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Expanding the annotation of zebrafish microRNAs based on smallRNA sequencing

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Abstract

MicroRNAs (miRs) are short non-coding RNAs that fine-tune the regulation of gene expression to coordinate a wide range of biological processes. Because of their role in the regulation of gene expression, miRs are essential players in development by acting on cell fate determination and progression towards cell differentiation and are increasingly relevant to human health and disease. Although the zebrafish *Danio rerio* is a major model for studies of development, genetics, physiology, evolution, and human biology, the annotation of zebrafish miR-producing genes remains limited. In the present work, we report deep sequencing data of zebrafish smallRNAs from brain, heart, testis, and ovary. Results provide evidence for the expression of 56 un-annotated *mir* genes and 248 un-annotated mature strands, increasing the number of zebrafish *mir* genes over those already deposited in miRBase by 16% and the number of mature sequences by 63%. We also describe the existence of three pairs of mirror-*mir* genes and two mirtron genes, genetic features previously undescribed in non-mammalian vertebrates. This report provides information that substantially increases our knowledge of the zebrafish miRNome and will benefit the entire miR community.

Keywords

miRNA; zebrafish; teleost; smallRNA-seq; mirror-mir; mirtron

Introduction

MicroRNA (miRs) are short non-coding RNAs that fine-tune the regulation of gene expression to coordinate a wide range of biological processes (Ambros, 2004; Kosik, 2010). MicroRNAs are transcribed from *mir* genes and primary miR transcripts are processed to approximately 22 nucleotide single strand mature forms that function as repressors of

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transcript translation when bound to the 3'UTR of protein-coding transcripts in association with the RISC (Davis and Hata, 2009; Kim et al., 2009). Due to their role in the regulation of gene expression, miRs are essential players in development by acting on cell fate determination and progression towards cell differentiation (Ivey and Srivastava, 2010). the zebrafish *Danio rerio* is a major model for studies of development, genetics, physiology, evolution, and human health and disease, and many miR-related discoveries have come from zebrafish investigations (Giraldez et al., 2005; Wienholds et al., 2005; Giraldez et al., 2006; Flynt et al., 2007; He et al., 2009; Mishima et al., 2009; He et al., 2011a, 2011b). For instance, *mir140* was shown to regulate zebrafish palatal development by down-regulating the translation of Pdgfra protein in neural crest derived cells (Eberhart et al., 2008). Experiments stimulated by that discovery showed that a single nucleotide polymorphism in the human *MIR140* gene leads to improper mature *MIR140* production and contributes to nonsyndromic cleft palate susceptibility in patients (Li et al., 2010). Another example of successful transfer of discoveries from zebrafish to human is the discovery of the crucial role of *mir451* in zebrafish erythropoiesis (Pase et al., 2009), which was later confirmed in mouse (Rasmussen et al., 2010; Patrick et al., 2010) and recently in human (Kim et al., 2013), thereby highlighting the therapeutic potential of miR inhibitors. Despite the high impact of zebrafish research in many aspects of development and physiology, the annotation of zebrafish miR-producing genes remains limited.

This limitation in our knowledge is a broad problem because the annotation of the zebrafish genome, the most complete among fish (Howe et al., 2013), often drives annotation in all teleost fish, which constitute half of all vertebrate species (Nelson, 2006). Moreover, a largely incomplete characterization of the miR repertoire compromises studies on miRNA evolution, their emergence and retention in vertebrates, especially in the rayfin fish lineage following the teleost genome duplication. Furthermore, uncovering and transferring evolutionarily conserved miR functions from fish models to human health depends on recognizing true fish orthologs of human miRs. Thus, the entire miR community would benefit substantially by a better understanding of the zebrafish miRNome.

In the present report, we provide extensive miRNA sequencing data from several zebrafish tissues demonstrating the expression of new *mir* genes and the expression of previously un-annotated mature strands. Further, we describe for the first time in non-mammalian vertebrates, the expression of mirror-mirs (Scott et al., 2012; Tyler et al., 2008) and mirtrons (Chung et al., 2011; Ladewig et al., 2012; Okamura et al., 2007).

Materials and methods

Small RNAs were extracted using the Norgen microRNA purification kit from four different organs (brain, heart, testis and ovary). Animals were actively reproductive adult "AB" strain zebrafish (*Danio rerio*) obtained from the University of Oregon fish facility. From two males, we sampled brain, heart, and testes and made six libraries, three for each individual; from two females, we sampled ovaries and made two libraries, one for each individual. Eight tissue-specific sequencing libraries were prepared and barcoded using the BiooScientific NEXTflex smallRNA Sequencing Kit which uses a 3' adenylated adapter that ligates onto miRs and other small RNAs with a 3' hydroxyl group, following the

manufacturer's instructions. Libraries were subsequently sequenced by Illumina HiSeq2500 at the University of Oregon Genomics Core Facility and raw single-end 50nt long reads were deposited in the NCBI Short Reads Archive under the accession number SRP039502. All animal work was performed according to the University of Oregon IACUC approved protocol (#09–1BRRA).

Bioinformatic processing involved removing 3' adapter sequences and filtering reads based on quality; reads with any base Q<30 were removed using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). A custom pipeline was created to remove reads outside of the targeted size range (<15 and >28), count the number of occurrences of each read, and remove reads with low counts (<30 summed across all eight libraries). The script then aligns reads to the Zv9 version of the zebrafish reference genome using blastn (Altschul et al., 1990) allowing up to five mismatches, one gap, and alignments at up to 20 locations in the genome; finally, the script groups reads together based on their genomic locations. Aligned groups were annotated against mature and hairpin sequences already annotated in miRBase (release 20)(Kozomara and Griffiths-Jones, 2013) as well as zebrafish noncoding RNA sequences annotated by Ensembl (release 74)(Flicek et al., 2012).

Putative new mirs were predicted based on read presence in our experimental dataset and secondary structures were computed on the RNAfold web server (Zuker and Stiegler, 1981) using default parameters except for changing the calculation of minimum free energy from 37°C to 28°C, the temperature at which zebrafish are reared (Westerfield, 2000). A tentative name was assigned to newly recognized sequences based on identity with deposited sequences in miRBase (Kozomara and Griffiths-Jones, 2013) for zebrafish or other species.

Results/discussion

The sequencing of small RNAs with 3' hydroxyls from several tissues of adult zebrafish yielded a total of 59.5 million reads that passed all filtering criteria, among which 47.5 million (79.8%) could be directly annotated from mature zebrafish entries in miRBase due to sequence identity. Among remaining reads, sequences mapping perfectly to the zebrafish reference genome at fewer than 20 locations were further studied for putative new *mir* genes and/or for mature strand annotation.

The annotation pipeline identified 56 *mir* genes that do not appear in miRBase, although 43 have of them have been bioinformatically predicted as "Novel miRNA" genes in Ensembl (Flicek et al., 2012); thus, our work increases the number of annotated zebrafish *mir* genes by 16% compared to the most recent miRBase (Release20) (Kozomara and Griffiths-Jones, 2013) (Table 1). Most of these new *mir* genes (54/56) are clear homologs to genes belonging to known mir families or described in related species and could thus be easily added to their corresponding group.

Two of the miR-encoding genes we identified in our experimental data, tentatively named *dre-mir-733-2* and *dre-mir-733b*, are paralogs of *dre-mir-733* (MI0004778) and originate from vaultRNAs, and a third miR, tentatively named *dre-mir-735b*, a paralog of *dre-mir-735* (MI0004781), actually originates from a Y_RNA gene. Human orthologs (*hsa-mir-886* and

hsa-mir-1979 respectively) of these zebrafish genes were withdrawn from miRBase after their biogenesis was understood (Stadler et al., 2009; Meiri et al., 2010). The role of vaultRNA and Y_RNA short fragments in translation repression is, however, not fully understood and they may act in a "mir-like" manner (Meiri et al., 2010; Persson et al., 2009; Nicolas et al., 2012), as do some snoRNA-derived or lncRNA-derived miRs (Yang and Lai, 2011). If the vaultRNA and Y_RNA derived short sequences do act like miRs, then production of short RNA fragments from vaultRNA and Y_RNA would be a new non-canonical way of generating miRs (Verhagen and Pruijn, 2011).

Our zebrafish smallRNA-seq dataset contained two completely new *mir* genes that showed no similarity with sequences yet deposited in miRBase for any species. These two new *mirs*, along with the 54 *mir* genes in our experimental dataset that are predicted in Ensembl but not referenced in miRBase, are described in the Supplemental File, which gives a tentative name, genomic localizations, predicted hairpin sequences, corresponding secondary structures, minimum free energy calculated at 28°C, the localization of mature sequences on the hairpin sequence, and secondary structure.

The breadth and depth of our sequencing dataset also allowed us to provide annotation for a total of 248 mature strands, 158 of them corresponding to previously annotated *mir* genes and 90 from the set of 56 new *mir* genes not present yet in miRBase and described here above, increasing the total number of annotated zebrafish mature sequences from 391 to 639 (a 63% increase) (Table 1). The Supplemental File gives the sequence, and genomic location of each newly identified miR.

Among the newly annotated structures are miRs with several interesting features. The zebrafish smallRNA-seq dataset clearly contained three pairs of mirror-mirs (Table 1), which are defined as a pair of *mir* genes originating from overlapping genomic regions but on opposite strands (Tyler et al., 2008; Scott et al., 2012). For example, the expression of *mir3120*, mirror-mir of *mir214*, has been described in mammals but in no other species (Scott et al., 2012; Desvignes et al., 2014). Here we show that *miR3120*, a mature fragment originating from the mirror-mir of *mir214*, is expressed in adult zebrafish. We also found the pair *mir7547* and *mir7553*, which had been previously identified as independent *mirs* in the channel catfish *Ictalurus punctatus* (MI0024671 and MI0024685 respectively)(Xu et al., 2013), but had not been described as a mirror-mir pair, probably due to the lack of a reference genome. The newly annotated zebrafish *mir7552b* and its mirror gene, *mir7552bOS*, had also not previously been annotated in zebrafish, even though a homolog of *mir7552b* is annotated in the channel catfish (MI0024684)(Xu et al., 2013). This finding is of particular interest given that, to our knowledge, mirror-mirs have been described so far only in *Drosophila* and mammals (Tyler et al., 2008; Scott et al., 2012). Evidence given in this report demonstrating the expression of mirror-mirs in zebrafish suggests that mirror-mirs are likely a type of gene featured in all vertebrates.

Finally, we also report the presence and expression of a pair of 3'-tailed mirtron paralogs located in *gria3* ohnologs (*gria3a*, ENSDARG00000032737; and *gria3b*, ENSDARG00000037498) (Table 1). These *mir* genes are co-orthologous mirtrons to the predicted human mirtron gene *AL356213.1* (ENSG00000265082), which is located in

GRIA3 (ENSG00000125675); they lie at orthologous positions in both zebrafish ohnologs; and they show high similarities with tetrapod *mir2985*, which is deposited in miRBase in zebra finch *Taeniopygia guttata*, platypus *Ornithorhynchus anatinus* and rat *Rattus norvegicus* as intronic *MiRs* of the *Gria2* genes (Kozomara and Griffiths-Jones, 2013). Similar to mirror-mirs, to our knowledge, mirtrons have only been shown in *Drosophila*, the nematode *Caenorhabditis elegans* and mammals (Okamura et al., 2007; Chung et al., 2011; Ladewig et al., 2012). This report is thus the first example of mirtrons in a non-mammalian vertebrate.

Conclusion

The present report analyzing smallRNA deep sequencing data expands the annotation of microRNA genes and mature strands in the laboratory model zebrafish *Danio rerio*, whose *mir* gene repertoire remains understudied and poorly characterized in comparison to other species, such as *Drosophila*, worms, mouse, or human, despite its relevance for microRNA research. Increasing the annottion of zebrafish miRs will contribute to a better understanding of the evolution of *mir* genes in vertebrates and within the rayfin fish lineage, will help annotate *mir* genes in other teleost species, and will increase confidence in the annotation of each gene thanks to the description of both mature strands, considered as a criterion for *mir* gene annotation confidence (Kozomara and Griffiths-Jones, 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations list

miR	microRNA
smallRNA-seq	small RNA sequencing
UTR	untranslated region
RISC	RNA-induced silencing complex

Highlights

- Annotation of 56 new miRNA genes in zebrafish
- Annotation of 248 new mature miRNA strands in zebrafish
- First description of mirror-miR pairs outside of *Drosophila* and mammals
- First description of 3’-tailed mirtrons outside of *Drosophila*, worms and mammals

Table 1

Summary of improvements to zebrafish annotations in miRBase.

Zebrafish	miRBase Release 20	Present study	% increase	Total
mir genes	346	56	16	402
mature sequences	391	248	63	639
mirror-mir pairs	0	3	N/A	3
mirtrons	0	2	N/A	2