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Short communication

Extreme conservation of miRNA complements in opisthorchiids

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ABSTRACT

MicroRNAs (miRNAs) are important gene regulators that are key players in animal development and diseases. They are excreted in extracellular vesicles and because they were shown to be taken up by host cells they have been proposed as mediators of parasite-host communication, and potential biomarkers for the detection of parasitic infections from host blood. Consequently, it is crucial to precisely know the miRNA complements of medically important agents such as the liver flukes of the Opisthorchiidae. Using publicly available and new datasets we curated and reannotated the surprisingly small and variable miRNA complements previously described for *Opisthorchis viverrini*, *O. felineus* and *Clonorchis sinensis*. We find three highly similar miRNA complements with 53 identical and two miRNA genes with species specific sequences that signify a set of potential biomarkers and promising candidates for further investigations.

Liver flukes are parasitic flatworms that parasitise many different species of economic relevance and also infect humans. The medically most important group of liver-flukes is the Opisthorchiidae with Eurasian wide distributed species *Opisthorchis felineus*, *Opisthorchis viverrini* and *Clonorchis sinensis* (Fig. 1) [1]. All three of these species are agents of human infections with Clonorchiasis/Opisthorchiasis transmitted by raw or undercooked fish and it is estimated that at least 1.2 million people worldwide are infected with *O. felineus*, 10 million with *O. viverrini* and 35 million with *C. sinensis* [2]. In humans, the infections are characterized by long durations, can occur with frequent exacerbations or without symptoms, and they may contribute to liver cancer development [3,4]. Because opisthorchiids are classified as bio-carcinogens, they came into the focus of various “OMICS”-studies that aimed at their characterization and, ultimately, identification of biomarkers towards the development of treatments [5,6]. One important class of potential biomarkers is microRNAs (miRNAs) and their importance in host-parasite communication and immune-modulation was proposed by a number of studies [7–9]. miRNAs are small non-coding RNAs that are post-transcriptional gene regulators with important roles in many biological processes [10]. Previously, miRNA complements of the 3 opisthorchiids were published and they showed a surprisingly variable number of miRNA genes (between 16 and 18 conserved and between 20 and 43 novel genes) that was unexpected [11,12]. The number of identified genes was also very low given the predictions from studies on miRNA evolution in flatworms [13] but most importantly no abundantly expressed miRNA was identified that showed differences in their sequences in all 3 species. The goal of our study was therefore first to curate and reannotate the miRNA complements of *O. viverrini*, *O. felineus* and *C. sinensis* and second to compare the complements for similarities and differences in expression and most importantly in sequence composition. Finally we describe expression differences in *O. felineus* stages for both miRNAs and mRNAs, identifying miRNA:mRNA interactions of possible importance for the development of *O. felineus*.

We analyzed a total of 1 billion small RNAseq reads of sixteen published miRNA NGS datasets for all 3 opisthorchiids [11,12], the references genomes for *O. viverrini* [14], *C. sinensis* [15] and the draft assembly of *O. felineus* (Ershov et al. in prep) were used. For RNAseq data we used annotations and expression levels directly from Pomanznoy et al. [5]. Briefly, small RNAseq reads of the projects PRJNA270708 [11] and PRJNA127731 [12] were downloaded from the Sequence Read Archive (SRA) and processed as described before [11,16] (Mapping statistics see Supplementary Table 1). Genomic references were downloaded from http://parasite.wormbase.org/ (PRJDA72781 & PRJNA222628) and made available by Ershov et al. respectively. Using the miRNA prediction algorithm MirMiner [17] & (Fromm et al. in prep) that applies consistent set of criteria for the prediction and annotation of miRNA genes [18], we reanalyzed and reannotated opisthorchiids' miRNAs. The annotation criteria were 1) two 20–26 nt long reads expressed from each of the two arms derived from a hairpin precursor with 2-nt offsets between the 5p and 3p arms; 2) 5′end homogeneity of expression; 3) at least 16-nt complementarity between the two arm sequences; 4) the loop sequence is at least 8 nt in

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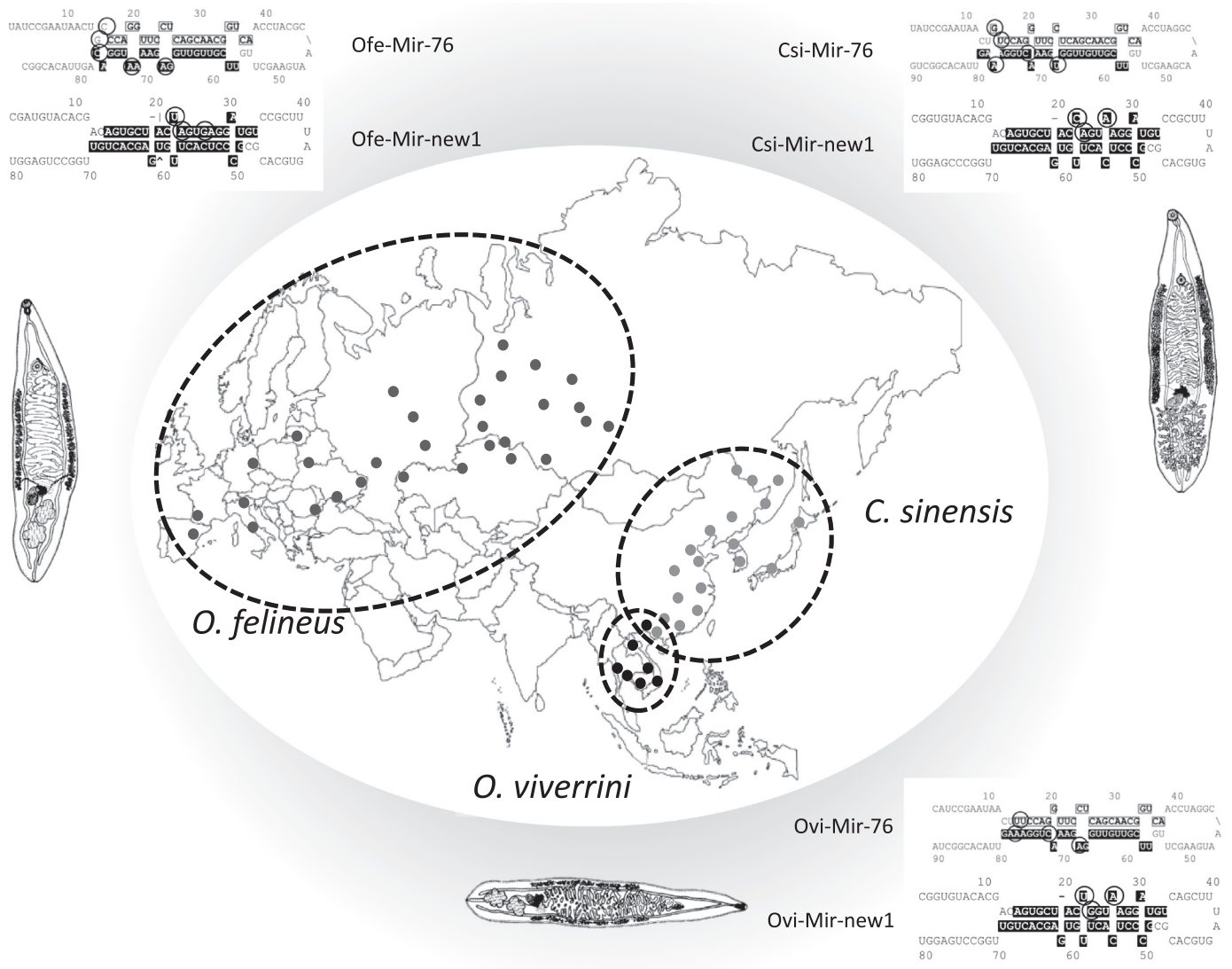

Fig. 1. Distribution of opisthorchiids in Eurasia and how they differ in the two variable miRNA loci. Light grey dots indicate locations where *O. felineus* was detected, dark grey dots –*C. sinensis*, black dots – *O. viverrini*. Mature miRNAs are highlighted by black color. Differences in mature and star sequences are indicated by black circles.

length; the maximum length of the loop is ~ 40 nt but there is no apparent maximum.

We found that the miRNA complements of the three opisthorchiids are very similar and much larger than presumed: they are composed of 55 conserved miRNAs (34 families) shared by the three flatworms and only found support for 1 novel miRNA (Supplementary Tables 2 and 3, Supplementary File 1). Our prediction algorithms found 35 previously missing conserved miRNA genes that belong to 22 conserved miRNA families and 1 novel miRNA gene. Further we rejected 96 previously described novel miRNA genes because they did not fullfil annotation criteria for *bona fide* miRNA genes [18] (Supplementary Table 3). A noteworthy finding is that Mir-76 and the Mir-Novel-1 show sequential differences between the 3 species while having abundant expression levels (Fig. 1). The complete complements were submitted to MirGeneDB [18].

When we compared the expression patterns of all miRNAs in the adult worm datasets of all three species we found that they are very similar, too. The top three expressed miRNAs in adults of all three species were Mir-10-P2a, Mir-71-P1 and Mir-281. It is worth noting that we were not able to detect mature expression of Mir-12 in *O. viverrini* and very low passenger strand expression in *O. felineus* but because the sequence is identical to the very little expressed version of Mir-12 in *C. sinensis* we included it for both species, too (Supplementary Table 3, asterisks).

Nevertheless, the homogenous pattern of miRNA expression we observed among the different species was not found when we compared miRNA abundance in the different stages available for *O. felineus*. We found highly distinct miRNA expression patterns between the datasets for metacercariae and the adults (Fig. 2, Supplementary Table 5, Supplementary Fig. 2). Remarkably, we were unable to detect Mir-76, Mir-10-P3 and Mir-2160-P1 in the metacercariae datasets. Because miRNA regulate gene-expression on the mRNA level we asked if we can observe a connection between the reported mRNA level differences between adult and metacercarians and the miRNA level differences we observe between the adult *O. felineus* and metacercarian stages. Previously, the transcriptome analysis of two *O. felineus* stages identified 12,665 distinct transcripts of those 903 were metacercariae specific and 648 adult specific [5]. In total, seven pathways were significantly enriched for differentially expressed genes (Lysosome, Neuroactive ligand–receptor interaction, Phagosome, Riboflavin metabolism, ECM–receptor interaction, Tyrosine metabolism and Arginine and proline metabolism). Consequently, we performed bioinformatics miRNA target prediction on the 3′UTR sequences of mRNA downloaded from GenBank. To ensure that we identify highly likely targets we used the intersection of three widely used programs (RNAhybrid, PITA and TargetScan) and identified 291 mRNA-targets for 46 miRNAs of *O. felineus*

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