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Helminth of Japanese urodelans: a parasitological approach to the alien species problem and challenges in systematic confusion

by
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Abstract

Japan has abundant amphibian fauna and the number of described species has recently increased dramatically; nevertheless, they face population decline due to habitat destruction, over-collection, and biological invasion. Their parasite fauna is one of the important biotic indicators to evaluate the current ecological status of host animals. The species diversity and host-parasite association are still little known in parasites of amphibians, especially urodelans. Hence, intensive studies were conducted for helminths parasitizing Japanese urodelans.

First, I investigated the current parasitic helminth fauna of the introduced and hybrid giant salamanders. As a result, one trematode species, *Liolope copulans* Cohn, 1902, and three nematode species, *Amphibiocapillaria tritonispunctati* (Diesing, 1815), *Falcaustra* sp., and *Spiroxys hanzaki* Hasegawa, Miyata and Doi, 1998, were reported from the digestive tract of hybrids between Japanese and Chinese giant salamanders (*Andrias japonicus* × *Andrias* sp.) as well as introduced Chinese giant salamander (*A. davidianus*) in Kyoto Prefecture. These species except *Falcaustra* have been recorded from Japanese giant salamanders in Japan. For this reason, the spillback of native parasites on the introduced and hybrid giant salamanders was suggested. In the second chapter, two kathlaniid species, *Falcaustra hanzaki* Tsuchida, Urabe and Nishikawa, 2023 and *Urodelnema takanoensis* Tsuchida, Urabe and Nishikawa, 2023 were described morphologically and molecularly, which were recovered from the digestive tract of hybrids between Japanese and Chinese giant salamanders in Kyoto Prefecture. *Megalobatrachonema nipponicum* Yamaguti, 1941 was also redescribed morphologically and its molecular data was first provided, which was recovered from the digestive tract of Japanese and hybrid giant salamanders in Kyoto and Mie prefectures. Those kathlaniid species morphologically differed from those reported from Chinese giant salamanders in China. Furthermore, the phylogenetic analysis strongly indicated the systematic confusion between the superfamily Cosmocercoidea and Seuratoidea. In the final chapter, the validity of *Mesocoelium* species parasitizing Japanese amphibians was elucidated. *Mesocoelium brevicaecum* Goto and Ozaki, 1929 (unintentionally available) was widely distributed over Japanese amphibians both Urodela and Anura, while *M. monas* (Rudolphi, 1819) was locally found in hynobiid species occurred in Fukushima and Nagasaki prefectures. This result indicated that it is valid for key characteristics in this genus whether the ceca extend beyond the edge of the ovary or not. *Mesocolium elongatum* Goto and Ozaki, 1929, *M. lanceatum* Goto and Ozaki, 1929, *M. pearsei* Goto and Ozaki, 1930, *M. ovatum* Goto and Ozaki, 1930, and *M. minutum* Park, 1939 were considered as the junior synonyms of *M. brevicaecum*, and *M. japonicum* Goto and Ozaki, 1930 was regarded as the junior synonym of *M. monas*. Thus, the present study revealed that at least three *Mesocoelium* species (the third species: *Mesocoelium* sp. 1 by Waki et al. 2022) were distributed in Japan. The phylogenetic analysis supported well the monophyly of this genus and showed that the genetic divergence of this genus is dependent on geographic distance.

The present study showed the validity of the parasitological approach for estimating the potential risk by alien species in the current ecosystem concerning amphibians. This study also strongly indicated that the fundamental study in helminths is meaningful and essential to deep comprehension of ecology in amphibians as well as phylogeny in parasites.

Keywords

Amphibia, *Andrias*, Cryptobranchidae, helminth, hybridization, Hynobiidae, Japan, Kathlaniidae, molecular phylogeny, morphology, Nematoda, parasitology, Platyhelminthes, Salamandridae, taxonomy, biogeography, introduced species, spillback

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Contents

General introduction

1. Amphibians in Japan

Japan is one of the territories with the richest amphibian fauna in East Asia and over 90 % of those species are endemic to this country (Nishikawa, 2017). There are some phylogenetically old taxa like Cryptobranchoidea in Japanese amphibians (Browne et al, 2012). As Matsui (2000) predicted, the number of known amphibian species has rapidly increased in recent decades due to new molecular approaches. In fact, the number of described species in both Anura and Urodela dramatically increased since 2010 in Japan (Djong et al., 2011; Sekiya et al., 2012; Nishikawa and Matsui, 2014; Matsui et al., 2019; Eto et al., 2022; Yoshikawa and Matsui, 2022). Besides, recent studies also revealed the restricted distribution and regional genetic diversity in Japanese amphibians (Yoshikawa et al., 2008; Tominaga et al., 2010, 2013; Fukutani et al., 2022).

On the other hand, the global decline in amphibians is one of the pressing environmental problems in recent decades, which is derived from habitat destruction, over-collection, disease pandemic, and biological invasion (Daszak et al., 1999; Nishikawa et al., 2017). Without exception, a lot of Japanese amphibians face a crisis of extinction. The alien species problem is one of the significant factors driving the situation directly and indirectly. The direct effect is, for instance, predation and competition, while the indirect effect is trophic cascades and interaction modification (White et al., 2006). It is impossible to exclude introduced populations and impacts if the alien species could be widespread and establish populations like *Lithobates catesbeianus* (Shaw, 1802), *Xenopus laevis* (Daudin, 1802), and the Chinese giant salamander [mainly *Andrias davidianus* (Blanchard, 1837)] (Maeda and Matsui, 1999; Kuraishi et al., 2009; Fukumoto et al., 2015). However, the national and local conservation activities do not catch up with the current systematic change in amphibians yet.

2. Parasitic helminths of amphibians in Japan

In Japan, more than 100 parasitic helminth species have been hitherto reported from amphibians, mainly anurans, while the described parasite diversity was extremely limited in urodelans (Uchida and Ooi, 2018; Uchida et al., 2019; Sata et al., 2020a, b; Tsuchida et al., 2022). Host-parasite associations between described helminth species and amphibian hosts were little known. However, several species are known endemic to a single host species (Cohn, 1902; Hasegawa et al., 1998). Thus, it is expected that more endemic species would be found if more parasitological studies would be conducted.

The parasitological survey is helpful to understand the host ecology. A parasite connects various invertebrate and vertebrate organisms in their life history. For example, digeneans (Platyhelminth: Trematoda) generally have a complex life history; the first host for them is a mollusk, the second various invertebrates or vertebrates, and the final a vertebrate (Cribb et al., 2014). The type of parasites and the timing of infection can provide useful information to comprehend the host trophic network and seasonal movement. This kind of study is also effective to detect and evaluate the potential risk held in the ecosystem by invasive alien species (Chinchio et al., 2020). A parasite is one of the biotic factors to facilitate the establishment of alien species and to construct a novel ecosystem after introduction through direct or indirect effects (Dunn et al., 2012). Since parasites can become novel pathogens and mediate the competitive interaction between indigenous and non-indigenous hosts, the parasitological study can help us with detecting the net impact of alien species. Thus, a fundamental study is essential to reveal species diversity and infection status in parasites in Japanese amphibians. Furthermore, we need to reconsider the host-parasite association under the updated host taxa. Because of lacking detailed local data in previous parasitological studies, it is often extremely difficult to estimate the current host species in amphibians.

3. Objectives of this study

Hence, the present study focuses on two main themes, (1) parasitological evaluation of alien species problems in amphibians and (2) elucidation of taxonomical confusion in parasites. In terms of theme 1, the current parasite fauna of giant salamanders was revealed in Chapter 1, which suffers the serious genetic introgression of Chinese giant salamanders. Furthermore, the detailed species comparison between Japanese and Chinese kathlaniids was conducted in Chapter 2 and this part was related to both themes. In Chapter 3 regarding theme 2, taxonomic status of the digeneans of *Mesocoelium*, one of the most complicated parasites taxa, in Japanese amphibians, was elucidated.

Chapter 1:

Current helminth fauna of giant salamanders in Japan

This section is mainly based on the manuscript:

- “The first survey for helminths parasitic in hybrid and introduced giant salamanders, genus *Andrias* (Amphibia: Caudata: Cryptobranchidae) in Kyoto, Japan” by Karin Tsuchida, Misako Urabe, and Kanto Nishikawa published in ***Current Herpetology*** 40 (2021) 109–119, 10.5358/hsj.40.109.

1.1. Introduction

The Japanese giant salamander, *Andrias japonicus* (Temminck, 1836) (Amphibia: Cryptobranchidae), is endemic to the western and central Japanese Archipelago and listed as both a special natural monument in Japan and Near Threatened species on the IUCN Red List (Kaneko and Matsui, 2004; Yoshikawa et al., 2012; Matsui, 2014). A few surveys have been conducted for the helminths parasitizing *A. japonicus*. In the early 20th century, *Liolope copulans* Cohn, 1902 (Trematoda: Liolopidae) and *Kamegainema cingula* (as *Filaria cingula*) (Linstow, 1902) Hasegawa, Doi, Araki and Miyata, 2000 (Nematoda: Micropleuridae) were first found from *A. japonicus* transported to Europe (Cohn, 1902; Linstow, 1902). Decades later, Yamaguti (1936, 1939, 1941) reported the following species from *A. japonicus* in Kyoto City, Japan: *Diplodiscus japonicus* (Yamaguti, 1936) (Trematoda: Diplodiscidae), *Pseudoacanthocephalus lucidus* (as *Acanthocephalus*) (Van Cleave, 1925) (Acanthocephala: Echinorhynchidae), and *Megalobatrachonema nipponicum* Yamaguti, 1941 (Nematoda: Kathlaniidae). Hasegawa et al. (1998) described *Spiroxys hanzaki* Hasegawa, Miyata and Doi, 1998 (Nematoda: Gnathostomatidae) recovered from *A. japonicus* in Hyogo Prefecture, Japan. Hasegawa et al. (2000, 2002) also reported the parasite fauna of *A. japonicus* in Osaka and Hyogo prefectures, Japan, as follows: *L. copulans*, *S. hanzaki*, *K. cingula*, *Amphibiocapillaria tritonispunctati* (Diesing, 1851) (Nematoda: Trichuridae), *Dioctophyme renale* (Goeze, 1782) (Nematoda: Dioctophymatidae) and Kathlaniidae gen. sp. (Nematoda). In addition, Physalopteroidea gen. sp. (Nematoda) was recovered but was considered a pseudoparasite that was accidentally acquired through ingesting parasitized fish (Hasegawa et al., 2002). Tanaka et al. (2016) documented similar parasite species to Hasegawa et al. (2002) in zoo-bred *A. japonicus* in Hiroshima Prefecture, Japan.

The genetic introgression of Chinese *Andrias* species into the native population of *A. japonicus* has been serious in Japan, particularly in Kyoto Prefecture (Yoshikawa, 2011). A recent molecular study concluded that several *Andrias* species occur in China, including *A. davidianus* (Blanchard, 1871) and *A. sligoi* (Boulenger, 1924) (Turvey et al., 2019). These species were introduced to Japan in the 1970s, leading to the ongoing hybridization with *A. japonicus* (Fukumoto et al., 2015). To fully evaluate the impacts of alien species, it is essential to examine whether parasites are also introduced with novel vertebrate species (e.g. Dunn et al., 2012). Introduced species can increase parasite transmission via spillover or spillback. Spillover occurs when a reservoir host species that was introduced transmits novel parasites to a native species (Hatcher et al., 2012). Alternatively, an introduced species can become a new reservoir for native parasite infection, which can increase infection in native hosts through spillback (Hatcher et al., 2012). In Kyoto Prefecture, the current parasite fauna on giant salamanders is unclear because parasitological surveys have not been conducted in almost 80 years since Yamaguti (1936, 1939, 1941).

In this study, I documented the current parasite fauna of giant salamanders, especially of introduced and hybrid individuals, the latter of which is now dominant in the rivers of Kyoto Prefecture. Based on the results, I discuss whether the introduction of Chinese giant salamanders affected the parasite fauna of *A. japonicus* via spillover or spillback.

1.2. Materials and Methods

1.2.1. Collection

A total of 27 giant salamanders were euthanized by the injection or immersion in 2-phenoxyethanol (Fig. 1.1, Tables 1.1 and 1.2). All dissections were approved by the Culture Bureau of Kyoto City. Because each *Andrias* species is difficult to identify by morphology, all collected salamanders were analyzed genetically (Yoshikawa et al., 2012). As a result, I identified 25 “hybrids” between *A. japonicus* and Chinese *Andrias* species (species not identified) and two *A. davidianus*, which is redefined by Turvey et al. (2019) (Nishikawa, unpublished). The alimentary canal, liver, lungs, and skin of each salamander were examined to collect parasites. All *Andrias* specimens used for this study were deposited at the Graduate School of Human and Environmental Studies, Kyoto University (KUHE57580, 57582–57583, 57647–57648, 57651, 57653–57655, 58714–58716, 58902–58904, 58924–58926, 58937, 59036–59039, 59464, 59470).

1.2.2. Morphological study

Collected nematodes were fixed in 70% ethanol, cleared in undiluted glycerin or mounted in glycerin-gelatin. Some collected trematodes were pressed between a coverslip and glass slide, fixed in alcohol-formol acetic fixative (AFA), mordanted in 4% ammonium iron (III) sulfate solution, stained with Heidenhain's iron hematoxylin, differentiated in 4% ammonium iron (III) sulfate solution, dehydrated in ethanol series, cleared in creosote, replaced in xylene, and mounted in Canada balsam. These specimens were observed using a light microscope for morphological study. *Liolope copulans*, *Spiroxys hanzaki*, and *Amphibiocapillaria tritonispunctati* were identified based on the morphological descriptions in Baba et al. (2011), Hasegawa et al. (1998) and Moravec (1982, 1986), respectively. All measurements are given in micrometres unless otherwise stated, as range followed by mean ± standard deviation in parentheses. All specimens studied were deposited in the Zoological Collection of Kyoto University (catalog no. KUZ Z3908–Z3912).

1.2.3. Molecular study

Genomic DNA was extracted from specimens fixed in 70 or 90 % ethanol using Wizard® SV Genomic DNA Purification System (Promega Corp., Madison, WI). Polymerase chain reaction (PCR) was performed to amplify the internal transcript spacer (ITS) 1 region of *S. hanzaki*. The PCR was performed using 50 µl PCR reaction mixture containing 5 µl of 10×KOD-Plus-Neo Buffer, 5 µl of dNTPmix (2 mM), 3 µl of MgSO4 (25 mM), 1 µl of KOD-Plus-Neo (TOYOBO Co., Ltd., Osaka, Japan), 1.5 µl of forward primer SSU24HF (5'-AGAGGTGAAATTCGTGGACC-3') (10 µM) and of reverse primer AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') (10 µM) (Li et al., 2014), and 33 µl of each template. The PCR process was conducted using 2720 Thermal Cycler (Applied Biosystems Inc., Waltham, MA), with thermocycling profile as follows; 30 s at 94°C, 40 cycles of 10 s at 94°C, 30 s at 50°C, 1 min at 72°C, and the final extension for 7 min at 72°C. To amplify the partial 18S rDNA region of *Am. tritonispunctati*, PCR was performed in 20 µl PCR reaction mixture containing 13.8 µl of Milli-Q water (MQW), 2 µl of 10×Ex Taq Buffer, 1.6 µl of dNTP mixture, 0.1 µl of Ex Taq (Takara Bio Inc., Shiga, Japan), 1 µl of forward primer NSF4/18 (5'-CTGGTTGATCCTGCCAGT-3') (10 µM) and of reverse primer SSU18R (5'-TGATCCTTCYGCAGGTTCAC-3') (10 µM) (Tamaru et al., 2015), and 0.5 µl of each template. Thermocycling profile was as follows: 30 s at 94°C, 40 cycles of 10 s at 94°C, 30 s at 50°C, 1 min at 72°C, and the final extension for 7 min at 72°C. To amplify the partial 28S rDNA region of *Falcaustra* sp., PCR was performed in 20 µl PCR reaction mixture containing 7.1 µl of MQW, 10 µl of 2×Gflex PCR Buffer, 0.4 µl of Tks Gflex DNA Polymerase (Takara Bio Inc.), 1 µl of forward primer 28S-F (5'-AGCGGAGGAAAAGAAACTAA-3') (10 µM) and of reverse primer 28S-R (5'-ATCCGTGTTTCAAGACGGG-3') (10 µM) (Nadler and Hudspeth, 1998), and 0.5 µl of each template. Thermocycling profile was as follows: 1 min at 94°C, 40 cycles of 10 s at 94°C, 15 s at 50°C, 1 min at 68°C, and the final extension for 7 min at 68°C. PCR products were visualized on electrophoresis gels with 1 µl Midorigreen Direct (NIPPON Genetics Co., Ltd, Tokyo, Japan) and purified using the Wizard® SV Gel and PCR Clean-up System (Promega Corp.). Sequencing was outsourced to FASMAC Co., Ltd. (Kanagawa, Japan). The quality of returned sequences was checked using the Applied Biosystems™ Sequence Scanner Software v2.0. All high-quality sequences were aligned using ClustalW implemented in MEGA 7 (Kumar et al., 2016). BLAST searches were performed in GenBank to compare obtained and registered sequences and identify sequences with the lowest E-values and highest similarities.

1.3. Result

1.3.1. Morphological study

Nematoda

Family Gnathostomatidae Railliet, 1895

Subfamily Spiroxyinae Baylis and Lane, 1920

Spiroxys hanzaki **Hasegawa, Miyata and Doi, 1998**

Measurement

Male (based on 10 adult specimens): body 13.9–30.0 (21.2 ± 5) mm long and 0.4–0.6 (0.5 ± 0.1) mm wide. Esophagus 2.9–6.6 (4.6 ± 1) mm long and 133–280 (211 ± 54) wide near posterior end. Nerve ring, excretory pore, deirids 534–947 (753 ± 133), 634–1234 (889 ± 176) and 1207–1367 (1309 ± 61), respectively, from anterior extremity. Spicules 760–1234 (1005 ± 124) long and 40–53 (47 ± 5). Tail 227–334 (293 ± 30) long.

Female (based on 12 adult specimens): body 21.9–40.2 (29.9 ± 5) mm long and 0.4–0.9 (0.7 ± 0.1) mm wide. Esophagus 2.7–6.5 (4.8 ± 1) mm long and 173–320 (235 ± 44) wide near posterior end. Nerve ring, excretory pore, deirids 667–1121 (845 ± 157), 867–1254 (1005 ± 118) and 1301–1934 (1431 ± 194), respectively, from anterior extremity. Vulva 12.7–24.2 (19.4 ± 4) mm from anterior extremity. Eggs 76–88 (80 ± 4) by 45–76 (58 ± 6) (n=25). Tail 367–667 (519 ± 93) long.

Taxonomic summary

Host: hybrid *Andrias* between *A. japonicus* (Temminck, 1836) and Chinese *Andrias* species.

Infection site: stomach.

Stage: adults and third stage larvae.

Locality: Kyoto City, Kyoto Prefecture, Japan: Kamo River (35°03'33" N, 135°45'00" E) [site no. 1 in Fig. 1.1], Kurama River (35°06'23" N, 135°45'52" E) [2], Katsura River (35°12'19" N, 135°44'32" E; 35°14'58" N, 135°45'56" E; 35°15'57" N, 135°44'34" E) [6], Teratani River (35°13'58" N, 135°47'35" E) [7], and Kiyotaki River (35°03' N, 135°46' E) [9].

Studied specimens: KUZ Z3910.

Remarks: general morphology agreed with Hasegawa et al. (1998). This work provides the first measurements of this species parasitizing giant salamanders in Kyoto.

Family Trichuridae (Ransom, 1911)

Subfamily Capillariinae Railliet, 1915

Amphibiocapillaria tritonispunctati **(Diesing, 1815) Moravec, 1982**

Measurement

Female (based on 2 specimens): body 9.2– 9.7 mm long and 67–87 wide. Esophagus 203–266 long. Stichocytes and vulva at 3.7– 4.2 mm and 4.6–4.9 mm, respectively, from anterior extremity. Nuclei 110–112 in stichosome. Eggs 52–60 (56 ± 3) by 27–30 (30 ± 1) (n=24). Rectum 79–88 long.

Taxonomic summary

Host: *Andrias davidianus* (Blanchard, 1871) and hybrid between *A. japonicus* and Chinese *Andrias* species.

Infection site: intestine and rectum.

Stage: adults.

Locality: Kyoto City, Kyoto Prefecture, Japan: Kamo River (35°06'46" N, 135°43'12" E) [1], Kurama River (35°06'23"

N, 135°45'52" E) [2], Nakatsu River (35°06'41" N, 135°43'27" E) [5], and Takano River (35°06'03" N, 135°49'32" E) [8].
Studied specimens: KUZ Z3911.
Remarks: general morphology consistent with Moravec (1982, 1986). This work provides the first measurements of this species parasitizing giant salamanders in Kyoto.

Family Kathlaniidae Lane, 1914

Subfamily Kathlaniinae Lane, 1914

***Falcaustra* sp.** (see Chapter 2)

Description

General: body elongate. Three well-developed lips present. Esophagus consisting of three distinct parts; esophageal corpus, short isthmus and esophageal bulb. Tail tapering.
Male (based on 10 specimens): body 7.8–12.3 (9.7 ± 1) mm long and 250–434 (334 ± 50) wide in midbody. Lips 27–33 (29 ± 2) by 55–67 (61 ± 4). Pharyngeal part 55–79 (71 ± 8) long and 39–52 (46 ± 5) wide. Esophageal corpus 1.2–1.5 (1.4 ± 0.9) mm long and 67–87 (73 ± 6) wide, short isthmus 100–120 (108 ± 6) long and 73–113 (91 ± 11) wide, esophageal bulb 139–193 (163 ± 17) long and 147–220 (178 ± 21) wide. Nerve ring and excretory pore at 279–349 (318 ± 21) and 1201–1414 (1306 ± 65), respectively, from anterior extremity. Single pseudosucker consisting of 13–15 pairs of muscles, 1.2–2.5 (1.9 ± 0.4) mm from cloaca. Spicules two, elongate, pointed; left spicule 547–727 (614 ± 45) long and 20–40 (31 ± 7) wide, right spicule 600–700 (635 ± 27) long and 21–40 (32 ± 7) wide. Gubernaculum 91–127 (108 ± 10) by 30–47 (37 ± 7). Tail 320–434 (386 ± 32) long.
Female (based on 10 specimens): body 9.8–14.0 (11.6 ± 1) mm long and 313–534 (399 ± 62) wide in midbody. Lips 24–36 (31 ± 4) by 36–70 (59 ± 10). Pharyngeal part 58–82 (72 ± 7) long and 24–58 (47 ± 11) wide. Esophageal corpus 1.3–1.9 (1.5 ± 0.2) mm long and 67–87 (78 ± 6) wide, short isthmus 73–120 (100 ± 13) long and 87–127 (103 ± 12) wide, esophageal bulb 147–193 (172 ± 17) long and 173–220 (194 ± 16) wide. Nerve ring and excretory pore at 306–427 (347 ± 41) and 1234–1581 (1393 ± 123), respectively, from anterior extremity. Vulva 6.2–8.8 (7.3 ± 0.8) mm long from anterior extremity. Eggs oval, with a layer, 61–73 (65 ± 3) by 42–55 (48 ± 3) (n=62). Tail 239–1134 (689 ± 227) long.

Taxonomic summary

Host: *Andrias davidianus* (Blanchard, 1871) and hybrid between *A. japonicus* and Chinese *Andrias* species.
Infection site: intestine and rectum.
Stage: adults and larvae.
Locality: Kyoto City, Kyoto Prefecture, Japan: Kamo River (35°01'16–52" N, 135°46'14–17" E; 35°03'33" N, 135°45'00" E; 35°06'46" N, 135°43'12" E) [1], Kurama River (35°05'52" N, 135°45'47" E; 35°06'23" N, 135°45'52" E) [2], Shizuhara River (35°05'52" N, 135°46'20" E; 35°06'14" N, 135°46'51" E) [3], Myozin River (35°03'27" N, 135°45'17" E) [4], Nakatsu River (35°06'41" N, 135°43'27" E) [5], Katsura River (35°12'19" N, 135°44'35" E; 35°15'57" N, 135°44'34" E) [6], Teratani River (35°13'58" N, 135°47'35" E) [7], Takano River (35°06'03" N, 135°49'32" E) [8], Kiyotaki River (35°03' N, 135°46' E) [9].
Studied specimens: KUZ Z3912.
Remarks: the specimens examined showed morphological features consistent with the genus *Falcaustra* as defined by Chabaud (2009) in the structure of lips and esophagus. Compared to the native congeneric species previously reported in Japan, *Falcaustra* sp. differed as follows: (1) single pseudosucker present instead of plural pseudosuckers present in males

of *F. odaiensis* Hasegawa and Nishikawa, 2009, (2) spicules (547–727 long) shorter than those (1.2–1.3 mm long) in *F. japonensis* (Yamaguti, 1935) (Yamaguti, 1935; Hasegawa and Nishikawa, 2009). *Falcaustra* sp. also differed from the introduced congeneric species reported in Japan as follows: (1) spicules (547–727 long) longer than those (277–314 long) in *F. catesbeianae* Walton, 1929, (2) pseudosucker consisting of 13–15 pairs of muscles instead of elongate pseudosucker consisting of 41–44 pairs of muscles in *F. wardi* (Mackin, 1936) (Baker, 1986; Hasegawa, 2006).

Trematoda

Family Liolopidae

***Liolope copulans* Cohn, 1902**

Measurement

Adult (based on 9 specimens): body 2.3–3.7 (3.1 ± 0.5) mm by 1.4–1.9 (1.7 ± 0.2) mm. Oral sucker 107–200 (160 ± 26) by 173–247 (210 ± 23). Pharynx 73–113 (95 ± 14) by 80–167 (124 ± 23). Ventral sucker 193–260 (230 ± 24) by 280–340 (307 ± 16). Anterior testis 173–567 (387 ± 117) by 334–494 (417 ± 54), posterior testis 273–614 (391 ± 127) by 287–534 (409 ± 74). Cirrus pouch 400–754 (631 ± 107) by 400–714 (588 ± 91). Seminal vesicle 400–700 (594 ± 94) by 160–300 (223 ± 37). Ovary 187–293 (254 ± 29) by 200–293 (253 ± 30). Eggs 12–26 (19 ± 4) in uterus, 140–147 (145 ± 3) by 73–80 (78 ± 3) (n=38).

Taxonomic summary

Host: *Andrias davidianus* (Blanchard, 1871) and hybrid between *A. japonicus* and Chinese *Andrias* species.

Infection site: stomach and intestine.

Stage: adults.

Locality: Kyoto City, Kyoto Prefecture, Japan: Kamo River (35°01'16–52" N, 135°46'14–17" E; 35°03'01–33" N, 135°45'00–29" E; 35°06'46" N, 135°43'12" E) [1], Kurama River (35°05'52" N, 135°45'47" E; 35°06'23" N, 135°45'52" E) [2], Shizuhara River (35°05'52" N, 135°46'20" E; 35°06'14" N, 135°46'51" E) [3], Myozin River (35°03'27" N, 135°45'17" E) [4], Nakatsu River (35°06'41" N, 135°43'27" E) [5], Katsura River (35°12'19" N, 135°44'32" E; 35°14'58" N, 135°45'56" E; 35°15'57" N, 135°44'34" E) [6], Teratani River (35°13'58" N, 135°47'35" E) [7], Takano River (35°06'03" N, 135°49'32" E) [8], Kiyotaki River (35°03' N, 135°40' E) [9].

Studied specimens: KUZ Z3908–Z3909.

Remarks: general morphology agreed with Baba et al. (2011). This work provides the first measurements of this species parasitizing giant salamanders in Kyoto.

1.3.2. Molecular study

The ITS1 region of *S. hanzaki* was successfully sequenced for 1,551 bp (accession no. LC605542). The BLAST search showed the highest similarity (99%) with a sequence of *S. hanzaki* from *A. japonicus* (Japan) (KF530326: Li et al., 2014). The partial 18S rDNA of *Am. tritonispunctati* was successfully sequenced for 786 bp (accession no. LC605543). The BLAST search showed the highest similarity (94%) with a sequence of *Aonchotheca putorii* (Rudolphi, 1819) (Nematoda: Trichuridae) (LC052349: Tamaru et al., 2015). The partial 28S rDNA of *Falcaustra* sp. was successfully sequenced for 596 bp (accession no. LC605539–LC605541). The BLAST search showed the highest similarity (98%) with a sequence of *Megalobatrachonema terdentatum* (Linstow, 1890) (Nematoda: Kathlaniidae) (MN444706, Chen et al., 2020a). The haplotype of larval *Falcaustra* sp. differed from those of adult *Falcaustra* sp. by 0.2–0.3% (*p*-distance). Two haplotypes of adult *Falcaustra* sp. differed by 0.2% (*p*-distance).

1.4. Discussion

The parasite fauna of *Andrias* populations in Kyoto Prefecture consisted of *Liolope copulans*, *Spiroxys hanzaki*, *Amphibiocapillaria tritonispunctati*, and *Falcaustra* sp. *Liolope copulans* and *Falcaustra* sp. were found in specimens at all of the study sites and were the most abundant species in the helminth fauna of giant salamanders in Kyoto Prefecture. No parasite species documented by Yamaguti (1936, 1939, 1941) were found in this study. Molecular data of *S. hanzaki* confirmed the species-level identification of the specimens based on morphology. Molecular studies for *Am. tritonispunctati* and *Falcaustra* sp. also supported the subfamily-level identifications based on morphology. Genetic differentiation between the haplotype of larval *Falcaustra* sp. and those of adults were similar to genetic differentiation between those of two adult nematodes; therefore, we concluded that larval *Falcaustra* specimens were the same species as adult *Falcaustra* specimens. *Spiroxys hanzaki* and *Am. tritonispunctati* are considered native parasites in Japan because *S. hanzaki* has been only reported parasitizing *A. japonicus* in Japan (e.g. Hasegawa et al., 2002). *Amphibiocapillaria tritonispunctati* is widely distributed over the Holarctic region (Moravec, 1986); however, this species has been recorded from multiple different species of Caudata in Japan for many years (Uchida et al., 2019). These facts permit us to regard them as helminths not derived from other countries. Therefore, it was concluded that the introduced *A. davidianus* could act as spillback reservoirs for native parasites in Kyoto Prefecture. It suggests that "enemy release" could not be found in introduced *A. davidianus* in Kyoto Prefecture, unlike the case demonstrated in Torchin et al. (2003). It is unclear whether such spillback affects the host-parasite relationship between the native populations of *A. japonicus* and parasites. This study is the first record of *Falcaustra* sp. found in giant salamanders in Japan. Further taxonomical studies for this genus were shown in Chapter 2.

1.5. Tables and figures

Table 1.1 Summary for the examined hybrid *Andrias* and their parasites: TL total length (mm).

Locality	Capturing date	Euthanizing date	Host TL	*S. hanzaki*	*Am. tritonispunctati*	*Falcaustra* sp.	*L. copulans*
Kamo River							
	2011Nov30		1075	1		1	149
	2016Dec03	2017Jan24	1007			9	1332
	2016Nov05		781			50	95
	2016Nov05		754			19	29
	2010May20	2017Mar14	413			5	1
Kurama River							
	2011Oct19	2017Mar02	880			3	16
	2011Jul17	2017Apr05	1017	1	2	1	12
Shizuhara River							
	2016Dec03	2017Jan24	1085			23	262
	2011Jul17	2017Mar14	895			2	150
	2014Sep11	2017Apr11	1139			176	289
Myozin River							
	2009Oct13	2017Mar14	979			2	4
	2010Jun14		917			6	7
Nakatsu River							
	2017Jun24	2017Jun28	508			6	
			348		1	69	47
Katsura River							
	2016Aug31	2017Mar02	931	7		25	358
	2013Oct12		770	7			6
	2014Sep08		993	45		6	88
	2012Aug06	2017Mar24	991			1	495
	2015Apr09		901	10		2	996
	2012Feb09	2017Apr11	1079	6			23
	2014Sep08		1119	11			33
Teratani River							
	2011Nov04	2017Mar02	880	10		17	557
		2017Mar24	1014			2	705
Takano River							
	2012Oct18	2017Mar02	812		1	49	25
Kiyotaki River							
	2011Oct27	2017Mar02	840	3		4	334
	Prevalence (%)			40	12	88	96
	Mean Intensity			10	1	22	251

Table 1.2 Summary for the examined *A. davidianus* and their parasites: TL total length (mm)

Locality	Capturing date	Euthanizing date	Host TL	*Am. tritonispunctati*	*Falcaustra* sp.	*L. copulans*
Kamo River						
	2009Jul10	2017Apr05	1131	17	114	8
			1018			32

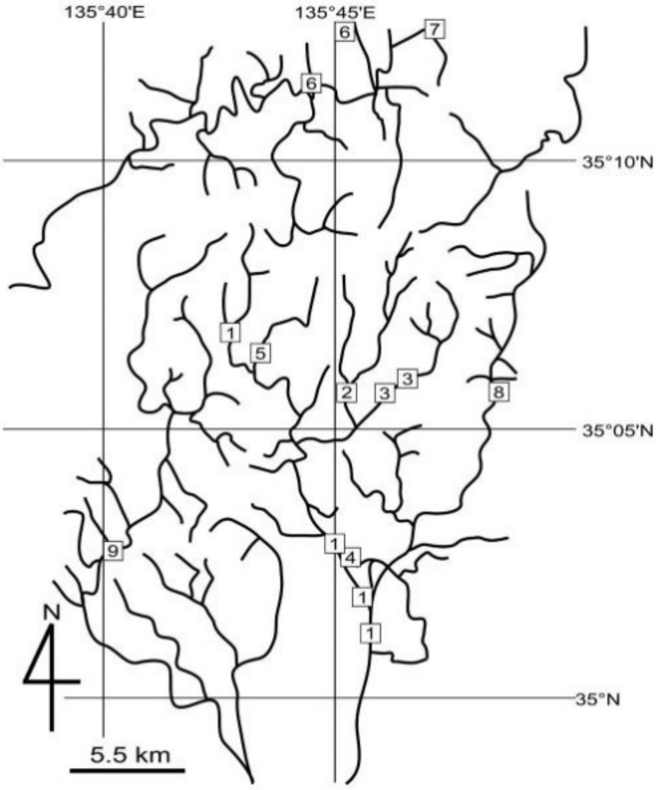

Fig. 1.1 Map of sampling sites for *Andrias* surveyed for internal parasites in Kyoto City (1, Kamo River; 2, Kurama River; 3, Shizuhara River; 4, Myozin River; 5, Nakatsu River; 6, Katsura River; 7, Teratani River; 8, Takano River and 9, Kiyotaki River).

Chapters 2 and 3 will be made public in journals in the future.

General summary

Chapters 1 and 2 provided a good empirical model for theme 1, which locates the parasitological evaluation of alien species problems in amphibians. In the case of giant salamanders, host introduction could trigger the latent risk against indigenous populations by indirect effects through the native parasite spillback. The present study proved the validity of the parasitological approach to comprehend the net impact of biological introductions. On the other hand, I could not conclude the presence or absence of alien parasite species in their populations due to the lack of parasitological studies for them in both Japan and China. The finding of new species in Chapter 2 permits us to consider that there remains room for fundamental study in parasites of Japanese giant salamanders. Neither local specificity nor host endemicity of parasite fauna is little known in them. Therefore, further parasitological study in various local populations is essential to understand the local specificity and continuous monitoring of parasite dynamics is desirable to detect the long-term impact of host hybridization. The conservation policy for giant salamanders is still unstable and under construction in Japan (Nishikawa, 2017). Hence, the parasitological study can help us with establishing a sustainable protection policy and realizing proper species conservation for native populations. In the case of giant salamanders, the comprehension of the spillback effect can be referred to decide the maximum number of hybrids in water systems and contribute to realize long-term conservation.

Besides, species conservation should be performed in an inclusive ecosystem with parasites. Endangered species face the risk of extinction in both their own population and their endemic parasite populations (Harris et al., 2013). Parasites are also a valuable biological resource for the ecosystem and human society, the same as host animals, due to their important role in biological interactions and food webs (Lafferty et al., 2008). A developed study for estimating host-parasite association provides useful information for such expanded conservation.

In terms of theme 2, elucidation of taxonomical confusion in parasites, Chapter 2 showed the systematic problem of higher classification in kathlaniid nematodes. Current molecular studies showed a mismatch between morphological base systematics and genetic base one in parasitic helminths. The present study challenged the boundary between superfamilies Cosmocercoidea and Seuratoidea and suggested the mismatch between the evolutional hypothesis by morphological features and the phylogenetic relationship based on the molecular approach. Chapter 3 also showed taxonomical confusion in trematodes derived from unstable morphological key characteristics. *Mesocoelium* species is cosmopolitan and has extremely low host specificity with vertebrate hosts. These biological features can cause a morphological variation in organ sizes and positions within an identical species. Direct comparison between examined specimens and holotypes or vouchers is often difficult in cosmopolitan species because of their accessibility, which causes many synonyms, which may be turned out to be invalid later. On the other hand, taxonomical study with only a molecular approach holds difficulty because of the necessity to define the border whether intra- or inter-specific divergence in genotypes. Simultaneously, it can be essential to confirm the morphological variety of a certain species. Both perspectives enable us to evaluate the species status. Therefore, a combination study of both morphological and molecular approaches can become further significant to resolve the systematics in parasite taxa than ever.

Parasites hold a lot of possibilities as a biotic parameter to assess the regional ecosystem. There remains extensive room to study in taxonomy, life history, interaction, and ecology in parasitic helminths in amphibians. It can be expected that the parasitological approach would contribute to finding out the ecology and environmental problem in amphibians in the future.

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