Parasitology International 67 (2018) 651–658

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Review

Gyrodactylus medaka n. sp. (Monogenea: Gyrodactylidae) parasitic on wild and laboratory-reared medaka *Oryzias latipes* (Beloniformes: Adrianichthyidae) in Japan

Masato Nitta[a,b,*], Kazuya Nagasawa[a]

[a] Graduate School of Biosphere Science, Hiroshima University, 1-4-4 Kagamiyama, Higashi-Hiroshima, Hiroshima 739-8528, Japan
[b] Graduate School of Science, Kobe University, 1-1 Rokkodai-chō, Nada, Kōbe, Hyōgo 657-8501, Japan

ARTICLE INFO

Keywords:
Gyrodactylus medaka n. sp.
Monogenea
Medaka
Oryzias latipes
Laboratory animal
Ammonium picrate-glycerin

ABSTRACT

Gyrodactylus medaka n. sp. (Monogenea: Gyrodactylidae) is described from the skin, fins, and gills of medaka *Oryzias latipes* (Beloniformes: Adrianichthyidae) from Japan. This new species was collected from wild medaka in Hiroshima, Aichi, Saga, and Kumamoto prefectures, and laboratory-reared medaka in Chiba and Aichi prefectures. The small marginal hook sickle (≤ 4 μm) and the length of the marginal hook of the new species are the diagnostic morphological characters differentiated from other gyrodactylids reported from Asia. The pairwise sequence divergences for the interspecific variation in ITS regions and the phylogenetic analysis suggest that the populations of *G. medaka* n. sp. may have a similar genetic variation as the medaka populations in Japan. *Gyrodactylus medaka* n. sp. and *Dactylogyrus oryziasi* (Monogenea: Dactylogyridae) can maintain their populations in laboratory aquaria using medaka as their hosts, and these monogeneans and medaka have the potential as experimental model animals for clarifying various aspects of their host-parasite relationships. In addition, we report the composition of modified ammonium picrate-glycerin (APG) and show it is advantageous for monogenean taxonomy.

1. Introduction

The medaka *Oryzias latipeparints* (Temminck and Schlegel, 1846) (Beloniformes: Adrianichthyidae) is a freshwater fish endemic to the Japanese Archipelago [1]. The fish is one of the most popular ornamental fishes in Japan and one of the most important vertebrate model organisms to study genetics, development, environmental research, and diseases [2–4].

The medaka experimentally reared in Japan is known to be infected by a gyrodactylid monogenean which was previously identified as *Gyrodactylus elegans* von Nordmann, 1832 [3, 5, 6] and *Gyrodactylus* sp. [4]. However, these identifications were not based on the morphology or genetic study [3–6], and the past host records of *G. elegans* have been considered as erroneous [7]. Currently, the gyrodactylid of medaka has been treated as an unidentified species and regarded as the most important parasite in research facilities rearing medaka [8]. We found the infection of this monogenean on wild and laboratory-reared populations of medaka in Japan and herein describe it as *Gyrodactylus medaka* n. sp.

The method of fixation for monogeneans using ammonium picrate-glycerin (APG) was originally reported by Malmberg [9]: its composition is ammonium-picrate:glycerin = 1:1, and it has been commonly used for monogenean studies until now. Nevertheless, Ergens [10] indicated that specimens stored in Malmberg's APG for over a year sometimes became difficult to observe their morphological structures because of the overstaining and evaporation and crystallization of the APG. Subsequently, Lim [11] and Lim and Gibson [12] improved the APG composition: they greatly increased the content of glycerin and added formalin. Their modified APG was used in some studies [e.g. 13–15], but they did not mention the quantitative composition nor the effects on monogenean specimens. Thus, we herein report the composition of modified APG and its effects.

2. Materials and methods

2.1. Collection and preparation of parasite specimens

A total of 110 wild medaka was collected at eight localities in five prefectures (two irrigation canals of the Nuta River, the Mitsugi River, and the Harada River in Hiroshima Prefecture; an irrigation canal of the Sonose River in Tokushima Prefecture; an irrigation canal of the Shiota River in Saga Prefecture; the Umeda River in Aichi Prefecture; and an

* Corresponding author at: Graduate School of Science, Kobe University, 1-1 Rokkodai-chō, Nada, Kōbe, Hyōgo 657-8501, Japan.
E-mail address: licht.bsn.mono@gmail.com (M. Nitta).

https://doi.org/10.1016/j.parint.2018.06.010
Received 7 February 2018; Received in revised form 28 May 2018; Accepted 21 June 2018
Available online 22 June 2018
1383-5769/

Table 1
Sampling localities of *Oryzias latipes*.

No.	Sampling locality			Date	Number of fish examined	Standard length range and mean (mm)
	Prefecture	Site	Longitude/latitude			
1	Hiroshima	An irrigation canal of the Nuta River	34°23′20″N, 133°02′02″E	4 June 2013	2	27.0–32.3 (29.7)
2			34°23′23″N, 133°01′57″E	14 November 2017	33	13.2–29.6 (20.1)
3		The Mitsugi River (the Nuta River system)	34°23′43″N, 132°58′00″E	7 October 2013	11	20.2–24.9 (22.2)
4		The Harada River	34°14′26″N, 132°52′51″E	28 April 2013	7	24.7–33.3 (29.2)
5	Tokushima	An irrigation canal of the Sonose River	34°02′17″N, 134°31′15″E	18 September 2013	20	13.0–20.9 (17.8)
				6 December 2013	9	19.1–22.7 (20.9)
6	Saga	An irrigation canal of the Shiota River	33°06′26″N, 130°04′57″E	26 September 2013	10	18.3–20.8 (19.5)
7	Aichi	The Umeda River	34°42′55″N, 137°24′23″E	7 November 2013	9	21.2–28.5 (24.6)
8	Kumamoto	An irrigation canal of the Midori River	32°43′50″N, 130°40′37″E	17 April 2017	9	19.5–25.6 (23.1)
9	Chiba	The Atmosphere and Ocean Research Institute (AORI), the University of Tokyo		27 March 2013	3	No data
10	Aichi	National Institute for Basic Biology (NIBB)		17 October 2016	6	21.0–24.5 (22.3)

irrigation canal of the Midori River in Kumamoto Prefecture), and nine laboratory-reared medaka were sampled at two localities in two prefectures (Atmosphere and Ocean Research Institute (AORI), the University of Tokyo in Chiba Prefecture; and the National Institute for Basic Biology (NIBB) in Aichi Prefecture) (Table 1). All the wild medaka and the laboratory-reared medaka from AORI were transported alive to the laboratory of Hiroshima University for parasitological examination. The medaka reared at NIBB were examined on site. The fish were killed by icing, identified based on Senou [1], and then examined for monogeneans on the skin, fins, and gills under a dissecting microscope. Prevalence and intensity of infection are those defined by Bush et al. [16]. The scientific names of fishes used in this paper follow Froese and Pauly [17].

Monogeneans were picked up using small needles and fixed in 99% ethanol or modified ammonium picrate glycerin (10 mL of commercial formalin, 90 mL of glycerin and 5 mL of saturated picric acid) under coverslip pressure for up to one week to 3 years and 3 months. For molecular analysis, the bodies of some ethanol-fixed specimens collected from Hiroshima, Tokushima, and Kumamoto prefectures, and AORI were cut from the haptors using needles and preserved in 99% ethanol. The rest of the haptors were flattened under coverslip pressure and stored in APG or digested by Proteinase K method [18] for morphology analysis. All specimens for morphology analysis were dehydrated through a graded ethanol series, cleared in xylene, and mounted in Canada balsam.

2.2. Morphological analysis

The haptoral hard parts and male copulatory organs were studied on the images taken by a CANON EOS 70D digital camera and drawing tube fitted on an Olympus BX51 light microscope. Measurements were made on the images of the haptoral hard parts using ImageJ software (version 1.48i). The basic method of measuring employed herein follows Christion et al. [19]. Measurements, in micrometers, are expressed as the mean ± standard deviation followed in parentheses by the range of structure measurements and the number (n) of specimens examined. Specimens are deposited in the Platyhelminthes (Pl) collection of the National Museum of Nature and Science, Tsukuba city, Ibaraki Prefecture (NSMT-Pl).

2.3. DNA analysis

DNA was extracted from five individual bodies collected from Hiroshima (locality 2 and 3 in Table 1), Tokushima (locality 5), Kumamoto (locality 7) prefectures and AORI (locality 9) using a DNeasy® Blood and Tissue Kit (Qiagen). The DNA was amplified by polymerase chain reaction (PCR) using the primer pairs S1 primer (5′-ATT CCG ATA ACG AAC GAG ACT-3′) [20] and IR8 primer (5′-GCT AGC TGC GTT CTT CAT CA-3′) [21], and ITS1A (5′-GTA ACA AGG TTT CCG TAG GTG-3′) and ITS2 (5′-TCC TCC GCT TAG TGA TA-3′) [22] to amplify a fragment spanning the 3′ end of the 18S rRNA subunit, ITS1 and 2, the 5.8S rRNA and the 5′ end of the 28S rRNA. Amplification reaction was carried out in 25 μL volume containing 0.125 μL of Takara Ex Taq DNA polymerase (Takara Bio), 2.5 μL of PCR buffer (Takara Bio), 2.0 μL of dNTP mixture (2.5 mM each dNTP) (Takara Bio), 1.0 μL of 10 μM each primer, 2.0 μL of extracted DNA and 16.375 μL of distilled water. PCR was carried out with the following protocol: 94 °C for 60 s followed by 35 cycles of 94 °C for 60 s, 56 °C for 60 s, and 72 °C for 60 s, and a final extension step at 72 °C for 10 min. PCR products were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) and sequenced with a 3130X Genetic Analyzer (Applied Biosystems) with the same primers that generated the PCR products. Genetic distances of ITS1 and ITS2 sequences according to the Kimura 2-parameter model were calculated in Mega 7 [23]. The obtained nucleotide sequences of 5.8S and ITS1 + ITS2 were subjected to a BLAST (http://www.ncbi.nlm.nih.gov/) search on 21 November 2017 for comparison with other sequences deposited in GenBank. The phylogenetic position of the new species was estimated by aligning five newly obtained sequences and the ITS1-5.8S-ITS2 fragments of 27 sequences (Fig. 2) that retrieved a 100% hit by the BLAST search with the 5.8S sequence using Mega 7. Alignment was performed with ClustalW using the default parameters. The phylogenic tree was constructed with the neighbor-joining (NJ) method under the K2 model, and with the maximum likelihood (ML) method under the GTR + G + I model, which was determined to be the best-fit model using the Akaike's information criterion. All phylogenies were tested with 1000 bootstrap repeats.

3. Results

3.1. Fields and laboratories surveys

In total, 85 specimens of gyrodactylid were collected from the skin, fins, and gills of wild medaka at all the eight localities sampled. Its prevalence and mean intensity on the wild medaka from those localities ranged from 9.1–55.6% and 1.0–7.4, respectively (Table 2). As many as about 1000 worms were found on each of the three medaka (prevalence, 100%) examined from AORI, and seven worms were collected from four of the six medaka (66.7%) examined at NIBB. In addition to the gyrodactylid, *Dactylogyrus oryziasi* Nitta and Nagasawa, 2017 (Monogenea: Dactylogyridae) was found on the gills of medaka in an irrigation canal of the Sonose River (locality 5 in Table 1), Tokushima Prefecture [14]. One specimen of dactylogyrid from the gill of medaka reared at NIBB (NSMT Pl-6376) was also identified as *D. oryziasi* based on Nitta and Nagasawa [14].