

1 **Characterization of microRNAs expressed in the cystic legion of the liver of *Mus***
2 ***musculus* perorally infected with *Echinococcus multilocularis* Nemuro strain**

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26

27 **Abstract**

28 Alveolar echinococcosis (AE) is a zoonosis caused by the metacestode of
29 *Echinococcus multilocularis*. The published genome of *E. multilocularis* showed that
30 approximately 86% of its genome is non-coding. Micro RNAs (miRNAs) are small non-
31 coding regulatory RNAs, and recent studies on parasitic helminths expect miRNAs as a
32 promising target for drug development and diagnostic markers. Prior to this study, only a
33 few studies reported the *E. multilocularis* miRNA profiles in the intermediate host. The
34 primary objective of this study was to characterize miRNA profiles via small RNA-seq
35 in *E. multilocularis* Nemuro strain, a laboratory strain of Asian genotype, using mice
36 perorally infected with the parasite eggs. The data were then compared with two

37 previously published small RNA-seq data. We identified 44 mature miRNAs as *E.*
38 *multilocularis* origin out of the 68 mature miRNA sequences registered in the miRNA
39 database miRbase. The highest quantities of miRNAs detected were miR-10-5p, followed
40 by bantam-3p, let-7-5p, miR-61-3p, and miR-71-5p. The top two most abundant miRNAs
41 (miR-10-5p and bantam-3p) accounted for approximately 80.9% of the total parasite
42 miRNAs. The highly expressed miRNA repertoire is mostly comparable to that obtained
43 from the previous experiment using secondary echinococcosis created by an
44 intraperitoneal administration of metacestodes. A detailed characterization and functional
45 annotations of these shared miRNAs will lead to a better understanding of parasitic
46 dynamics, which could provide a basis for the development of novel diagnostic and
47 treatment methods for AE.

48

49 **Keywords**

50 *Echinococcus multilocularis*, alveolar echinococcosis, microRNA, non-coding RNA,
51 high-throughput sequencing

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56 ***Main text***

57 *Echinococcus multilocularis* is the causative agent of alveolar echinococcosis
58 (AE), a neglected tropical disease acknowledged by the World Health Organization.
59 Humans are infected by *E. multilocularis* as accidental intermediate hosts. After an
60 asymptomatic incubation period of 5–15 years, patients start to show symptoms including
61 cholestatic jaundice, abdominal pain, fatigue, and weight loss, which can ultimately lead
62 to death once left untreated [10]. Diagnosis is performed by radiography or serological
63 tests such as enzyme-linked immunosorbent assays (ELISA) [10], but these methods can
64 only diagnose AE after a certain development of the metacestode stage. Difficulty in the
65 detection of early stage *E. multilocularis* infection causes patients to be diagnosed at
66 advanced stages, allowing fatal symptoms to occur.

67 Intermediate hosts are infected with the parasite by ingesting infective eggs
68 containing oncospheres, which hatch and develop into metacestodes in the liver. The
69 metacestode consists of an inner germinal layer that bud to form a brood capsule, which
70 grows protoscolex inside [14]. *Echinococcus multilocularis* grows aggressively as it buds
71 exogenously, infiltrating and colonizing surrounding tissues. It can also spread to adjacent
72 locations or distant organs owing to its metastatic nature [9].

73 The *E. multilocularis* Europe genotype genome was sequenced [20], and
74 preliminary analyses show that approximately 86% of the genome is non-coding, with
75 further non-coding RNA analyses, including microRNA (miRNA) awaiting exploration.
76 miRNAs have been identified in numerous organisms over diverse clades, including
77 helminths, and their relevance as repressors of gene expression is now broadly recognized
78 [1, 3]. The existence of the RNA induced silencing complex, which is needed for miRNA
79 utilization, has been demonstrated in *E. multilocularis* by *in silico* analysis [8].
80 Subsequently, the presence of *E. multilocularis* miRNA has been confirmed
81 experimentally using mice infected with the parasite [7]. However, this experiment was
82 conducted using secondary echinococcosis created by an intraperitoneal administration
83 of sieved metacestodes, which may result in atypical miRNA profiles compared to a
84 natural *E. multilocularis* infection. Furthermore, *E. multilocularis* is divided into three
85 genotypic clades: Asia, Europe, and North America [18] and previous studies on *E.*
86 *multilocularis* miRNA solely used European genotypes [7, 8]. Hence experimental data
87 using *E. multilocularis* with different genetic backgrounds, are necessary for a
88 comprehensive understanding of miRNA profiles.

89 The primary objective of this study was to characterize miRNA expression
90 profiles of *E. multilocularis* using Nemuro strain (Asian genotype), which has been

91 maintained in a cotton rat-dog cycle under laboratory conditions, in mice perorally
92 infected with the parasite eggs. Furthermore, the obtained data were compared with two
93 previously published *E. multilocularis* miRNA profiles using European strains.

94 *Echinococcus multilocularis* Nemuro strain was obtained from a male *Myodes*
95 *rufocanus bedfordiae* caught in Nemuro city (43°16'57"N 145°30'57"E), Hokkaido,
96 Japan in 1987. The parasite has been maintained in a specially designed safety facility
97 (Biosafety Level 2 Enhanced) at the Hokkaido Institute of Public Health, Hokkaido, Japan.
98 Six-week-old DBA/2 mice (Japan SLC, Japan) were infected with *E. multilocularis*
99 Nemuro strain by perorally administrating parasite eggs (2,000 eggs/mouse). To
100 characterize miRNAs expressed after cyst development, the samples were collected at 16
101 weeks post infection which allows protoscolex maturation in DBA/2 mice [16, 19]. The
102 infected mice were euthanized by cervical dislocation under isoflurane anesthesia and
103 their livers including *E. multilocularis* cysts were obtained and stored at -80°C until
104 RNA extraction. All procedures performed in this study involving animals were in
105 accordance with the Guidelines for Animal Experimentation of the Japanese Association
106 for Laboratory Animal Science, and the protocol for the animal experiments was
107 approved by the ethics committee of the Hokkaido Institute of Public Health (study ID
108 K26-3).

109 Total RNA was isolated from three *E. multilocularis*-infected mouse livers (30
110 g) using the Nucleospin® miRNA Kit (Machery-Nagel, Germany). RNA concentration
111 was measured with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, USA). A
112 Truseq® Small RNA Sample Preparation Kit (Illumina, USA) was used to prepare the
113 Illumina sequencing library from the pooled total RNA (500 ng) of three mice. Two
114 Illumina libraries, replicate nos. 1 and 2, were made as technical replicates. In brief,
115 SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) was used to perform
116 reverse transcription to adapter ligated small RNAs. The cDNAs obtained were then
117 amplified and purified by extracting amplicons that accounted for RNA lengths between
118 20 and 33 bp. Agencourt AMPure XP (Beckman Coulter, USA) was used to further
119 purify the amplicons. A High Sensitivity DNA Assay Kit (Agilent, USA) on an Agilent
120 2100 (Agilent Technologies, Santa Clara, CA, USA) was used to determine the quality
121 of the purified library. The library's quantity was adjusted after being measured with a
122 Kapa Library Quantification Kit (Kapa Biosystems, USA) and 7500 Fast real-time PCR
123 system (Applied Biosystem, USA). After the measurement, the library was adjusted to 4
124 nM with a 1% spike-in of phiX control library (Illumina) and sequenced on the HiSeq
125 2000 sequencing system (Illumina). All procedures were carried out according to the
126 manufacturers' protocols. Raw sequence data in this study have been deposited in

127 DDBJ Sequence Read Archive with an accession number of DRA010502.

128 miRNA sequences of mouse (*Mus musculus*) and *E. multilocularis* were
129 obtained from the public database miRBase version 22.1 (<http://www.mir-base.org/>).
130 All analyses were performed with CLC Genomics Workbench version 9.5.1 (Qiagen,
131 Denmark). The reads were trimmed by removing index sequences, selected between 18
132 and 24 bp, and merged and sorted according to the same sequences using the “extract
133 and count” command. The merged reads were annotated as *M. musculus* or *E.*
134 *multilocularis* miRNAs using the “annotating and merging small RNA samples”
135 command.

136 A total of 7,064,228 and 6,854,600 reads were initially obtained from replicate
137 nos. 1 and 2, respectively. Of these reads, 3,313,066 and 3,629,090 reads were filtered as
138 selected reads. An initial overview of sequencing results annotated 3,010,447 (90.9%)
139 and 3,274,942 (90.2%) of the selected reads as pre-miRNA. Among them, 2,983,927
140 (99.1%) and 3,247,608 (99.2%) reads were of mouse origin, while 26,520 (0.9%) and
141 27,334 (0.8%) reads were of *E. multilocularis* origin. A list of the *E. multilocularis* mature
142 miRNAs detected using the *E. multilocularis* miRbase database is shown in
143 Supplementary Data 1. Further analysis was carried out using replicate no. 1 as this library
144 detected more *E. multilocularis* miRNAs. Out of 36 precursors and 68 mature miRNAs

145 registered in miRbase for *E. multilocularis*, a total of 32 and 44 were detected from the
146 Nemuro strain, respectively. The most abundant mature miRNA detected was miR-10-5p
147 (n = 9,320), followed by bantam-3p (n = 6,213), let-7-5p (n = 1,859), miR-61-3p (n =
148 506), and miR-71-5p (n = 257). The top two most abundant miRNAs (miR-10-5p and
149 bantam-3p) accounted for approximately 80.9% of the total parasite miRNAs (Figure 1).

150 A total of 555 mouse-derived mature miRNAs were detected (Supplementary
151 Data 1). The most highly expressed mouse-derived mature miRNA was miR-22-3p (n =
152 609,309), followed by miR-192-5p (n = 140,335), miR-21a-5p (n = 104,063), miR-26a-
153 5p (n = 73,797), and miR-148a-3p (n = 60,906) (Figure 1). miR-22-3p consisted 39.3%
154 of all mouse-derived miRNAs and top 10 most abundant miRNAs accounted for 78.6%
155 of the total mouse miRNAs (Figure 1).

156 Next, we compared our data with miRNA profiles obtained from two other
157 studies. The details of each experiment (experimental animal strain, origin of *E.*
158 *multilocularis*, infection route, and time of sampling) are summarized in Table 1. One
159 study conducted by Cucher et al. [7] analyzed *E. multilocularis* miRNAs in mice with
160 secondary echinococcosis, while the other study conducted by Boubaker et al. [2]
161 reported miRNA profiles in the liver of the mice orally infected with *E. multilocularis*
162 eggs. Since the latter study characterized only mouse miRNAs, we searched for parasite

163 miRNAs in the deposited sequence data (SRA nos. of SRX7826699-SRX7826701) using
164 the pipeline mentioned above, leading to the detection of 6 *E. multilocularis* miRNAs
165 (bantam-3p, let-7-5p, miR-1-3p, miR-9-5p, miR-61-3p, and miR-3479b-3p).

166 All 44 matured miRNAs obtained in the present study were found in the study
167 of Cucher et al. [7], since the present miRbase database for *E. multilocularis* is structured
168 using the result of Cucher et al. [7, 8]. A total of 24 *E. multilocularis* miRNAs registered
169 in the database were not detected in the Nemuro strain (Figure 2). This could be attributed
170 to the difference in the number of miRNA sequences obtained for each study; a total of
171 53,854 reads mapped to *E. multilocularis* miRNAs in this study and 1,497,226 and
172 2,169,374 reads mapped to *E. multilocularis* miRNAs in Cucher et al. [7]. In fact most of
173 the miRNAs absent in this study showed only 0.042% or lower (1~623 reads) in
174 proportion, implying that increased reads can detect the lowly expressed miRNAs. The
175 only exception was miR-190-5p, which accounted for 0.508~0.558% of reads, and was
176 ranked in 15th highest expressed *E. multilocularis* miRNA out of the 68 [8]. This may
177 imply that miR-190-5p plays different roles in the cyst between genotypes or parasite
178 infection routes. Since more than 99% of miRNA reads obtained in this study were mouse
179 origin, further analysis using only cysts instead of liver tissue with cysts may lead to
180 increased detection of lowly expressed parasite miRNAs.

181 Boubaker et al. [2] extracted RNA from the liver tissue of peri-parasitic area,
182 precisely 3 to 4 mm adjacent to parasite lesions, in *E. multilocularis*-infected mice.
183 Nonetheless, we could detect six *E. multilocularis* miRNAs from their sequencing data.
184 The six miRNAs were also detected in the present study and 5 were highly expressed,
185 suggesting that these miRNAs share necessary roles throughout the *E. multilocularis* cyst
186 development between different strains. As excretory/secretory vesicles of *E.*
187 *multilocularis* have been inferred to carry parasite miRNAs, these miRNAs could
188 possibly have been transported to surrounding murine tissue.

189 *Echinococcus multilocularis* miRNA functions are beginning to be assessed [24],
190 but most remain unknown. However, since some miRNAs are shared among different
191 organisms, their functions can be speculated. In particular, miR-10-5p, the most
192 abundantly expressed miRNA in *E. multilocularis* Nemuro strain, also exists in mice
193 (mmu-miR-10a-5p) and humans (hsa-miR-10a-5p). This miRNA is upregulated during
194 cancer in humans [15], and recent studies showed that miR-10a may have a causal role in
195 metastases in gastric cancer [5] and glioma [23]. In the case of glioma, the overexpression
196 of miR-10a causes epithelial–mesenchymal transition to occur, enabling glioma cells to
197 be transferred to other parts of the body [23]. Because *E. multilocularis* infection causes
198 tumor-like metastases in mice and humans, *E. multilocularis* miR-10 may play roles in

199 the expansion of metacestodes across host organs. As modulation of miRNA activities
200 has been studied as a novel therapeutic approach in the field of cancer research [11, 17],
201 parasite miRNAs including miR-10 could be potential drug targets. Nonetheless, since *E.*
202 *multilocularis* miRNAs are expressed inside the cyst enclosed by the laminated layer, a
203 proper delivery system should be developed in the drug design process.

204 microRNA can also be present in blood, and its usage as a tumor-specific marker
205 is being considered in the field of oncology [21]. As parasite-derived miRNAs have
206 recently been found in the host blood stream [4, 6, 12, 22], the use of miRNAs as
207 biomarkers of parasite infection should be considered. In fact, a recent study revealed the
208 presence of several *E. multilocularis* miRNAs in experimentally infected mice sera [12],
209 indicating that certain miRNAs may serve as potential AE diagnostic biomarkers.

210 In conclusion, we detected 44 mature miRNAs of *E. multilocularis* Nemuro
211 strain. The data obtained in this study will provide a reliable resource to supplement
212 existing knowledge on *E. multilocularis* miRNAs. Further comparative experiments are
213 required to reveal the effect of parasite strain, infected animal (species and strain), and
214 mode of infection on the expression patterns of *E. multilocularis* miRNAs in the
215 intermediate host. Nonetheless, this study showed that certain miRNAs are shared even
216 among different infection conditions. Further characterization and functional annotation

217 of these miRNAs will lead to a better understanding of the parasite and host interactions
218 which could enlighten new strategies to tackle AE.

219 **Conflict of interest**

220 The authors declare no conflict of interest.

221 **Acknowledgements**

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308 **Table 1. Details of experimental design of *Echinococcus multilocularis* miRNA studies.**

309

	Year	Mouse strain	<i>E. multilocularis</i> origin	Infection route/ Parasite material	Sampling time
This study ¹	2020	DBA/2	Hokkaido, Japan	Oral/Egg	16 weeks after infection
Cucher et al. ²	2015	CF1	Europe	Intraperitoneal/Cyst	4 months after infection
Boubaker et al. ³	2020	C57BL/6	Switzerland	Oral/Egg	1 month after infection

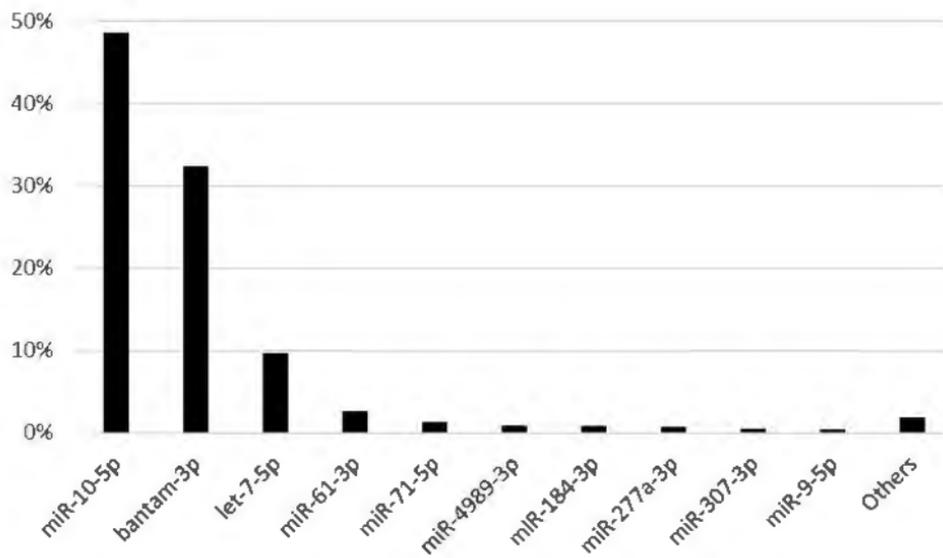
310 ¹ Eggs obtained from experimental gerbil-dog cycle; ²Infection route not clearly mentioned in paper; ³Eggs obtained from hunted fox.

311

312 **Figure legends**

313 **Figure 1. Percentage of most abundant miRNAs found in *Echinococcus multilocularis*-**
314 **infected mice liver.** Each graph shows the top 10 miRNAs that are expressed in *E.*
315 *multilocularis* infected mouse. (A) is of *E. multilocularis* origin and (B) is of *M. musculus*
316 origin. miR-10-3p and bantam-3p accounted for most (approximately 80%) expression in *E.*
317 *multilocularis*. Data from replicate no. 1 are used.

318 **Figure 2. Comparison of *Echinococcus multilocularis* miRNAs observed in two different**
319 **studies.** The numbers stand for the ranks when listed according to the miRNA abundance in
320 each small RNA-seq. The list of miRNAs not detected in this study is shown on Supplementary
321 Data 2. *miRNAs found in Boubanker et al., [2]. ND: Not detected.

A**B**