GENETICS, EVOLUTION, AND PHYLOGENY - SHORT COMMUNICATION

Evaluating the ontogenetic external morphology of an ectoparasitic *Torix tukubana* (Hirudinida: Glossiphoniidae), with records of its new host amphibian species

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Abstract

Torix is a leech genus containing freshwater proboscidate species, and several members of this taxon are ectoparasites specific to amphibians. *Torix tukubana* inhabits mountain streams in Japan, and only two frog species are known to be hosts. We collected this leech from two other amphibians, *Onychodactylus japonicus* (Japanese clawed salamander) and *Rana ornativentris* (montane brown frog), for the first time. This finding suggests that the host specificity of *T. tukubana* is low. The immature individuals of *T. tukubana* were also collected and identified based on DNA data. This is the first juvenile record of this species confirmed by its DNA barcode sequences. Several morphological characters known from large individuals and used as diagnostic characteristics in taxonomic keys were not observed in the juveniles, suggesting that these are ontogenetic traits.

Keywords Leech · *Onychodactylus japonicus* · *Rana ornativentris* · *cox1* · Japan

Introduction

Torix is a leech genus classified within the proboscidate family Glossiphoniidae, and the genus now contains seven nominal species, which are mainly distributed in East Asia (Sawyer 1986). Three Japanese species—*T. orientalis* (Oka 1925a), *T. tagoi* (Oka 1925b), and *T. tukubana* (Oka 1935)—inhabit mountain streams in Japan and are ectoparasitic on multiple amphibian species (Oka 1925a, b; Sasaki 2015). However, further elucidation of their host specificities and their true taxonomic accounts has been hampered by a lack of sufficient information regarding their hosts, developmental stages, and life cycles.

Torix tukubana is characterized by its possession of developed papillae on the dorsal surface (Oka 1935) and can thus be identified based on its external characteristics. To our knowledge, only two Japanese brown frogs—*Rana japonica* Boulenger 1879 and *Rana tagoi* Okada 1928—have been reported as host amphibians of this leech (Kikuchi and Fukatsu 2005; Yoshida 2009). However, we recently found *T. tukubana* individuals that were parasitic on other mountainous Japanese amphibians. Here, we report those amphibian species as new hosts of *T. tukubana*. In addition, we briefly report the morphological differences between the large individuals and juveniles of this leech species according to mitochondrial DNA identification and observation of their external morphology.

Materials and methods

During faunal investigations of Mt. Kanmuriyama in Hiroshima Prefecture, Honshu, Japan (34°28′07″ N, 132°04′33″ E), that were carried out from March 2016 to June 2018, we observed amphibians in the mountain streams. Host amphibians were not collected but were identified by the first

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Table 1 Specimens collected and used in this study

Voucher	Collection date	Host	Stage	Ventral annulation	*Cox1* INSDC accession no.
KUZ Z2016	23 Mar 2016	*Rana tagoi, Rana ornativentris*	Juvenile	Uniannulate	LC413905
KUZ Z2017	18 Jun 2017	*Onychodactylus japonicus*	Large	Triannulate	LC413906
KUZ Z2019	17 Dec 2017	Free-living	Large	Triannulate	LC413907
KUZ Z2020	07 Jan 2018	Free-living	Large	Triannulate	LC413908
KUZ Z2025	19 May 2018	*Rana tagoi*	Juvenile	Biannulate	LC413909
KUZ Z2026	19 May 2018	*Rana tagoi*	Juvenile	Triannulate	LC413910
KUZ Z2028	26 May 2018	*Onychodactylus japonicus* (Larvae)	Juvenile	Biannulate	LC413911
KUZ Z2056	02 Jun 2018	Free-living	Large	Triannulate	LC413912

author in the field. Leeches attached to these amphibian hosts and free-living individuals from the same locations were collected and used for identification. In total, eight lots that comprise 53 individuals were obtained. All leeches were directly preserved in 99% ethanol and deposited in the Zoological Collection of Kyoto University (KUZ) (Table 1).

The leeches were identified preliminarily by their external morphologies based on Oka (1935). Mitochondrial cytochrome *c* oxidase subunit I gene (*cox1*) sequences, known as a usable DNA barcoding marker for animals and often used for phylogenetic estimation of leeches (Siddall and Burreson 1998; Tessler et al. 2018), were then determined from the specimens. The partial *cox1* sequences for the leeches were obtained using the methods described in Nakano (2012) using the primer sets (LCO1490 and HCO2198: Folmer et al. 1994), and the total DNA of each specimen was extracted from its caudal sucker. The sequence data collected in this study were deposited in the International Nucleotide Sequence Database Collaboration (INSDC) through the DNA Data Bank of Japan (Table 1). The *cox1* sequences were aligned by MAFFT version 7 (Katoh and Standley 2013) and their uncorrected *p*-distances were calculated using MEGA6 (Tamura et al. 2013).

Results and discussion

In total, 53 (four large and 49 small) glossiphoniid leeches were collected. Among them, three individuals were free-living, and the other 50 specimens were ectoparasitic on amphibians. On 18 June 2017, four Japanese clawed salamanders, *Onychodactylus japonicus* (Houttuyn 1782), gathered for breeding in a headstream where the water was gushing from the bedrock. We discovered one large *Torix tukubana* attached to the right forefoot of one of these salamanders and collected this leech (KUZ Z2017). Three large free-living *T. tukubana* individuals were collected (KUZ Z2019, Z2020, and Z2056) at the same time.

On 23 March 2016, we found many small leeches clinging to two other amphibians, *Rana tagoi* and *R. ornativentris* Werner 1903 (montane brown frog), submerged under the

rocks. Twenty-two leeches were attached to the first of these frogs and six leeches to the second; the leeches were preserved (KUZ Z2016). Similarly, we collected 34 and seven small leeches from distinct individuals of *R. tagoi* on 19 May 2018 (KUZ Z2025 and Z2026, respectively), and one small individual was isolated from a larva of *O. japonicus* on 26 May 2018 (KUZ Z2028). Because these leeches lacked key morphological traits for species identification, we analyzed their *cox1*.

We amplified and sequenced the partial *cox1* region (645 bp in length) using the eight specimens (four large *T. tukubana* and four unidentified small leeches) from Mt. Kanmuriyama. The *cox1* sequence of one of the small leeches (KUZ Z2016) dovetailed perfectly with those of the several large individuals of *T. tukubana* (KUZ Z2017, Z2019, and Z2020) (Table 2), and thus, we concluded that the small leeches from *R. tagoi* and *R. ornativentris* were the juveniles of *T. tukubana*. Although few *cox1* divergences (*p*-distance: 0.1–0.9%) were observed among other specimens (including both juveniles and large individuals), the values fall in the range of intraspecific *cox1* divergences for other leeches (e.g., de Carle et al. 2017).

It is known that *T. tukubana* is distinguishable from other congenic species by the possession of numerous papillae on each annulus and a unique mid-body annulation pattern (dorsally biannulate and ventrally triannulate: Fig. 1). In addition,

Table 2 Uncorrected *p*-distance of the *cox1* sequences of *Torix tukubana*

Voucher	1	2	3	4	5	6	7	8
1. KUZ Z2016								
2. KUZ Z2017	0.000							
3. KUZ Z2019	0.000	0.000						
4. KUZ Z2020	0.000	0.000	0.000					
5. KUZ Z2025	0.004	0.004	0.004	0.004				
6. KUZ Z2026	0.007	0.007	0.007	0.007	0.006			
7. KUZ Z2028	0.004	0.004	0.004	0.004	0.000	0.006		
8. KUZ Z2056	0.009	0.009	0.009	0.009	0.007	0.001	0.007	

Fig. 1 Dorsal (a-d) and ventral (e-h) views of *Torix tukubana* collected in this study. Large individual (KUZ Z2020: a, e) and juveniles; ventrally uniannulate (KUZ Z2016: b, f), biannulate (KUZ Z2025: c, g), and triannulate (KUZ Z2026: d, h). Scale bar 5 mm
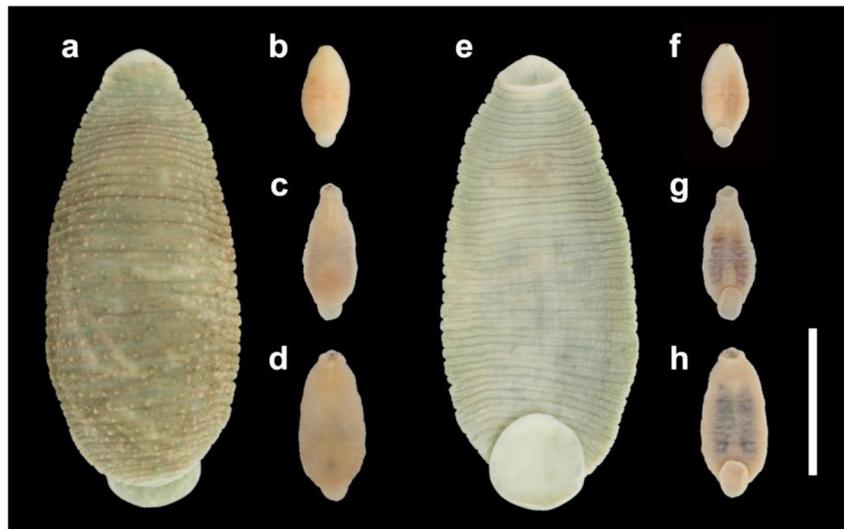
one pair of eyes and a small caudal sucker (with a diameter less than half the body width) are recognized as the diagnostic characteristics of this species (Sawyer 1986). Some of these characteristics were commonly observed in both the large and small individuals collected here. However, dorsal papillae, which are quite prominent in large individuals, were not observed in juveniles. Furthermore, there were some variations in the ventral annulus structures of their mid-body segments (uniannulate: KUZ Z2016; biannulate: Z2025 and Z2028; triannulate: Z2026) (Table 1). These morphological characters are assumed to be ontogenetic traits, and the morphologies will be developed along with the growth. In addition, the characteristics of no papillae on the back and mid-body segments biannulate dorsally and ventrally are consistent with the description of *T. tagoi* in Sawyer (1986). As a result, it is possible that the juveniles of *T. tukubana* have been confused with *T. tagoi*. Further taxonomic studies will be needed to clarify whether there is a genetic or morphological difference between the two.

Although only *R. tagoi* and *R. japonica* are known hosts of *T. tukubana* so far, our observations added new host records for this leech. Because host *Onychodactylus* salamanders and *Rana* frogs belong to distinct amphibian orders, the host specificity of *T. tukubana* seems to be low. Moreover, the leech was also found on a larva of *O. japonicus*, suggesting that all amphibians that use stream environments, regardless of their growth stage, can be potential hosts of *T. tukubana*.

It is known that some glossiphoniid leeches including *T. tukubana* are sometimes infected by *Rickettsia* spp. (Kikuchi et al. 2002; Kikuchi and Fukatsu 2005). Meanwhile, there are many unknown parts in the life cycle of *Rickettsia*, including their degree of host specificity and the mechanism used to maintain the population, and these are usually unique to each *Rickettsia* species. In the terrestrial ecosystem, *Rickettsia* is known to enlarge its population through horizontal transmission via the vertebrate hosts of blood-sucking arthropods (Dasch and Weiss 1992). Although it is plausible that amphibians, the host of *Torix* leeches, are also involved in the maintenance of *Rickettsia*, no data on *Rickettsia* within amphibians are available yet. To assess the amphibian-leech-*Rickettsia* relationship, it is first necessary to ascertain the range of host leech taxon for each *Rickettsia* species and the availability of amphibians as a secondary host. Consequently, further study into precise identification of the leeches, phylogenetic estimation of *Rickettsia* from each leech species, and a survey of *Rickettsia* in amphibians will be required.

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