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A Highly Efficient Computational Strategy for Unraveling MicroRNA Profiles in the Aquatic Larvae of the Mosquito Disease Vector *Culex quinquefasciatus*

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ABSTRACT

In the intricate life cycle of *Culex quinquefasciatus*, characterized by an aquatic phase, the mosquito undergoes key developmental stages in water bodies. The study emphasizes the significance of this aquatic environment, shedding light on the molecular intricacies of the mosquito's larvae, a pivotal aspect in the broader context of its life cycle and ecological interactions. MicroRNAs (miRNAs), the small non-coding RNA sequences, play critical roles in cell differentiation and development in insects, including processes such as moulting, behaviour, metamorphosis, embryogenesis, and insect vector-pathogen interactions. Mosquitoes are implicated as vectors of many serious diseases, including malaria, Dengue fever, and other neglected tropical diseases. One of the most notable characteristics of miRNAs is their high conservation across highly divergent species. In this study, we employed two homology search methods to identify new miRNAs in the genome of the *Culex quinquefasciatus* mosquito. By using the fruit fly *Drosophila melanogaster* known miRNAs as reference sequences, we successfully identified 51 candidate miRNAs, along with their corresponding pre-miRNAs in *Cx. quinquefasciatus*. Among these candidates, 29 predicted pre-miRNAs exhibited similarity to pre-miRNAs already known in other insect species, ranging from 80% to 100% in similarity. Additionally, our homology search approach led to novel discovery of three new pre-mirs (mir-263b, mir-9c, mir-307) and their respective positions in the entire genome of *Cx. quinquefasciatus*. The identification of these newly discovered miRNAs in *Cx. quinquefasciatus*, opens possibilities for utilizing these small molecules in the development of novel control approaches. By expanding our knowledge of miRNAs repertoire in this mosquito species, we can potentially target these molecules to develop more effective strategies for mosquito-borne diseases control.

INTRODUCTION

Mosquitoes, ubiquitous insects with a global presence, are recognized for their significant impact on public health due to their role as vectors for various infectious diseases, including malaria, Dengue fever, and other neglected tropical diseases (El-Bahnasawy *et al*., 2013). While much attention has been given to the adult mosquito's ability to transmit

diseases, it is crucial to understand their life cycle, which includes a distinctive aquatic stage. Mosquitoes lay their eggs in water, and the larvae develop and mature in aquatic environments before emerging as flying adults (Rejmánková *et al*., 2013). This unique life history underscores the importance of water bodies, such as stagnant pools and containers, in mosquito breeding. Interestingly, these aquatic stages also make mosquitoes vulnerable to predation and serve as a crucial component in the intricate balance of aquatic ecosystems (Vasquez *et al*., 2020). Numerous organisms, ranging from insects to amphibians, act as both predators and prey during the mosquito's aquatic phases (Roux and Robert, 2019). Understanding the interactions within these ecosystems provides valuable insights into the ecological dynamics that influence mosquito populations. In this context, exploring the molecular aspects of mosquito biology, particularly the role of microRNAs (miRNAs), becomes imperative. By shedding light on the molecular intricacies of mosquito biology, this research not only expands our understanding of their life cycle but also holds promise for innovative approaches in controlling mosquito-borne diseases.

MicroRNAs (miRNAs) are small non-coding RNA sequences of 19-25 nucleotides in length that have proven effective in regulating gene expression during post-transcription in a variety of organisms, including insects (Lucas and Raikhel, 2010). In recent years, there has been a rapid recognition of the significance of MicroRNAs (miRNAs) as a crucial class of short endogenous non-coding RNAs after Lee and his colleagues (1993) discovered it for the first time (Eddy, 2001; He and Hannon, 2004; Choudhuri, 2010; Ma *et al*., 2021). These miRNAs play a vital role in post-transcriptional regulation of gene expression. They achieve this regulation by binding to specific target sites within messenger RNAs (mRNAs) through base-pairing interactions (Lucas and Raikhel, 2013; Chipman and Pasquinelli, 2019; Abbas *et al*., 2023). MicroRNAs exert control over various cellular pathways, encompassing processes from development to carcinogenesis in both animals and plants. Within the cell's cytoplasm, miRNAs predominantly exist in their mature form, comprising RNA molecules of 19-25 nucleotides (nt) in length.

In most cases, the miRNA "seed sequence," consisting of 2-8 nucleotides at the 5' end of the miRNA, which binds to the 3' untranslated regions (3'UTR) of mRNA molecules. This binding event leads to the regulation of mRNA translation or mRNA degradation, thereby influencing gene expression (Bartel, 2004). Notably, the first microRNA, lin-4, was characterized in 1993 by Ambros and colleagues in the microscopic worm *Caenorhabditis elegans* (Lee *et al*., 1993). The name "lin" was derived from "lineage" highlighting its role in regulating cell lineage patterns of division in *C. elegans* (Lee *et al*., 2004). The full significance of miRNA regulation came to light with the discovery of the highly conserved miRNA called let-7 (*Lee et al*., 1993). It was found that let-7 is conserved across a wide range of animals, including flies and humans. The name "let" was chosen because worms deficient in this gene exhibited premature lethality (Reinhart

et al., 2000). Since the discovery of let-7 and its conservation, numerous tools and techniques have emerged, enabling the identification of new miRNAs in various organisms. These advancements have paved the way for further exploration of miRNA functions and their roles in different biological processes.

The experimental detection of miRNAs can be accomplished through conventional techniques such as cloning, northern blotting, and microarray analysis (Varallyay *et al*., 2008; Cissell *et al*., 2009; Kim *et al*., 2010; Niu *et al*., 2015; Akmal *et al*., 2017; Ye *et al*., 2019). However, these methods have demonstrated limitations in terms of their effectiveness and time consumption. Moreover, one of the challenges in the experimental validation of miRNAs is that their expression may be induced only in response to specific signals or during certain developmental stages. This dynamic nature of miRNA expression poses difficulties in capturing their presence and activity using traditional experimental approaches.

Therefore, alternative methods and strategies have been developed to overcome these challenges and improve the detection and validation of miRNAs (Cacheux *et al*., 2019; Rojo and Busskamp, 2019). To address the limitations of conventional techniques, researchers have turned to next-generation sequencing (NGS) as a powerful tool for miRNA detection (Liu *et al*., 2011). Next-generation sequencing encompasses a range of sequencing platforms and algorithms that have demonstrated high sensitivity and reliability in identifying miRNAs and uncovering their roles in regulating various biological processes (Kolanowska *et al*., 2018). Unlike traditional methods, NGS allows for the precise identification of non-conserved or weakly expressed miRNAs, which may have been challenging to detect using previous approaches (Buermans *et al*., 2010). The application of NGS technology has significantly advanced our understanding of miRNA biology and has contributed to the discovery of novel miRNAs and their functional characterization (Saini *et al*., 2021). By leveraging the capabilities of NGS, researchers can explore miRNA expression profiles comprehensively and gain insights into their regulatory functions in diverse biological contexts (Tam *et al*., 2014; Saini *et al*., 2021).

The advent of high-throughput sequencing (NGS) has significantly contributed to the accumulation of miRNA-related data, leading to the discovery of thousands of miRNAs in various organisms, including plants, animals, and insects. The exponential growth of miRNA data has prompted the development of bioinformatics tools (TargetScan, miRanda, and PicTar) and databases (miRbase, miRDB, mirWalk, mirTarBase, etc.) to effectively manage and analyze this wealth of information and harness the large numbers of genomes sequenced in recent years, especially in insects (Moore *et al*., 2016; Bellato *et al*., 2019). These resources primarily focus on two computational challenges: 1) miRNA prediction and validation in a genome, and 2) identification of miRNA targets.

In miRNA prediction, two main approaches are commonly employed: a) homology modeling and b) machine-learning (Quillet *et al*., 2021; Guzmán-Lorite *et al*., 2023). **Homology modeling** relies on the knowledge "learned" from previously identified miRNAs in related organisms' genomes. While this approach can effectively predict miRNAs based on existing information, its limitation lies in its inability to identify novel miRNAs. On the other hand**, machine-learning** approaches are specifically designed for the organism under study, utilizing algorithms derived from that organism's own data rather than relying on information from unrelated organisms. This approach allows for the discovery of miRNA genes without additional prior knowledge (Yousef *et al*., 2016).

The emergence of these computational tools and databases has revolutionized miRNA research by facilitating the extraction and analysis of miRNA-related data. They provide valuable resources for both predicting and validating miRNAs in genomes, as well as identifying their targets, enabling researchers to gain deeper insights into the regulatory roles of miRNAs in various biological processes (Guzmán-Lorite *et al*., 2023).

In the prediction of miRNAs, several key features are taken into consideration. These include sequence conservation across different species and specific structural characteristics such as hairpin structures and minimal folding free energy (Ha *et al*., 2008; Riffo-Campos *et al*., 2016). Early bioinformatics tools primarily relied on conserved intragenic sequences that have the potential to form hairpin structures. Examples of such tools include MiRscan (Lim *et al*., 2003) and MiRseeker (Lai *et al*., 2003), which were initially developed for nematodes and flies, respectively. However, one drawback of this approach is its limited ability to identify novel miRNAs. To address this limitation, machine learning algorithms have been developed (Parveen *et al*., 2019, Ben Or and Veksler-Lublinsky, 2021). These algorithms leverage information from previously validated miRNAs, using them as positive standards. By training on these known miRNAs, machine learning models can learn patterns and features indicative of miRNA sequences. This approach enhances the ability to identify novel miRNAs that may not exhibit the same level of conservation or structural characteristics as the previously known ones (Parveen *et al*., 2019; Ben Or and Veksler-Lublinsky, 2021).

Machine learning algorithms offer the advantage of flexibility and adaptability (Sætrom and Snøve 2007; Stegmayer *et al*., 2019). They can incorporate diverse features and utilize sophisticated classification techniques to improve the accuracy of miRNA prediction. These algorithms are trained on datasets that include both positive miRNA examples and non-miRNA examples, allowing them to distinguish between true miRNA sequences and other non-functional sequences. By integrating machine learning approaches into miRNA prediction, researchers have been able to overcome the limitations of purely conserved sequence-based methods. This has greatly expanded the scope of miRNA discovery, enabled the identification of previously unknown miRNAs and advanced our understanding of their roles in gene regulation (Sætrom and Snøve,

2007; Stegmayer *et al*., 2019; Parveen *et al*., 2019; Ben Or and Veksler-Lublinsky, 2021; Azari *et al*., 2023).

In miRNA prediction, various algorithms are employed, including Hidden Markov Model (HMM), Naïve Bayes Classifier (NBC), and Support Vector Machine (SVM) (Shen *et al*., 2012; Akhtar *et al*., 2016). In this study, the HMM approach is utilized for miRNA prediction, specifically using the HMM-based tool ProMiRII (Nam *et al*. 2006). This tool incorporates several filtering criteria, such as C/C ratio, conservation score, entropy, and free energy, to enhance the accuracy of miRNA prediction.

By adjusting these filtering criteria, ProMiRII can predict both conserved and non-conserved miRNA genes. The integration of multiple methods in miRNA prediction aims to detect hairpin or stem-loop structures while reducing the number of false positive results. Many algorithms prefer shorter nucleotide sequences (less than 500 nts) for predicting RNA secondary structures due to the faster folding capabilities of shorter sequences.

The availability of the *Culex quinquefasciatus* (*Cx. quinquefasciatus*) genome has provided a valuable resource for computational homology searches of miRNA genes. Using mature miRNAs from *Drosophila* (Gesellchen and Boutros, 2004) as a reference against the genome of *Cx. quinquefasciatus*, researchers have employed computational approaches to predict miRNAs in mosquitoes. These approaches rely on sequence information and the structural characteristics of previously identified miRNAs.

Studies on the computational identification of mosquito miRNAs have thrived since Wang and his colleagues' work in 2005, relying on both structure and sequence alignment for the identification process. Since then, an increasing number of miRNAs have been discovered (Winter *et al*., 2007; Li *et al*., 2009; Puthiyakunnon *et al*., 2013; Dritsou *et al*., 2014; Jain *et al*., 2015; Lei *et al*., 2015; Feng *et al*., 2018; Xu *et al*., 2021).

The aim of this study is to computationally predict different miRNAs in *Cx. quinquefasciatus* as a preliminary step for further work to understand the expression patterns of miRNAs in different mosquito tissues provides valuable insights into their roles and regulatory functions. So, the researchers can unravel the specific roles of miRNAs in mosquito development, physiology, and response to environmental stimuli. These findings contribute to our knowledge of mosquito biology and have implications for vector control strategies and disease transmission.

MATERIALS AND METHODS

Data collection (Gather relevant genomic data from the target organism)

The genome of the southern house mosquito (*Cx. quinquefasciatus*), Johannesburg strain (Arensburger *et al*., 2010), was used for this study. The entire genome was sequenced and assembled into 3,171 supercontigs using the ARACHNE whole genome assembly package (Batzoglou *et al*., 2002). Up to this point, none of these supercontigs have been

assigned to specific chromosomes. The current version of the whole genome shotgun project is identified by the accession number AAWU01000000.

Data Availability

All the curated and annotated data of the assembled *Cx. quinquefasciatus* genome sequence are publicly available at VectorBase (https://vectorbase.org/vectorbase/app) or EVuPathDB (https://veupathdb.org/veupathdb/app).

Genome Information

The estimated genome size of *Cx. quinquefasciatus* is 579.06 Mega base-pairs (Mb) (Mb= 1^3kb= 10^6bp) (Arensburger *et al*., 2010, VectorBase last rel. 49, 2020-NOV-05.). A total of 18,965 protein-coding genes have been identified in the genome. Additionally, there are 828 non-coding sequences that do not code for proteins, out of which only 90 have been identified as precursor miRNAs (https://vectorbase.org/vectorbase/app/search/organism/GenomeDataTypes/result?filterTerm=Culex%20quinquefasciatus%20Johannesburg) (Arensburger *et al*., 2010, VectorBase last rel. 49, 2020-NOV-05.).

Identification of miRNA Candidate Sequences

Sequence alignment tools and filtering criteria

To identify miRNA candidate sequences in the targeted *Culex* genome, a Basic Local Alignment Search Tool (BLAST) search was performed using previously identified pre-miRNAs as queries. The BLAST search was conducted with sensitive parameter settings, including a word-length of 7, an E-value cutoff of 0.001, a scoring matrix of BLOSUM 6.2, and low-complexity masking. In this study, the ProMiRII web server (http://cbit.snu.ac.kr/~ProMiR2/) was used for general miRNA prediction (Nam *et al*., 2006). Additionally, the length difference between the potential *Cx. quinquefasciatus* pre-miRNAs and their counterparts in the reference set were required to be less than 5 nucleotides, based on findings by He *et al*. (2008) and Singh & Nagaraju (2008).

Annotating and categorizing predicted microRNAs based on genomic location, target genes, and other relevant features.

The alignment provided insights into the evolutionary relationships, conservation patterns, and potential functional implications of the identified pre-miRNAs in *Cx. quinquefasciatus*. The ClustalW2 program, available from the European Bioinformatics Institute (EBI), offers a user-friendly interface for conducting sequence alignments and generating alignment results. It was accessed freely through the URL (http://www.ebi.ac.uk/Tools/clustalw2/index.html) to perform the necessary alignments and analyze the results in the context of their study.

The web-based tool is designed to search for potential miRNAs in a given sequence or in its vicinity within various model organisms, including D. melanogaster, which was used as a reference genome sequence in our study.

RESULTS

Direct computational prediction of miRNAs from whole *Cx. quinquefasciatus* genome

Target region preparation

To predict miRNAs, the genome of *Cx. quinquefasciatus* was divided into equal-sized segments called microcontigs (micons), each measuring 10 kilobases (Kb). To ensure overlapping coverage, each micon had a 50-nucleotide overlap at either end of each micon. For analysis, a specific region of the genome spanning 280 Kb was selected, starting from position 1 and ending at position 280,000 nucleotides. This region consisted of a total of 28 micons (Fig. 1, 2). Among these micons, 23 (81.1%) yielded 51 predicted candidate pre-miRNAs. The details of these predicted pre-miRNAs are provided in Table (1). However, the remaining five micons (17.9%) were not scanned for pre-miRNA detection due to the lack of specified criteria, including minimum free energy (MFE) with a default value of -25 Kcal/mol, G/C ratio ranging from 0.3 to 0.7, conservation score (C-score) ranging from 0 to 1, and entropy ranging from 0 to 2.

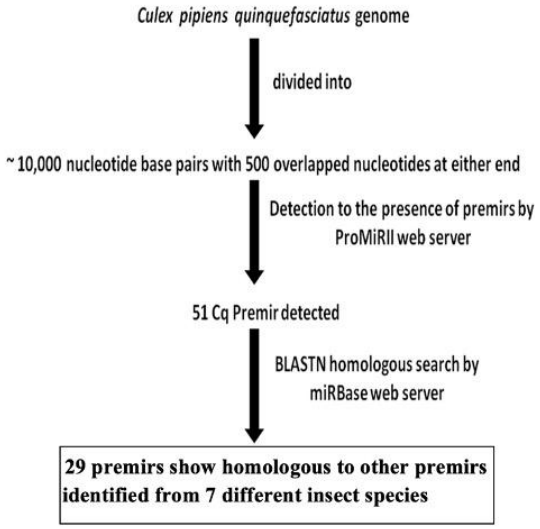

Fig (1): A flow-chart representing the computational strategy used for identifying *Cx. quinquefasciatus* miRNAs.

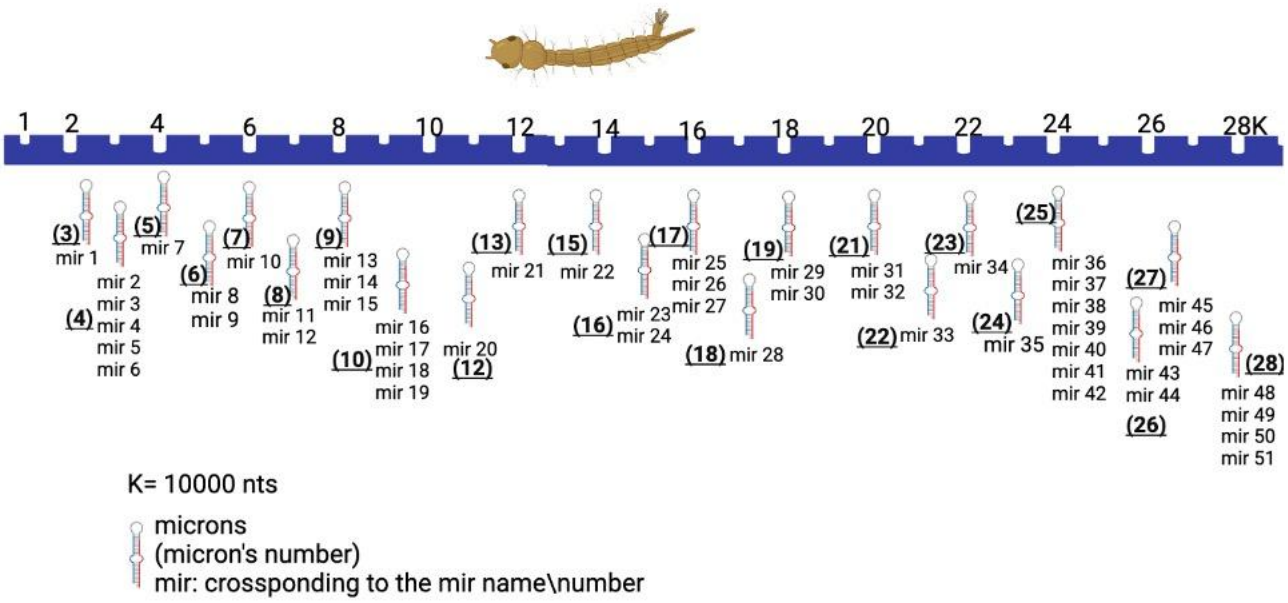

Fig (2): A sketch to the predicated pre-mir(s) mapped on the micons of target selected genomic region of *Cx. quinquefasciatus*.

Table (1): A list of the identified pre-miRNAs, along with their positions on the Micons of the target genomic region in *Culex quinquefasciatus* "Johannesburg strain" as a refence.

Micon number	**Micon genomic locus (start and end nt)**	**Micon length (nt)**	**CpqPre-mir Identified (This study)**
1	No pre-mir was predicated		
2	No pre-mir was predicated		
3	19844-29809	9965	CpqPre-1
4	29759-39719	9960	CpqPre-2 / CpqPre-3 CpqPre-4 / CpqPre-5 CpqPre-6
5	39669-49621	9952	CpqPre-7
6	49571-59531	9960	CpqPre-8 / CpqPre-9
7	59481-69441	9960	CpqPre-10
8	69391-79351	9960	CpqPre-11 / CpqPre-12
9	79301-89298	9997	CpqPre-13 / CpqPre-14 CpqPre-15
10	89248-99191	9943	CpqPre-16 / CpqPre-17 CpqPre-18 / CpqPre-19

11	No pre-mir was predicated		
12	109051-119011	9960	CpqPre-20
13	118961-128941	9980	CpqPre-21
14	No pre-mir was predicated		
15	138801-148624	9823	CpqPre-22
16	148574-158527	9953	CpqPre-23 / CpqPre-24
17	158477-168436	9959	CpqPre-25 / CpqPre-26 CpqPre-27
18	168386-178353	9967	CpqPre-28
19	178303-188263	9960	CpqPre-29 / CpqPre-30
20	No pre-mir was predicated		
21	198123-208083	9960	CpqPre-31 / CpqPre-32
22	208033-218015	9982	CpqPre-33
23	217965-227956	9991	CpqPre-34
24	227906-237866	9960	CpqPre-35
25	237816-247776	9960	CpqPre-36 / CpqPre-37 CpqPre-38 / CpqPre-39 CpqPre-40 / CpqPre-41 CpqPre-42
26	247726-257686	9960	CpqPre-43 / CpqPre-44
27	257636-267604	9968	CpqPre-45 / CpqPre-46 CpqPre-47
28	267554-277514	9960	CpqPre-48 / CpqPre-49 CpqPre-50 / CpqPre-51

Predication of the candidate pre-miRNAs

The identification of predicted pre-miRNAs in *Cx. quinquefasciatus* was followed by a search for their homologous mature miRNAs using the miRBase web server (http://www.mirbase.org/) (Release 22.1: October 2018) (Griffiths-Jones *et al*., 2008), which serves as a comprehensive repository of known miRNAs for sequence comparisons to the predicted pre-miRNAs homologues in other organisms deposited in miRBase.

Out of the 51 predicted pre-miRNAs in *Cx. quinquefasciatus*, 29 pre-miRNAs (approximately 57%) showed homology to pre-miRNAs in other insect species with a similarity of at least 80% (cpqPre-10 "this study" against ame-mir-190 "present in *Apis mellifera"*) and in some cases, the similarity reached 100% (cpqPre-6 "this study" against tca-mir-11632 "present in *Tribolium castaneum"*). This indicates that these 29 pre-miRNAs are conserved across different insect species, suggesting their conserved

potential functional importance. The remaining 22 pre-miRNAs may have homologous miRNAs in insects and/or other model organisms, but with a similarity percentage lower than 80% such as in (cpqPre-22 “this study” against bmo-mir-279c “of *Bombyx mori”*). Further investigation and comparative analysis may be required to determine the conservation and functional relevance of these pre-miRNAs in other organisms.

Homology search for the corresponding mature miRNAs against miRBase

The second approach used well-studied and previously known pre-mirs in many insect species as query sequences to search for homologous pre-mirs in *Cx. quinquefasciatus* Genomic Supercontigs (http://cpipiens.vectorbase.org/index.php) by using BLAST tool. These pre-mirs include mir-1, let-7, mir-263b, mir-276-1, mir-307 mir-315 mir-7, mir-9c, Bantam, mir-87 and as a training gene set. The sequences of these pre-mirs were collected from the miRNAs specific archive database miRBase (http://www.mirbase.org/).

Homology search for the corresponding mature miRNAs with the basic local alignment tool (BLAST)

BLAST homology search identified a homologous sequence in *Cx. quinquefasciatus* genome for each pre-mir in the training set. The predicted sequence was then used to search miRBase for all potential homologues in the mir family of each one of them.

By aligning the predicted *Cx. quinquefasciatus* pre-miRNAs with other identified miRNA sequences, we compared and analyzed the similarities and differences between the mature sequences.

Out of the 10 selected pre-miRs used as a pilot test in this study (Table 2), 7 pre-miRs (mir-1, let-7, mir-276, mir-315, mir-7, bantam, and mir-87) were found to be located on the same identified supercontigs that are currently available on Vector Base (rel. 49, 2020) (downloaded from the non-coding RNAs file) (last accessed in 2023). The successful identification of miRNAs in the genome of *Cx. quinquefasciatus*, as well as in other insect genomes like *Phlebotomus papatasi* (which had no previously identified miRNAs until 2023), highlights the effectiveness of the method used in this study. However, the remaining three predicted pre-miRs (mir-263b, mir-9c, and mir-307) were found at positions 3.31, 3.83, and 3.16, respectively, on the supercontigs. These newly identified three pre-miRs have not been identified in miRBase or VectorBase to-date (Table 2).

Table (2): A comparison of 10 selected microRNAs positions as reported on different supercontigs in VectorBase database and their correspondence predicated in this study (based on the homology search method)

Mirs name	Position of mirs on supercontig according to VectorBase	Position of mirs on supercontig according to homology search method
mir-1	3.78	3.78
Let-7	3.4	3.4
mir-263b	NOT IDENTIFIED	3.31
mir-276-1	3.136	3.136
mir-315	3.438	3.438
mir-7	3.1	3.1
mir-9c	NOT IDENTIFIED	3.83
bantam	3.65	3.65
mir-87	3.431	3.431
mir-307	NOT IDENTIFIED	3.16

DISCUSSION

In recent years, a group of small RNAs called microRNAs (miRNAs) has been discovered, and they are known to play vital roles in the post-transcriptional regulation of gene expression in various organisms, including insects (Eddy, 2001; He and Hannon, 2004; Choudhuri, 2010; Ma *et al*., 2021). Given their diverse functions in controlling biological and cellular processes, the identification and understanding of miRNAs have become crucial (Vidigal and Ventura, 2015). Various approaches have been employed to predict miRNAs, including forward genetics and direct cloning, as well as bioinformatics and computational prediction methods (Varallyay *et al*., 2008; Cissell *et al*., 2009; Kim *et al*., 2010; Niu *et al*., 2015; Akmal *et al*., 2017; Ye *et al*., 2019).

The computational prediction of miRNAs has gained significant attention as a primary tool for miRNA identification (Mendes *et al*., 2009; Liu *et al*., 2014). However, it is crucial to validate the predicted miRNAs using experimental approaches to ensure the accuracy of the identified miRNAs. Experimental validation is necessary to the avoid potential drawbacks of in silico identification, including false negatives (missed identification) or false positives (misidentification). Although combining multiple methods for miRNA identification is commonly preferred, computational prediction is often cited as the initial and sometimes the only approach used for identification.

In the case of insects, the reliance on computational prediction is of particular significance due to the incomplete understanding of miRNA presence in their genomes (Ma *et al*., 2021). By employing computational approaches, researchers can identify

potential miRNAs in insect genomes, opening avenues for subsequent experimental validation and further investigation. This computational exploration serves as a valuable starting point in unraveling the miRNA landscape in insects and advancing our knowledge in this field.

In comparison with the number of identified miRNAs (approximately 2000 mirs) in human and mouse, few "mirs" have been identified in mosquitoes representing only in three species (*Aedes aegypti*, *Anopheles gambiae* and *Cx. quinquefasciatus*) (Griffiths-Jones *et al*., 2008). The most identified miRNAs in Hexapoda (insects) are found in fruit flies identified from twelve *Drosophila* species with a high degree of confidence (Kozomara *et al*., 2014). Although, mosquito miRNAs data in miRBase (74 precursors, 91 mature) and VectorBase (90 mirs) shows a controversy between the numbers of miRNAs identified by wet experiments and those identified *in silico* by computational methods (Feng *et al*., 2018).

Since 2010, research on the identification of miRNAs from the mosquito vector *Cx. quinquefasciatus* has been scarce. Only one study, conducted by Skalsky et al. (2010), reported the identification of 77 miRNAs in *Cx. quinquefasciatus*. Interestingly, these miRNAs were found to be predominantly conserved in other insects, such as the mosquito An. gambiae and the fruit fly *D. melanogaster*. The limited research conducted thus far highlights the need for further exploration and understanding of miRNAs in *Cx. quinquefasciatus* and their potential implications in relation to other insect species.

Given the significance of *Cx. quinquefasciatus* as a major vector of filariasis and arboviral diseases, which pose significant health concerns globally and particularly in Egypt, our research aimed to investigate the miRNAs in this mosquito species. The availability of the complete genome sequence data for *Cx. quinquefasciatus* further supported our decision to focus on this species for miRNA identification. Through our study on *Cx. quinquefasciatus* miRNAs, we sought to enhance our understanding of its role as a disease vector and potentially uncover unique miRNAs specific to this species.

Our findings were consistent with the results reported by Skalsky *et al*. (2010). Utilizing a homologous alignment approach, we selected 10 previously identified miRNAs for prediction. Among these miRNAs, seven exhibited identical genomic loci in the supercontigs as reported by Skalsky *et al*. (2010). Furthermore, these miRNAs displayed a high degree of sequence similarity, ranging from over 85% to 100%, with miRNAs identified in other insect species such as *Anopheles gambiae*, *Drosophila melanogaster*, *Apis mellifera*, and *Bombyx mori*. Notably, these miRNA genes were distributed across various regions of the *Cx. quinquefasciatus* genome, namely mir-1, let-7, mir-276-1, mir-87, bantam, mir-7, and mir-315.

It is noteworthy that our study uncovered three previously unannotated miRNAs, namely mir-263b, mir-9c, and mir-307, which have not been reported in miRBase or characterized in other species. These miRNAs were specifically located on supercontigs 3.31, 3.83, and 3.16 within the *Cx. quinquefasciatus* genome. The identification of these novel miRNAs in *Cx. quinquefasciatus* suggests the existence of unique miRNA sequences in this mosquito species. Further exploration and characterization of these miRNAs hold significant potential for gaining insights into their functional roles. Such investigations would contribute to our understanding of miRNA diversity in *Cx.* quinquefasciatus and shed light on their implications in mosquito biology, including their involvement in vectorial capacity for disease transmission and the development of effective vector control strategies (Feng *et al*., 2018; Xu *et al*., 2021).

The main objective of our study was to highlight the successful computational identification of mir-263b, mir-9c, and mir-307 in the mosquito vector *Cx. quinquefasciatus.* It is noteworthy that, and to the best of our knowledge, only one other study by Liu *et al*. (2014) has reported on the differential expression of some of these miRNAs in males and females of the mosquito *An. anthropophagus*. However, these miRNAs have not been annotated or described in miRBase or VectorBase at the time of our study.

The identification of these miRNAs in our study adds to the growing body of evidence regarding their existence and potential functional roles in mosquitoes. The lack of annotation in existing databases emphasizes the importance of continued research and exploration of miRNA diversity in various species, including *Cx. quinquefasciatus.* Further investigations are warranted to elucidate the biological significance and regulatory mechanisms associated with mir-263b, mir-9c, and mir-307 in this mosquito species.

The study of the *Cx. quinquefasciatus* genome provides a valuable resource for investigating various mechanisms involved in mosquito development, survival, and vectorial capacity for disease transmission. The high sequence similarity between *Cx. pipiens* and other related organisms such as *An. gambiae* and the fruit fly *D. melanogaster*, which are all dipteran insects, allows for the identification of *Culex* miRNAs based on the conservation between their genomes. It is important to note that relying solely on computational prediction of miRNAs based on their structural characteristics would result in a high false positive rate.

Comparative bioinformatics and genomics approaches have played a crucial role in validating the computational predictions of miRNAs. These approaches utilize miRNAs identified in related organisms and leverage sequence and structural similarities to confirm the presence of miRNAs and enhance their accuracy. The field has witnessed the development of various computational tools that rely on comparative genomics, which

have proven to be effective in identifying regulatory elements and non-coding RNA genes, including miRNAs.

The value of these methods was exemplified by a study conducted by Lai *et al*. (2003), where several miRNAs were identified through the structural analysis of conserved genomic sequences between the nematodes *C. elegans* and *C. briggsae*. This study highlighted the importance of comparative analysis in elucidating the presence and characteristics of miRNAs, showcasing the utility and significance of these computational approaches in miRNA research.

In the present study, we depended on the identification of miRNAs from the *Cx. quinquefasciatus* genome on two different methods; considering the homology search method as a second line of evidence for the identity of those miRNAs identified by the machine learning method. From 280 kbp nearly 0.048% of the *Cx. quinquefasciatus* genome (~579Mbp), direct identification of 51 pre-mirs (Cpqmirs) were predicted. Out of 51 predicted Cpqmirs, 29 Cpqmirs showed high homology to other pre-mirs in different insect species. From our analyses of predicted miRNAs, 4 Cpqmirs have potential homologues in *An. gambiae* pre-mirs (Aga-mirs) from total 130 mirs predicted according to miRBase (Release 22.1: October 2018, last accessed on 2023) (Kozomara *et al*., 2014). For aga-mir-7, it has homology to both Cpq-pre-mir-22 and Cpq-pre-mir-51. Aga-mir-7 belongs to gene family mir-7, its chromosomal locus in *An. gambiae* genome is in intron 9 of 2L (left arm) (Biryukova *et al*., 2014). It has been evidenced by similarity to its homologue, dme-mir-7 in *Drosophila* according to Stark *et al*. (2003). Furthermore, the gene targets for mir-7 in *Drosophila* were identified by computational prediction followed by experimental validation. miR-7 regulates a family of Notch pathway targets including the Enhancer of split and Bearded complex genes Tom and m4, and the basic helix-loop-helix transcriptional repressors HLHm3 and hairy (Stark *et al*., 2003). For aga-mir-309, it belongs to gene family mir-3, its chromosomal locus is 3R (right arm) and intergenic region. It has been evidenced by similarity to dme-mir-309 (Griffiths-Jones, 2008). For mir-315, it belongs to the gene family mir-315, its chromosomal locus is in 2R/ intergenic. Its evidence has been notified by the similarity to dme-mir-315 (Lai *et al*., 2003). For mir-87, it occurs within chromosome X/intergenic region. Its gene family is mir-87. This mature miRNA was cloned from *An. stephensi*, and mapped to the *An. gambiae* genome (Mead *et al*., 2008). For the last homologue, mir-9c, it belongs to mir-9 gene family. Its chromosomal locus is 3R/ intron 5. This mir has been verified by its high similarity to dme-mir-9c. This mir sequence was predicted based on conservation in *D. pseudoobscura*, but its expression was not determined (Lai *et al*., 2003). Aravin and colleagues (Aravin *et al*., 2003) have described the small RNA profile of *D. melanogaster* as a function of development by cloning and sequencing over 4000 small RNAs, from which mir-9c was identified.

Compared to *Drosophila* species mirs, a total of 23 Cpq-premirs have potential homologues to mirs identified from 12 *Drosophila* species, which ranged from 71 mirs in *D. mojavensis* to 258 mirs in *D. melanogaster* (Release 22.1: October 2018, last accessed in 2023) (Kozomara *et al*., 2014). This fluctuation in the numbers of homologous mirs may refers to the updating genome annotation of more dependent-on model organisms such as D. *melanogaster*. Also, it may be due to the difference in the complexity of lifestyle and biology between the vector mosquito and the fruit fly.

According to *Culex* Cpq-premirs identified by the homology search method, many different Cpq-premirs are homologous to the same mirs predicted from miRBase using BLASTn. The high number of homologues to our predicted Cpq-premirs was found in both *C. elegans*/*C. briggase* and *D. melanogaster* with 35 and 24 pre-mirs, respectively, indicating that they are the uppermost studied organisms in the identification of miRNAs. Out of the 51 predicted Cpq-premirs, 2 mirs (Cpq-pre-mir22, Cpq-pre-mir51) have homologues in other insect species, such as tca-miR-7-5p and tca-miR-3831-3p respectively. For example, Mir-9c has been predicted by Lai *et al*. (2003) based on comparative conservation in *D. pseudoobscura*, and it belongs to mir-9 family.

Because miRNAs influence the stability and translation efficiency of mRNA, they play broad and key regulatory roles in many important biochemical processes and pathways such as cellular proliferation, development, fat metabolism, behavior and embryogenesis in different organisms as well as tumor genesis in humans (Bartel *et al*., 2004; Zhang *et al*., 2009). For insect metamorphosis, Gomez-Orte and Belles (2009) have studied miRNA-dependant metamorphosis in cockroaches by silencing Dicer ribonucleases that are involved in the production of mature miRNAs from pre-miRNAs. They found that the depletion of Dicer-1 resulted in extending the cockroach life span to seven nymphal instars instead of only six nymphal instars and the adult stage with the wings not well stretched. In *D. melanogaster*, the loss of Dicer-1 function has led to defects in embryogenesis and disruption of olfactory neuron morphogenesis. In flies, although they appear externally normal, the knockout of let-7 resulted in behavioral defects by affecting flight and motility processes, impaired fertility and weakening of the neuromuscular junctions (Gomez-Orte and Belles, 2009). According to Zhang et al. (2009), many silkworm miRNAs have been found to be differentially expressed and stage-specific, e.g. in eggs or the pupa stage, which suggests that silkworm miRNAs function in both embryogenesis and metamorphosis. Another study determined that the expression of mir-2943 is specific for egg stage suggesting its association with the development of mosquito eggs (Liu *et al*., 2014). In addition, identification of reaper, grim and sickle as targets suggests that mir-2 family miRNAs may be involved in control of apoptosis (Stark *et al*., 2003). From all the examples listed above, it is becoming clear that miRNAs are integrally involved in the complex regulatory networks that govern the

developmental, homeostatic and physiological processes of most organisms (Zhao & Srivastava, 2007; Feng *et al*., 2018).

The recent developments in mosquito genomics have provided good opportunities to enhance our understanding of mosquito genetics, physiology, and behavior. New insights in mosquito biology will facilitate the development of novel strategies to control the different mosquito-borne diseases. Identifying the biological functions of a novel class of gene regulatory molecules such as miRNAs in *Cx. quinquefasciatus* will lead to an investigation of the potential roles of this molecule in processes that are intimately linked to mosquito vectorial capacity. In some organisms, microRNAs serve as key regulatory molecules during embryonic development, stem cell division, neurogenesis, haematopoietic cell differentiation, and cell death. They are also included in the control of viral infections and cancer (Feng *et al*., 2018).

For mosquitoes, the identification of several mosquito-specific miRNA will represent a new and great research area in vector biology that could increase our current knowledge and provide potential targets for the control of vector-borne diseases. Li *et al*. (2009) indicated that the express in levels of several miRNAs change significantly after a blood meal. For example, in *An. anthropophagus*, mir-989 is highly expressed in female mosquitoes but shows no expression detection in males ensuring the specificity of this mir to female mosquitoes (Liu *et al*., 2014).

Determination of miRNA expression profiles in *Cx. quinquefasciatus* before and at different time points after a blood meal will serve as the foundation for future research. We will focus on identifying those miRNAs and their targets to regulate processes affecting blood meal within different tissues, especially in pathogen-infected vectors. Characterization of tissue-specific miRNA expression will reveal novel and differentially regulated miRNAs that have not been detected at the whole organism's level.

Overall, this future direction will facilitate vector research by starting to fill a major gap in our understanding of mosquito biology and contribute to novel strategies to control mosquito-borne infectious diseases.

CONCLUSION

Our understanding of the functions of mosquito miRNAs largely relies on studies conducted in other species, such as the fruit fly *D. melanogaster* and the worm *C. elegans*. However, it is important to note that while miRNAs are conserved between different organisms, the ecological and physiological differences between sugar- and blood-feeding mosquitoes and sugar- feeding fruit flies can lead to variations in the roles of even highly conserved miRNAs. Therefore, it is crucial to investigate and characterize miRNAs specifically in mosquitoes to gain a comprehensive understanding of their functions in these important vectors of disease.

ETHICAL APPROVAL

All experiments in this research were approved by the Ethics Committee of the Faculty of Science, Ain Shams University, Cairo, Egypt (Approval code: ASU-SCI/ENTO/ 2023/6/5).

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