Title: Profiling microRNAs and finding their targets in the earthworm *Perionyx excavatus* during epimorphosis regeneration.

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Introduction

Regeneration is a biological process that enables the restoration of damaged body parts. However, the extent and efficacy of regeneration kinetics vary significantly among species, highlighting their distinct abilities in this regenerative process(Allen et al., 2016). Hydra and planarians possess an extraordinary ability to regenerate entire organism from a small fragment of their body parts. This remarkable regenerative capability made themselves valuable models in the field of invertebrate regeneration research and regenerative medicine. They are known to possess abundant multipotent stem cells, known as interstitial cells or neoblasts, distributed throughout their bodies, enabling them to undergo morphallactic regeneration. In contrast, vertebrate models such as axolotls, newts, and teleost fish exhibit a more restricted regenerative capacity that relies on the dedifferentiation of specialized adult cells with predetermined fates (Joven et al., 2019).Earthworms are the eco-friendly and important soil fauna organisms of the earth. Rather than that, they provide significant scientific value by serving as useful animal models for research fields, such as regeneration and toxicology. Earthworm, *Perionyx excavatus* known for its regeneration ability and vermicomposting potential. These annelids are the invertebrate model organism that demonstrates regeneration through the process of epimorphosis (Bae et al., 2020). Earthworm regeneration is usually believed to be an epimorphosis, which is characterised by the dedifferentiation of adult tissue to form a highly proliferating cell mass known as a blastema, followed by its re-specification into appropriate cell types (Ribeiro et al., 2018). For example, adult earthworms undergo a straightforward mode of growth that takes place specifically at the damage sites, rather than throughout the entire body. To accomplish this, the earthworm must possess molecular mechanisms that allow it to precisely regenerate various types of cells and tissues from pluripotent or potentially totipotent blastemal cells(Yoon et al., 2020). microRNAs are the small, noncoding RNA which partially binds to the complementary sequences of target mRNAs and control translation, and repress the mRNA stability by various mechanism, depending on the cellular context and environmental conditions (Hammell, 2008; Michlewski and Cáceres, 2019). MicroRNA Lin-4 regulate embryogenesis by controlling the developmental timings (Huang et al., 2012; Lee et al., 1993). The processing of precursor miRNA molecules takes place both in the nucleus and cytoplasm, carried out by two endoribonucleases, DROSHA and DICER, respectively. These processing events result in the creation of mature miRNAs, which are then loaded onto the RNA-induced silencing complex (RISC) to carry out their biological functions (Michlewski and Cáceres, 2019). The miRNA

duplex is incorporated into RNA-induced silencing complex (RISC) through an ATP-dependent process, with the aid of HSC70/HSP90 chaperones, by forming a complex with the AGO2 protein (Iwasaki et al., 2010). Following the formation of the complex, AGO2 unwinds the RNA duplex, and discards the passenger strand, resulting in the creation of the mature RISC complex (Kobayashi and Tomari, 2016). Once activated, the RISC identifies a specific mRNA sequence through complementary base-pairing, leading to either inhibition of translation or degradation of the RNA (Iwakawa and Tomari, 2015). Thirty percent of protein and non-protein coding genes are regulated by thousands of miRNAs (Lewis et al., 2005). miRNAs are endogenous regulators where these sncRNAs are involved in cell growth, differentiation, and apoptosis (Wienholds and Plasterk, 2005). At the transcriptional, post-transcriptional, and epigenetic levels, miRNAs play critical roles in controlling gene expression. Previous studies have shown the involvement of miRNAs in a wide range of biological processes, including cell cycle, cell differentiation, invasion, and migration (Sastre et al., 2019; Sato et al., 2011)**.**

In order to have a deeper understanding of the miRNA expression patterns linked to the process of anterior epimorphosis regeneration, we conducted a high-throughput sequencing analysis to examine the miRNAs present in the earthworm, *P. excavatus*. Using poly-A-tail based qPCR validation, the expression profiles of a representative set of conserved miRNAs are assessed. The miRNAs that have been found and their corresponding targets have also been predicted. This study will help to comprehend the functions of miRNAs role in controlling the biological processes involved in regeneration biology.

Objectives

- To Sequence and profile the microRNAs in earthworm (Perionyx excavatus).
- To Study the specific miRNAs and their peculiar role in the pattern of regeneration process.
- To examine tissue-specific expression patterns of miRNA.
- To elucidate the molecular mechanism of regeneration from the perspective of miRNA.

Materials and methods

Bedding and maintenance of earthworm

Mature earthworms, *Perionyx excavatus* were used in this study. Briefly, worms were amputated at 10^{th} segment in the anterior region for anterior blastemal sample collection. Five days were dedicated to maintaining the amputated worms in the vermibed. On the fifth day, surgical blades (Surgical Blade No.22, Lister Surgical Blades) were used to cut down the regenerated blastema along with their adjacent segments for miRNA extraction. For control, worms were gut cleaned for 48 hours by incubating the worms with moist tissue paper and after 48 hours, 1^{st}-10^{th} anterior head segments were used for miRNA extraction.

Tissue homogenization and RNA isolation

RNA was extracted from earthworm tissue using TRIzol (Cat. 15596-018; Ambion). Briefly, tissues were transferred to tommy tubes with 4 stainless steel beads and homogenized in Tommy microsmash. TRIzol reagent was added to the homogenized tissue and last cycle in Tommy microsmash was completed. The tubes were incubated at room temperature for 5 min. The phase separation was performed by adding chloroform, followed by invert mix and centrifuge at 13,200 rpm for 15 min at 4°C. The aqueous phase was transferred to fresh microcentrifuge tube and RNA was precipitated overnight at -80°C, with equal volume of isopropanol and glycogen (20µg/µl). The pellet was recovered by centrifugation and washed with 70% ethanol. The air-dried pellet was re-suspended in nuclease free water. Concentration and purity of RNA was quantified using the Nanodrop Spectrophotometer (Thermo Scientific; 2000). The miRNA concentration was quantified using Qubit microRNA assay kit (Cat. Q32880).

Library Preparation and sequencing

Small RNA sequencing (smRNA-seq) libraries were constructed using QIAseq® miRNA Library Kit (Cat. 331502) protocol (Qiagen, Maryland, U.S.A.). Briefly, 63ng of Qubit quantified total RNA was used as starting material. 3’ adapters were ligated to the specific 3’OH group of microRNAs followed by ligation of 5’ adapter. Adapter ligated fragment was reverse transcribed with Unique Molecular Index (UMI) assignment by priming with reverse transcription primers. cDNA was enriched and barcoded by PCR amplification (17 cycles). The Illumina-compatible sequencing libraries were quantified by Qubit fluorometer (Thermo Fisher Scientific, MA, USA). The fragment size distribution of the libraries was analyzed on

Agilent 2200 TapeStation. These libraries were sequenced on Illumina NovaSeq 6000 using SE50 read length and sequencing chemistry.

Data Analysis

Illumina single-end sequencing was carried out for 50 cycles on Illumina NovaSeq 6000 High Output sequencing platform following manufacturer's instructions. The raw reads were processed for filtering of specific length of 16 –40 bases and processed reads were mapped to non-coding RNA database which is useful to exclude the other RNAs such as rRNA, tRNA and snoRNAs. The unmapped reads from Rfam alignment results, which should be only small RNAs were used for classification of known, novel miRNAs and target prediction analysis.

Raw data processing

The raw data was processed by sRNA-workbenchV3.0_ALPHA1 which was used to trim 3' adapter and performed length filtering (minimum length 16 bp and maximum 40 bp). The low quality and contaminated reads were removed on the following criteria to obtain final clean reads. Elimination of low-quality reads (<q30), Elimination of 3' adapters, Elimination of reads <16bp and >40bp, Elimination of reads matching to other ncRNAs (r, t, sn, and snoRNAs).

miRNA identification

Conserved miRNAs were identified using homology approach against mature *Capitella teleta* and *Caenorhabditis elegans* miRNAs database from miRBase22. Novel miRNAs were identified based on the secondary structure prediction tool.

Novel miRNA prediction

Sequences with no homology to known miRNAs were extracted and considered for prediction of potential novel miRNAs. The sequences were initially aligned to the mRNA sequences as a reference from *Perionyx excavatus* transcriptome project (SO_10555) using bowtie2. The aligned sequences were used for novel miRNA prediction using Mireap_0.22 b5. The prediction is based on the identification of stem loop structure and miRNA with such secondary structure were reported as potential novel miRNAs. For all the predicted novel miRNAs, stem-loop structures were reported. Secondary structures have been provided in dot and bracket format [Column M] which can be visualized using RNAfold-6.

Known miRNA prediction

The final clean reads were made unique and hence read count profile was generated. Further, homology search was performed for these miRNAs against *Capitella teleta* and *Caenorhabditis elegans* miRNA sequences retrieved from miRbase-223 using ncbi-blast2.2.304 with an e-value cut-off of e^{-4} and non-gapped alignment.

miRNA target prediction

The miRNAs with copy number >=5 was considered for target prediction for known and novel miRNAs. These miRNA sequences were used as input along with reference mRNA sequences from *Perionyx excavatus* transcriptome project (SO_10555) to miRanda7 tool.

Differential gene expression

Read counts across all known and novel miRNAs were generated by taking the count of reads aligning to a particular miRNA. This information is useful in understanding the expression pattern of miRNAs. Differential Expression analysis was carried out using DESeq8 tool.

GO and Pathway analysis

Gene Ontology annotation was performed against "*Annelida*" data from the Uniprot database. Genes were assigned with a homolog protein (uniprot) from Annelida protein sequences, if the match was found with an e-value less than e-5 and minimum similarity greater than 30%. Pathway analysis was performed using KAAS9. In the KEGG database, genes in complete genomes are annotated with the KEGG orthology (KO) identifiers, or the K numbers, based on the best hit information.

qPCR validation

Five known miRNAs with different expression patterns are selected for Poly-A-tail based qPCR amplification. Briefly, total RNA was isolated from the tissue using TRIzol based protocol. Then the RNA samples were Poly-A- tailed and converted into cDNA using Micro script microRNA cDNA synthesis kit. After obtaining the cDNA, it was further diluted by a factor of ten using sterile water. To determine the gene expression levels, real-time quantitative polymerase chain reaction (qPCR) was carried out using the SSO Advance Universal SYBR Green Master Mix from Bio-Rad (USA). The qPCR protocol consisted of the following conditions: an initial cycle at 94 °C for 2 minutes, followed by 40 cycles at 95 °C for 10 seconds

and 60 °C for 30 seconds. All the reactions were performed in triplicate for each sample. The miRNA expression levels were quantified relative to a reference using the equation = $2^{-\Delta\Delta Ct}$.

Statistical analysis

Data were analysed using GraphPad Prism v.9 and presented as the mean ± standard deviation (SD). Statistical analysis was performed using multiple unpaired t test method. The data were considered statistically significant at a p-value of less than 0.05 ($p<0.05$).

Results and Discussion

Small RNA sequencing and reads removal

Currently, there is a limited literature on miRNAs and their role in earthworm regeneration, particularly in the context of epimorphosis mode of regeneration process. In order to analyse the miRNA expression, small RNA libraries from regenerated and control worms were sequenced using Illumina high-throughput sequencing technology. After trimming the reads, removing < 15 nucleotide sequences and eliminating low-quality reads, we attained 16612383 and 10788356 small RNA reads, between the range of 16-40 nucleotides from the regenerated and control worm samples, respectively. rRNA, tRNA, snRNA, and snoRNA reads present in the regenerated worm (3.04%) and control worm (5.80%) were removed based on the alignment (Table 1). Unaligned reads were taken for the identification of known and novel miRNA prediction. Based on the results, the sequencing data were enriched in small RNA sequences. In addition, analysis of length distribution of these small RNA sequences across both samples revealed a consistent pattern. The bar graph indicating the total reads obtained from sequencing (from 16 to 40 nucleotides) were shown in the (Fig 1B). The complete workflow is depicted in the (Fig 2A).

Conserved miRNA identification in *P. excavatus*

For detecting known miRNAs in *P. excavatus*, we compared the valid reads against reference miRNA database. All small RNA sequences underwent a BLASTN search using the miRbase 21.0 database of reference miRNAs. In *P. excavatus*, 55 conserved miRNAs with 55 different precursor sequences were identified. According to the size distribution of the conserved sequences, there are more than 50 miRNAs in the 22 nt length group, which has the most miRNAs overall. There are 36 miRNA families composed of these conserved miRNAs. Based on miRbase, the conserved miRNAs that were identified have been categorized into 36 miRNA

families (Table S3). Among these families, miR10 was the largest with 4 members, while the majority (79.45%) of the families consisted of only one member. Exceptionally, 8 miRNAs were not identified as family members (Pex-miR-2000-P2_5p*, Pex-miR-1996-P1_3p, Pex-miR-1993-P1_5p*, Pex-miR-193-P1o_5p*, Pex-miR-1990-P3_5p*, Pex-miR-2693-P2_3p*, Pex-miR-2707-v1_3p*, Pex-miR-2687_3p*). The overall read count matrix was shown in the (Fig 1D).

Novel miRNAs

Advantages in high-throughput sequencing aid us to explore even the low expression levels of novel miRNAs in small RNA library (Juan et al., 2014). The MIREAP software was used to identify the possible stem-loop structures in the unannotated reads. Notably, the transcripts of *P. excavatus* was used as a reference to identify the novel miRNAs and their precursors (Unpublished data). Interestingly, 50 presumed novel miRNAs were predicted, on that 22 miRNAs were from 3' arm and 28 miRNAs from 5' arm. The top 20 Novel miRNAs were indicated in the (Table S2). The ancestors of these possible novel miRNAs were detected and exhibited with appropriate secondary hairpin structures, having free energies that ranged from -25.5 kcal·mol^{-1} to -59.2 kcal·mol^{-1} (with an average of -37.2 kcal·mol^{-1}). The 3' and 5' arm miRNA prediction attributing that gene regulatory networks might be controlled by species-specific miRNAs. The average length of putative precursors of novel miRNAs ranges between 64 to 96 nt and length of the mature miRNAs ranged from 20 to 24 nt, the data was similar to *Capitella teleta* and *Eisenia fetida* Precursor. The overall read count matrix was shown in the (Fig. 1D). Secondary structures of the precursor miRNAs with novel miRNAs (red colour marked) were shown in the (Fig 1C).

Target predictions of known and novel miRNAs

The identification of possible targets is important in understanding the regulatory network of miRNA. miRanda algorithm was used to predict the mRNA targets of miRNAs, total of 2395 target were identified for 27 differentially expressed miRNAs. On the list, most of the transcripts are highly targeted by Pex-miR-2b (59 targets), Pex-miR-1996b (35 targets), Pex-miR-279 (16 targets), Pex-miR-281 (15 targets), Pex-Nov-14-3p (452 targets), Pex-Nov-16-3p (377 targets), Pex-Nov-1-5p (286 targets), Pex-Nov-17-3p (274 targets) and Pex-Nov-19-5p

(244 targets). Our study revealed that a significant proportion of the predicted targets were Regeneration-upregulated protein 2, Anaphase-promoting complex subunit 1, Leucine-rich repeat-containing protein 14, FHA domain-containing protein, Tubulin-C domain-containing protein, DNA-dependent protein kinase catalytic subunit, Frizzled-4, AAA domain-containing protein, GATOR complex protein WDR24, Signal recognition particle subunit SRP68, Nudix hydrolase domain-containing protein and so on. The potential predicted targets identified in this study insights into the specific biological processes involved in the growth and development of *P. excavatus*. However, to establish the correlation between miRNAs and these putative targets, further experimental evidence is needed for validation. List of miRNAs and the number of their putative targets were shown in the (Fig 2B).

Gene Ontology enrichment

The primary objective of this study is to gain a comprehensive understanding on the physiological functions of miRNAs and their mRNA target, elucidation of the regulatory network of miRNAs. The predicted target genes of the discovered miRNAs were subjected to GO and KEGG pathway analysis for understanding the physiological processes and pathways regulated by specific miRNAs. Cellular Component (CC), Molecular Function (MF) and Biological Process (BP) were classified based on the GO analysis of miRNA targets. Totally, 96 cellular components, 184 molecular functions and 100 biological processes were classified by miRNA target genes. Overall, 15 significantly enriched GO terms were graphed (Fig. 2C).The genes are classified into subcategories based on their biological processes, with a substantial proportion of the targets comes under the following categories actin cytoskeleton organization, carbohydrate metabolic process, DNA replication, membrane budding, vesicle-mediated transport, pre-miRNA processing, regulation of cell differentiation, organ development, intracellular transport, microtubule-based movement and neurogenesis. Cellular components were integral component of membrane, cytoplasm, nucleus, microtubule, Golgi membrane, mitochondrion, endoplasmic reticulum membrane, synaptic vesicle and extracellular space. Molecular functions were ATP binding, actin filament binding, RNA binding, DNA binding, calcium ion binding, adenosine deaminase activity, Wnt-activated receptor activity, heme binding, growth factor activity, motor activity and transcription cofactor activity. Overall, 15 significantly enriched GO terms of Novel miRNAs were graphed (Fig. 2D).

KEGG pathway analysis

In order to gain a better understanding on the regulatory roles played by miRNAs in the epimorphosis regeneration of earthworm *P. excavatus*, an enriched KEGG analysis were performed on the target miRNAs of the identified miRNAs. The analysis revealed a comprehensive annotation of 148 pathway networks, providing a universal summary of orchestrating functions of miRNAs in this process. The pathway of cytoskeleton proteins exhibited the highest level of enrichment in relation to both the rich factor and the number of genes (14 genes), followed by exosome (13 genes), membrane trafficking (10 genes), domain-containing proteins not elsewhere classified (8 genes), mTOR signalling pathway (6 genes), chromosome and associated proteins (5 genes) and wnt signalling pathway (3 genes). The findings suggest that these potential targets may be associated with the processes of growth and development, immune response, wound healing, cell proliferation, cell migration, cell survival

Differential expressed miRNA analysis

To predict the biological functions of miRNAs during *P. excavatus* regeneration, analysing their expression patterns is crucial. Thus, differential expression analyses of miRNAs performed were between the two sample of interest - the anterior head (control) and anterior blastema. In this analysis, total 15 miRNAs were significantly changed in the control and during epimorphosis regeneration (10 up-regulated and 5 down-regulated). Out of these data, Pex-miR-125, Pex-miR-71, Pex-miR-365 and Pex-miR-36 (had a log2 fold change >1 and $P<0.05$) were highly up-regulated miRNAs in this profiling. Pex-miR-1, Pex-miR-7, Pex-miR-29a and Pex-let-7 were most significantly downregulated miRNAs in this data. In addition, around 17 miRNAs are neutrally expressed in both the samples.

Poly-A-tail based qPCR validation of differentially expressed miRNAs

It is important to explore the fundamental roles of miRNAs in earthworm epimorphosis regeneration to validate and quantify the miRNAs that are differentially expressed in the blastema samples. This process of validation is crucial as it sets the groundwork for further investigation in understanding the functions of these miRNAs. Regeneration specific five miRNAs (Bantam, Pex-Let-7, Pex-miR-125, Pex-miR-210b and Pex-miR-71) were validated using poly-A-tailing based qPCR.

The expression of all five miRNAs that exhibited differential expression is consistent with the high-throughput sequencing data, as they are expressed in both the samples. Except for Pex-

Let-7 miRNA, the rest of the miRNAs are upregulated in anterior blastema sample, which was very similar to the differential gene expression. The data were shown in the (Fig. 3E).

Impact of the research in the advancement of knowledge or benefit to mankind

A total of 55 known and 50 novel miRNAs were identified from the earthworm, *Perionyx excavatus*. This study is the initial comprehensive effort to find previously undiscovered microRNAs (miRNAs) derived from the regenerated earthworm. Poly A-tailed qPCR was used to confirm the presence of five miRNAs that were highly regulated during the formation of the blastema. Different miRNAs may play significant roles in the regeneration process of epimorphosis, according to the findings. Subsequently GO and KEGG pathway enrichment of miRNA targets indicated that these small non coding RNA involved in major pathways like Wnt signalling pathway, mTOR signalling pathway, exosome and membrane trafficking. This study offers evidence suggesting that microRNAs (miRNAs) and their target genes play a significant role in coordinating the process of regeneration. Therefore, our work establishes a foundation for the advancement of miRNA-based treatments in the field of regenerative medicine.

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Figure and Figure legend

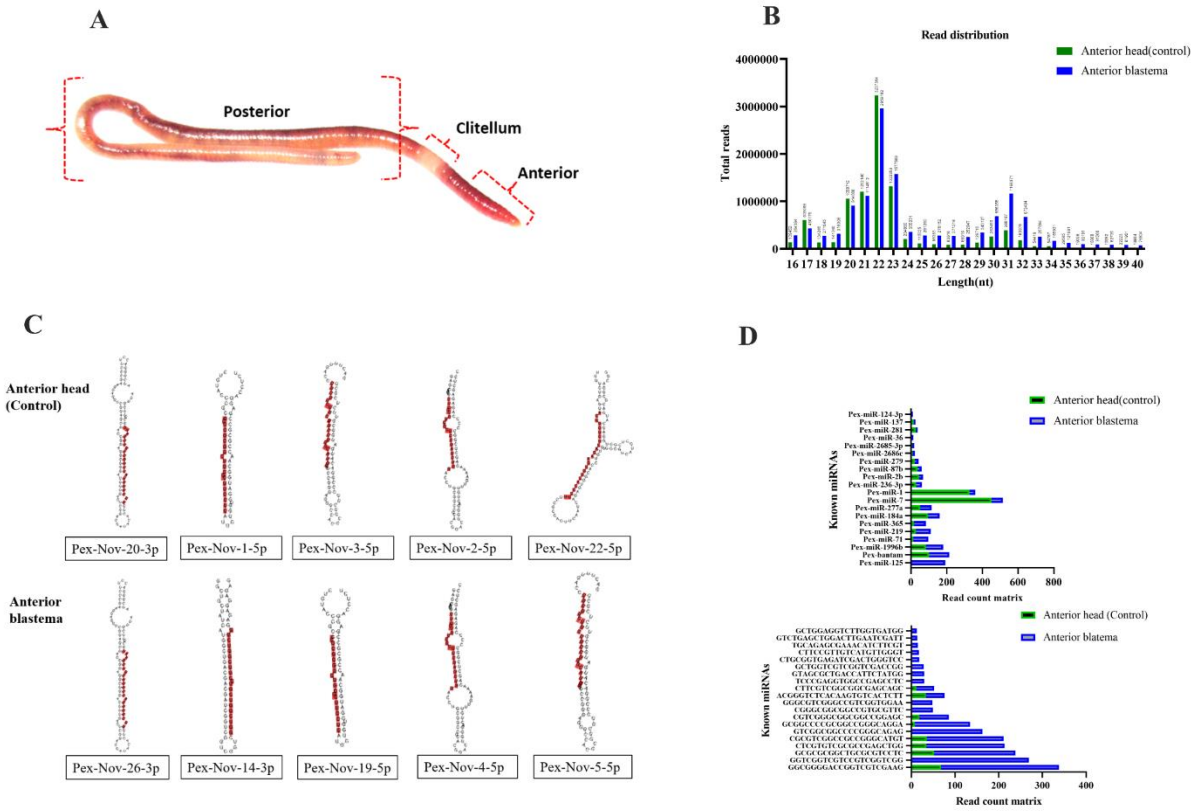

Fig. 1. A: Segmented adult earthworm *Perionyx excavatus.* **B**: Read distribution from the anterior head (Control) and anterior blastema samples. The graph indicates that sequenced reads were enriched in small RNA. **C**: The consensus mature miRNA is highlighted in the schematic representation of the secondary structure of novel miRNAs. **D**: Read count matrix of conserved (known) miRNAs and novel miRNAs.

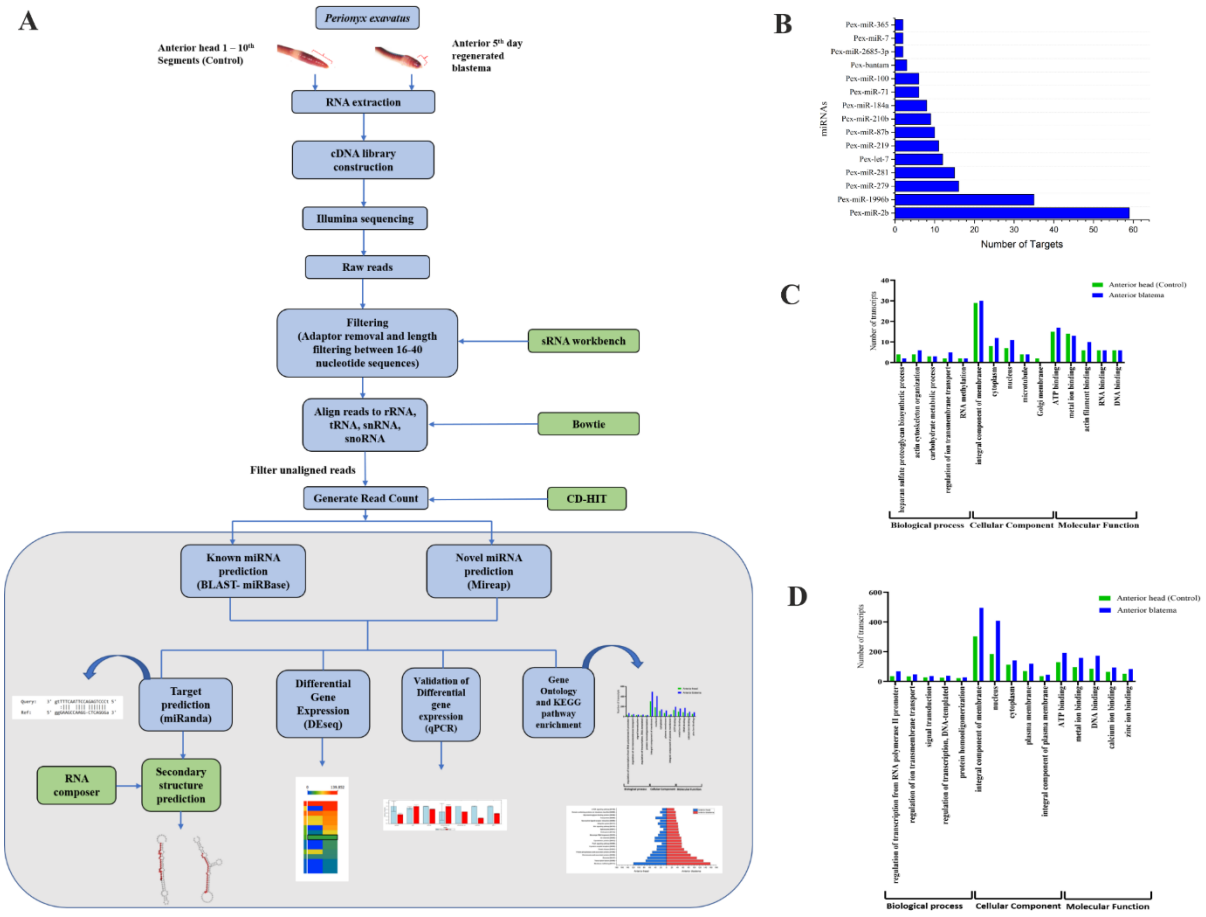

Fig. 2. A: Experimental design and bioinformatics workflow for earthworm, *Perionyx excavatus* miRNA identification. **B:** List of miRNAs and their putative targets in numbers. **C**: Bar chart representing the Gene Ontology (GO) distribution of known miRNAs of *P. excavatus*. **D:** Bar chart representing the Gene Ontology (GO) distribution of novel miRNAs of *P. excavatus*

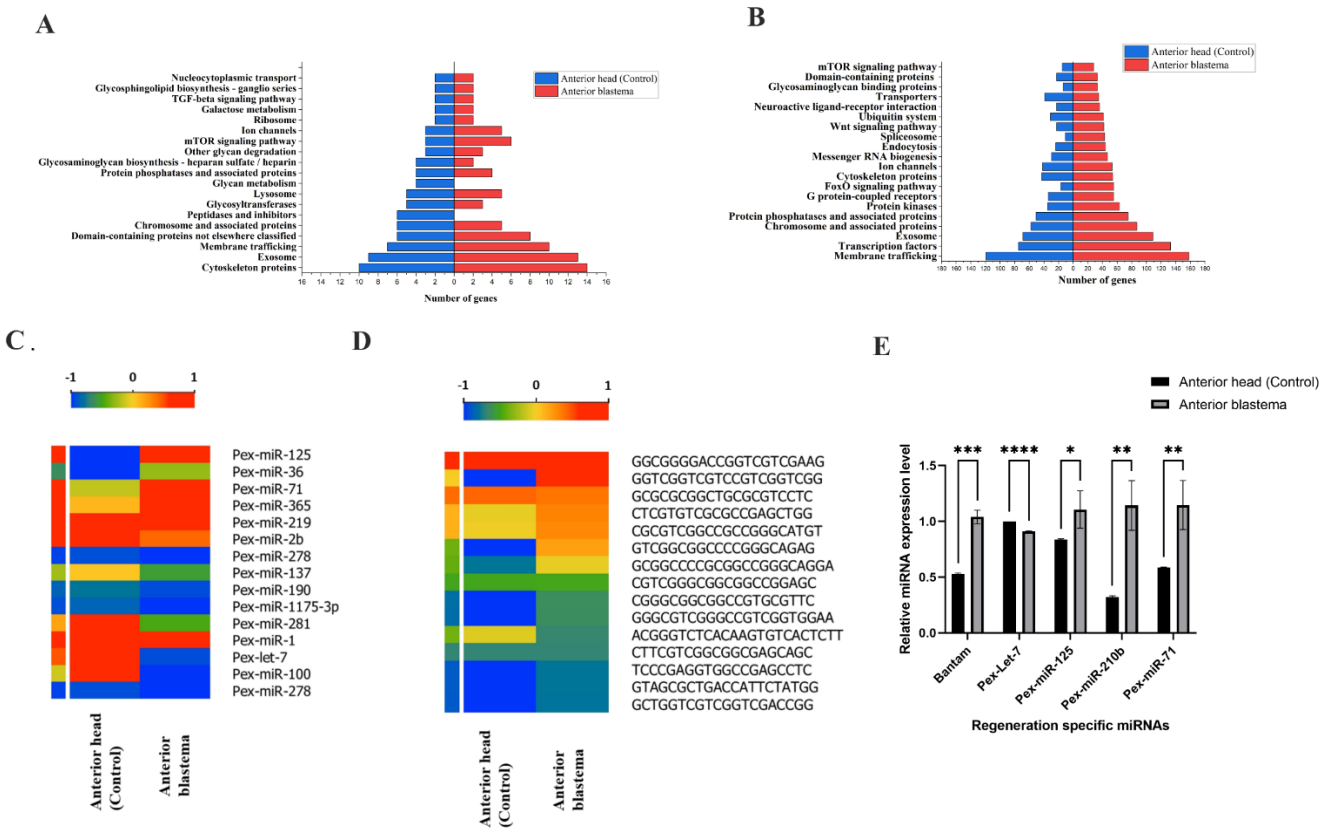

Fig. 3. Bar graph indicating the distribution of Kyoto Encyclopedia of Genes and Genomes (KEGG) functional categories linked to the *P. excavatus* miRNA targets. **A:** KEGG of known miRNAs. **B**: KEGG of Novel miRNAs. **C**: Heatmap clustering of the differentially expressed miRNAs between the control anterior segments and anterior regenerated blastema samples of earthworm *P. excavatus*. **D**: Heatmap clustering of the differentially expressed novel miRNAs between the control anterior head segments and anterior regenerated blastema samples of earthworm *P. excavatus.* **E**: Quantitative RT-PCR validation of the differentially expressed miRNAs. The qRT-PCR validation of the miRNA of *P. excavatus* were analysed in anterior head (Control) and anterior blastema.

Table 1: Top 20 pathways related to known miRNA targets.

S.No.	Pathway	Anterior head (control)	Anterior blastema
1	Cytoskeleton proteins [04812]	10	14
2	Exosome [04147]	9	13
3	Membrane trafficking [04131]	7	10
4	Domain-containing proteins not elsewhere classified [04990]	6	8
5	Chromosome and associated proteins [03036]	6	5
6	Peptidases and inhibitors [01002]	6	0
7	Domain-containing proteins not elsewhere classified [04990]	5	5
8	Glycosyltransferases [01003]	5	3
9	Lysosome [04142]	5	5
10	PATHWAY: Glycan metabolism	4	0

DECLARATION

I hereby declare that the particular given above are true to the best of my knowledge.

yours sincerely,

Place: Chennai, India

S. Ravichandran