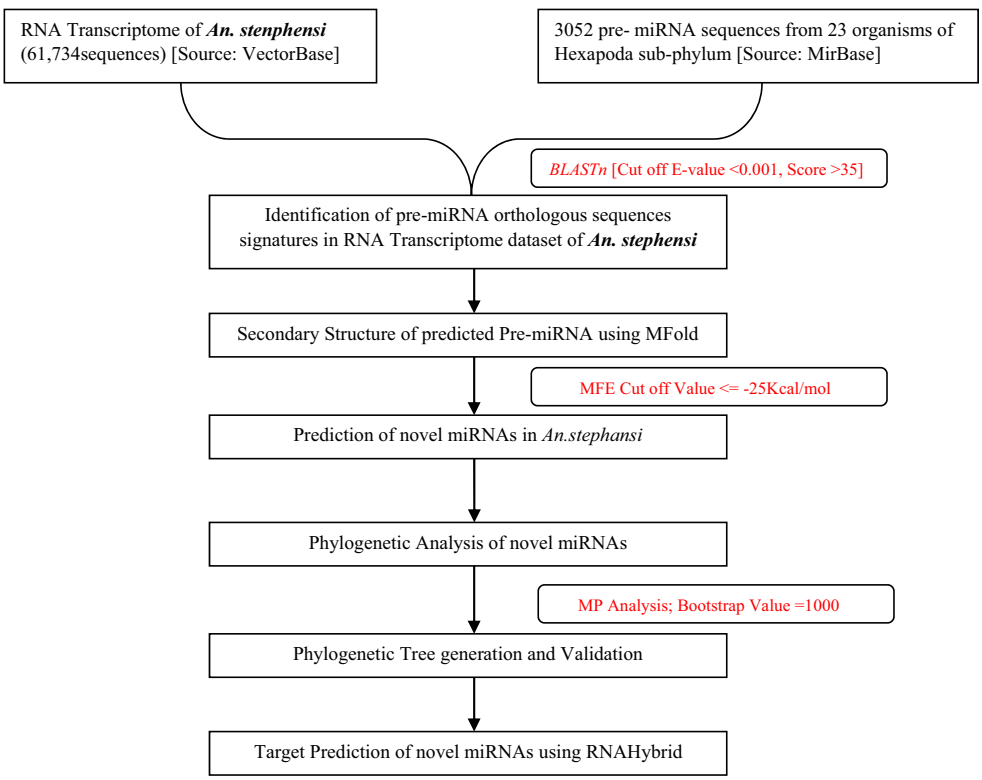

Fig. 1 Flowchart indicating the methodology for predicting novel miRNAs and their targets

Predicting novel mature miRNA genes in *A. stephensi*

The stem-loop sequences obtained from miRBase were considered as the *query* and RNAseq transcripts as the *subject* for performing BLAST to identify pre-miRNA stem-loop signatures in RNAseq transcripts of *A. stephensi*. BLAST 2.2.30 + program—(Altschul et al. 1997), BLASTn was used with default parameters. A stringent cut-off value was set with E-value <0.001; BLAST Score >35; %Identity >60. The sequences that satisfied the cut-off values were carefully screened. Validation of the identified secondary structures was performed using M-Fold tool based on thermodynamics metrics (Zuker 2003). The annotation of mature miRNA was performed based on the widely accepted criteria for selecting miRNAs from pre-miRNA sequences (Ambros et al. 2003; Xue et al. 2005). These were (a) presence of mature miRNA sequence of at least 18 nucleotide length on the stem including GU Wobble pairs (b) Minimum Free energy value < -15 kcal/mol (c) Absence of multiple loops (d) ability to fold to stem-loop/hair-pin structures. The nomenclature followed for naming the novel miRNA genes was identical to that followed in miRBase and prefixed with "ast" to denote *A. stephensi* (Mead and Tu et al. 2008).

Phylogenetic analysis of novel miRNAs in *Anopheles stephensi*

The novel microRNAs computationally predicted in *A. stephensi* were sent for phylogenetic analysis and compared against their closest relative mosquito species—*Anopheles gambiae, Culex quinquefasciatus* and *Aedes aegypti*. The source organisms of the novel microRNAs namely *Apis mellifera*, *Drosophila pseudoobscura* and *Tribolium castaneum* were also considered for analysis. Mature miRNA sequences of the organisms were extracted from miRBase v21 and Clustal Omega tool used for multiple RNA sequence alignment. We used Molecular Evolutionary Genetic Analysis—MEGA v6.0 (Tamura et al. 2013) for phylogenetic tree generation and analysis. Assuming a conserved evolution for microRNA evolution, as reported with most microRNAs, we used the Maximum Parsimony method and the Tree was statistically validated by bootstrap analysis, replicated 1,000 times over to ensure maximum accuracy. Inferences regarding the extent of conservation and divergence patterns of the novel genes were drawn.

Target prediction of novel microRNAs

Novel microRNAs identified in this study were followed up for target prediction, using in silico RNA–RNA hybridization approach to determine their ability to bind and form miRNA/target duplexes. RNAHybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) was used for miRNA-target prediction as it applies both thermodynamics metrics and statistical analysis to predict multiple potential binding sites of microRNAs in a large sequence of target RNA (Rehmsmeier et al. 2004). The program includes features such as exclusion of non-Watson–Crick base pairs, seed region specific binding sites (2–7 seed complementarities was set), maximum number of loops and internal bulges allowed (set at 1), total number of target hits per microRNA (set at maximum value). As a final step, potential targets were predicted by aligning the dataset generated by RNAHybrid, against the non-redundant protein database by BLASTx program with default threshold values to draw biologically relevant conclusions.

Results

Nucleotide BLAST of 3,052 stem-loop sequences with 61,734 resulted in 51 orthologous pre-miRNA signatures in

Table 1 List of novel miRNAs predicted in *A. stephensi*

S. no	Novel miRNA	Size	Source miRNA	Source organism	Strand	Minimum free energy values (kcal/mol)
1	>*ast-mir-2502* GCAGCAGCGCCAGCAACAGCU	21	dpe-mir-2502	*Drosophila pseudoobscura*	3′	−25.1
2	>*ast-mir-2559* GCACAUCAUUUUCCCCCUCCCUA	23	dpe-mir-2559	*Drosophila pseudoobscura*	5′	−42.0
3	>*ast-mir-3868* AGCAACUAAAGCGTTTAAC	19	tca-mir-3868	*Tribolium castaneum*	3′	−31.9
4	>*ast-mir-9891* CUUCGUCCUCGUCGUCGUCG	21	ame-mir-9891	*Apis mellifera*	3′	−28.5

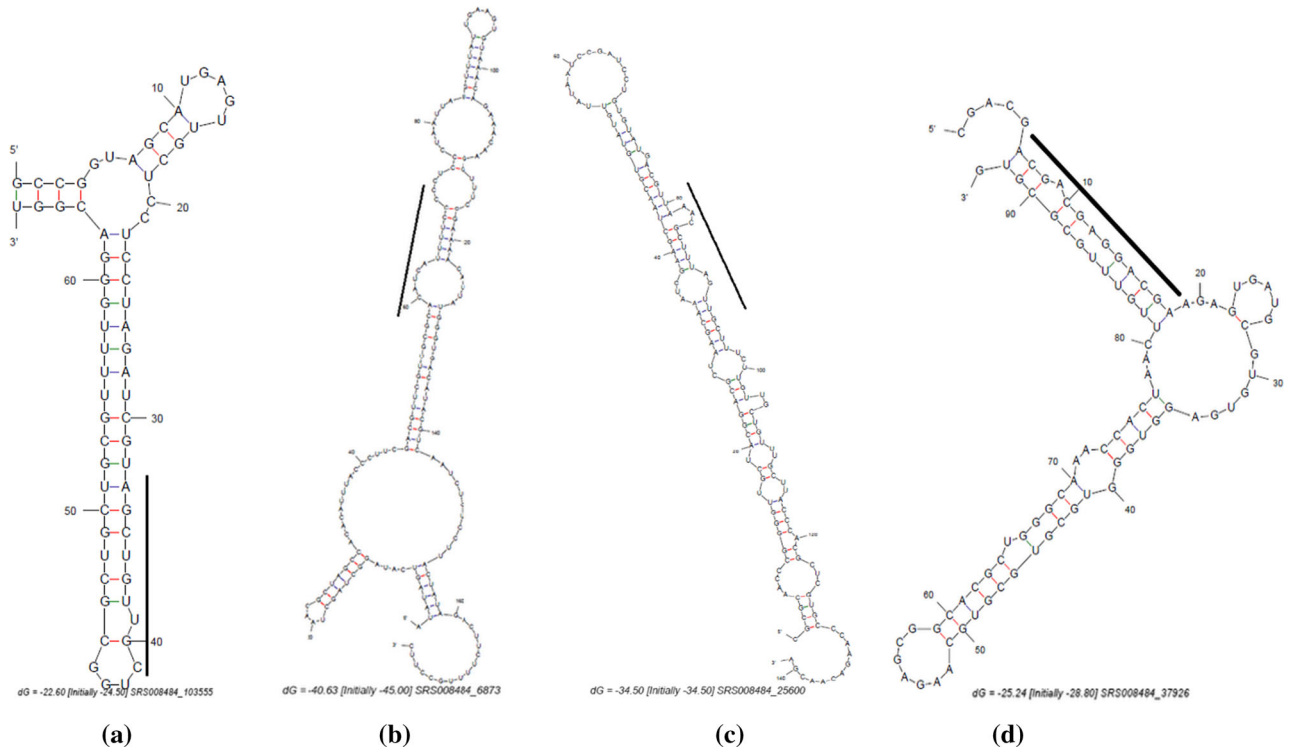

Fig. 2 Stem-loop sequences showing novel mature miRNA sequences. **a** Position of *ast-mir-250.* **b** Position of *ast-mir-2559.* **c** Position of *ast-mir-3868.* **d** Position of *ast-mir-9891*

17 different organisms (*Online Resource 1*). Following the flow chart outlined in Fig. 1, four novel microRNAs were identified in *A. tephansi* were named according to the mirBase naming convention as ***ast-mir-2502***, ***ast-mir-2559***, ***ast-mir-3868***, ***ast-mir-9891*** with an average minimum free energy value of −31.8 kcal/mol. Table 1 indicates the size and source of the four novel miRNAs and the positions of these mature miRNAs on the pre-miRNA sequences are denoted in Fig. 2a–d.

Phylogenetic analysis of these four microRNAs, as expected, showed closest proximity to their source organism (Fig. 3). The miRNA genes *ast-mir-3868* laid close not only to its source organism (dps-mir-3868), but also to the mosquito species *A. aegypti* miRNA gene, aae-mir-927 and *Tribolium castaneum* microRNA gene, tca-mir-927. It is also interesting to note that the *Tribolium castaneum* microRNA gene, tca-mir-3884 belonged to the same clade as the *Drosophila pseudoobscura* miRNA gene, dps-mir-2559 when the novel Anopheles miRNA *ast-mir-2559* seemed to show a slight divergence even from its source organism. All the genes showed high levels of seed region conservation which is key for miRNA-target interaction.

Target prediction for the four novel microRNAs was performed using RNAHybrid that generated novel protein-coding targets with significant biological roles (Table 2). A total of 26 potential targets that satisfied the minimum MFE cut-off values were predicted with 12 targets for ***ast-mir-2502***, 4 targets for ***ast-mir-2559*** and 9 targets for ***ast-mir-9891***. No targets among Hexapod protein coding regions were predicted for *ast-mir-3868*. The predicted targets were found to vary greatly with respect to their biological functions as detailed in the "Discussion" section.

Discussion

There have been increasing reports of insect resistance to insecticides in recent years (Nwane et al. 2014; Abdalla et al. 2014; Xu et al. 2014). Thus, a multipronged strategy is needed to combat malaria, involving both mosquito and human systems. Sufficient understanding of biological pathways in mosquito and human systems, is required to devise novel innovative therapeutic strategies.

This work is a progressive step towards adding useful gene regulatory data available in *A. stephensi*. Here we predict four novel miRNA namely *ast-mir-2502, ast-mir-2559, ast-mir-3868, ast-mir-9891* in *A. stephensi*, for the first time, to our best of knowledge. The miRNA functionality was studied based on the seed region conservation patterns. Since all the microRNAs shared the seed region with a maximum allowance of three mismatches, findings from the phylogenetic study of miRNAs indicate they

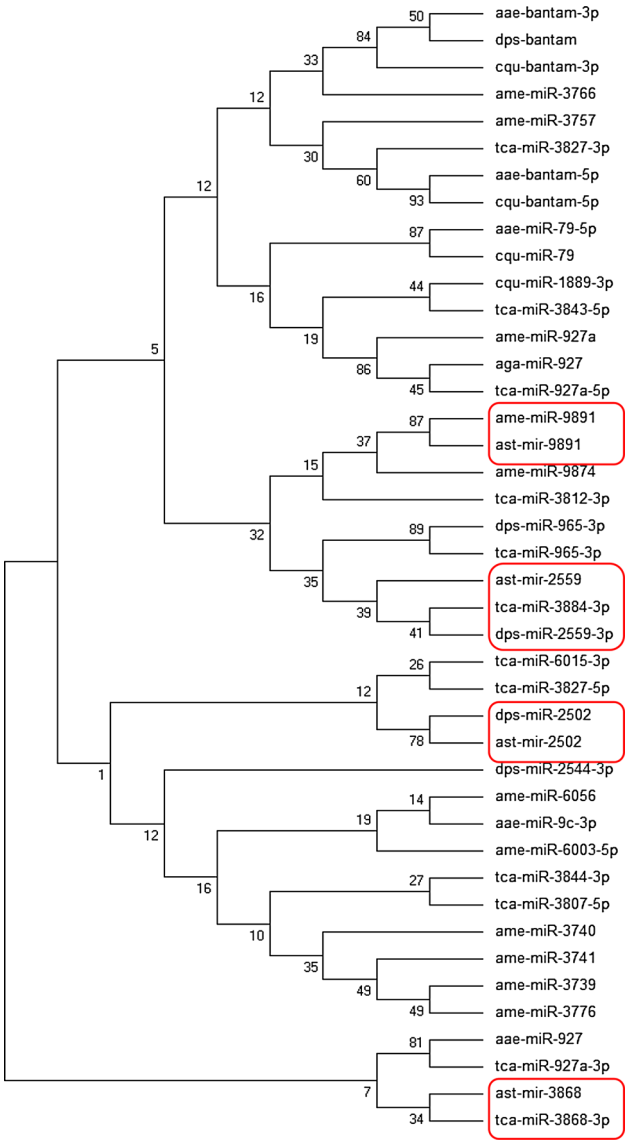

Fig. 3 Phylogenetic tree generated indicating functional seed region specific conservation pattern for the novel miRNAs

might possibly share targets as well. This is interesting because seed region conservation is important in understanding miRNA-target interactions. Organisms evolutionarily closest to each other have a higher tendency to share similar targets in their respective genomes. There is also a possibility of cluster association between microRNAs from the same taxa belonging to the same clade in the phylogenetic tree (*Example:* ame-mir-3739 and ame-mir-3776).

Here we identify 26 potential targets for three novel miRNAs with an exception of ast-mir-3868 thereby indicating that the gene that transcribes these sequences could be unique for *A. stephensi*. We were able to predict that *ast-mir-2502* and *ast-mir-9891* possibly target ankyrin repeats that are part of the spectrin-binding protein—ankyrins, known to act in association with other functionally active domains (Rubtsov and Lopina 2000). Ankyrins have been reported to play a crucial role in signal transduction, assembly of integral membrane proteins, associations with ion channels/pumps, calcium release channels and cell adhesion molecules (Bennett and Chen 2001).

The *ast-mir-2502* was found to interact with the Really Interesting New Gene (RING) domain that is an integral part of proteins mediators of ubiquitin ligase activity (Joazeiro and Weissman 2000) and epithelial development, protein folding, gene transcription and translation, mRNA trafficking, cytoskeleton organization, cell adhesion, chromatin remodeling, zinc sensing and so on (Laity et al. 2001). The RING finger gene family member, MGP is associated with mosquito gametogenesis (Zhao et al. 2000). Another important target of ast-mir-2502 is the metalloprotease–disintegrin (ADAM), which plays a key role in fertilization, proper axonal guidance, neural and insect wing development (Schlöndorff and Blobel 1999). The *ast-mir-2559* targets the Tweedle protein coding regions which is known to determine the body shape and directly linked to morphogenesis. These are found only in insects where the Tweedle protein in incorporated into the larval cuticular structures (Guan et al. 2006).

This study predicts significant outcomes for the *ast-mir-9891* binding with the predicted targets. For example, the eIF-2B (Eukaryotic Initiation Factor) is a protein translation initiator. Thus, the interaction of microRNA with eIF-2B target can lead to regulation of proteins that could be potentially fatal for the mosquitoes. Another important target is the *sine-oculis* gene that has molecular functions such as sequence-specific DNA binding transcription factor activity; RNA polymerase II distal enhancer sequence-specific DNA binding transcription factor activity; transcription factor binding; sequence-specific DNA binding; protein binding etc. The Regulator of Chromosome Condensation (RCC1) target is a eukaryotic protein that acts as a signaling molecule and sends inhibitory signals on detecting unreplicated DNA molecules as the cell cycle progresses from Synthesis phase to Mitotic phase [M phase] (Dasso 1993). All predicted targets have crucial biological roles ranging from insect morphogenesis to gametogenesis.

This study provides interesting leads on the mosquito specific microRNAs and their potential targets. In future, experimental studies will be required to validate these predictions. Our hope is to find novel mosquito specific targets and their binding molecular partners, towards effective control of malaria.

Table 2 List of predicted targets of three novel miRNAs with NCBI accession numbers and biological roles. No predictions were found for *ast-mir-3868*

Novel miRNA	Predicted target	NCBI Accession ID	Biological role
ast-mir-2502	Protein coding	NP_609025.1	Unknown
	Ankyrin-repeats; transient receptor	XP_001869048.1	Receptor for potential calcium channel and ankyrin repeats
	Adamts-7	XP_001650963.1	ADAMs (A disintegrin and metalloprotease)
	Protein coding	XP_002073043.1	Unknown
	Hypothetical protein TcasGA2_TC004092	EFA12299.1	Unknown
	Acid phosphatase-1	XP_001866993.1	Histidine phosphatase domain
	Conserved hypothetical protein	XP_001864938.1	Ion channel
	HL01250p	AAO39597.1	Lysophospholipid acyltransferases (LPLATs)
	GM22668	XP_002039548	Putative zinc finger motif, rRNA processing region
	GM23264 GF15935	XP_002042537 XP_001967304	Cytochrome P450; RING-finger (Really Interesting New Gene) domain
	Latent nuclear antigen	XP_001847559	The GH18 (glycosyl hydrolase, family 18) type II chitinases
	GE23967	XP_002098411	NAD(P) binding domain of glutamate dehydrogenase
ast-mir-2559	TweedleN	NP_733160	TweedleN
	Conserved hypothetical protein	XP_001870193	Hypothetical protein
	GK15325	XP_002065205	Nucleoside transporter
	GF21757	XP_001966998	RhoGAP_ARAP. ARAPs (also known as centaurin deltas); ankyrin repeat ras associating
ast-mir-9891	GK15450	XP_002065438.1	Leucine Rich site
	eIF2B-gamma protein	NP_001037654.1	eIF-2B is a eukaryotic translation initiator
	zinc finger DHHC domain	XP_001842726.1	Gene regulation
	GH12211	XP_001991169.1	Putative Rho binding site
	Uridine-cytidine kinase 1-like 1	NP_001167608.1	Uridine monophosphate kinase (UMPK, EC 2.7.1.48)
	Hypothetical protein AaeL_AAEL006116	XP_001651804.1	Regulator of chromosome condensation (RCC1) repeat regions
	AGAP011049-PA, partial	XP_560377.3	MAEBL protein coding
	AGAP012236-PA, partial	XP_001689248.1	Sine oculis-binding protein; Extensin-like region
	Hypothetical protein TcasGA2_TC001087	EEZ98574.1	Ankyrin repeats; leucine rich repeats

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Conflict of interest The authors declare that they have no conflict of interest.

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