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Author(s)	Kabayashi, Chiaki; Kurabayashi, Atsushi; Nakano, Takafumi
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1 SHORT COMMUNICATION

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

4

5 Chiaki Kambayashi<sup>1</sup> • Atsushi Kurabayashi<sup>2,3</sup> • Takafumi Nakano<sup>4</sup>

6

7 <sup>1</sup> Amphibian Research Center, Hiroshima University, Hiroshima, Japan

8 <sup>2</sup> Department of Bio-Science, Nagahama Institute of Bio-Science and Technology, Shiga,

9 Japan

10 <sup>3</sup> Unit for Environmental Sciences and Management, North-West University, Potchefstroom,

11 South Africa

12 <sup>4</sup> Department of Zoology, Graduate School of Science, Kyoto University, Kyoto, Japan

13

14 Corresponding author: Chiaki Kambayashi

15 [chkambayashi@gmail.com](mailto:chkambayashi@gmail.com)

16 +81-82-424-4494

17 ORCID IDs:

- 18 0000-0003-4820-9842 (Chiaki Kambayashi)
- 19 0000-0002-0381-5219 (Atsushi Kurabayashi)
- 20 0000-0001-6107-2188 (Takafumi Nakano)

21 **Abstract**

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

32

33 **Keywords** leech • *Onychodactylus japonicus* • *Rana ornativentris* • *cox1* • Japan

34

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38

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42

43 **Introduction**

44

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

52 *Torix tukubana* is characterized by its possession of developed papillae on the dorsal  
53 surface (Oka 1935), and can thus be identified based on its external characteristics. To our  
54 knowledge, only two Japanese brown frogs: *Rana japonica* Boulenger, 1879 and *Rana tagoi*  
55 Okada, 1928, have been reported as host amphibians of this leech (Kikuchi and Fukatsu  
56 2005; Yoshida 2009). However, we recently found *T. tukubana* individuals that were parasitic  
57 on other mountainous Japanese amphibians. Here, we report those amphibian species as new  
58 hosts of *T. tukubana*. In addition, we briefly report the morphological differences between the  
59 large individuals and juveniles of this leech species according to mitochondrial DNA

60 identification and observation of their external morphology.

61

## 62 **Materials and methods**

63

64 During faunal investigations of Mt. Kanmuriyama in Hiroshima Prefecture, Honshu, Japan  
65 (34°28'07"N, 132°04'33"E) that were carried out from March 2016 to June 2018, we  
66 observed amphibians in the mountain streams. Host amphibians were not collected, but were  
67 identified by the first author in the field. Leeches attached to these amphibian hosts and  
68 free-living individuals from the same locations were collected and used for identification. In  
69 total, eight lots that comprise 53 individuals were obtained. All leeches were directly  
70 preserved in 99% ethanol and deposited in the Zoological Collection of Kyoto University  
71 (KUZ) (Table 1).

72 The leeches were identified preliminarily by their external morphologies based on Oka  
73 (1935). Mitochondrial cytochrome *c* oxidase subunit I gene (*cox1*) sequences, known as a  
74 usable DNA barcoding marker for animals and often used for phylogenetic estimation of  
75 leeches (Siddall and Burreson 1998; Tessler et al. 2018), were then determined from the  
76 specimens. The partial *cox1* sequences for the leeches were obtained using the methods

77 described in Nakano (2012) using the primer sets (LCO1490 and HCO2198: Folmer et al.  
78 1994), and the total DNA of each specimen was extracted from its caudal sucker. The  
79 sequence data collected in this study were deposited in the International Nucleotide Sequence  
80 Database Collaboration (INSDC) through the DNA Data Bank of Japan (Table 1). The *cox1*  
81 sequences were aligned by MAFFT version 7 (Kato and Standley 2013) and their  
82 uncorrected *p*-distances were calculated using MEGA6 (Tamura et al. 2013).

83

## 84 **Results and discussion**

85

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



94           On 23 March 2016, we found many small leeches clinging to two other amphibians,  
95   *Rana tagoi* and *R. ornativentris* Werner, 1903 (montane brown frog), submerged under the  
96   rocks. Twenty-two leeches were attached to the first of these frogs and six leeches to the  
97   second; the leeches were preserved (KUZ Z2016). Similarly, we collected 34 and seven small  
98   leeches from distinct individuals of *R. tagoi* on 19 May 2018 (KUZ Z2025 and Z2026,  
99   respectively), and one small individual was isolated from a larva of *O. japonicus* on 26 May  
100   2018 (KUZ Z2028). Because these leeches lacked key morphological traits for species  
101   identification, we analyzed their *cox1*.

102           We amplified and sequenced the partial *cox1* region (645 bp in length) using the eight  
103   specimens (four large *T. tukubana* and four unidentified small leeches) from Mt.  
104   Kanmuriyama. The *cox1* sequence of one of the small leeches (KUZ Z2016) dovetailed  
105   perfectly with those of the several large individuals of *T. tukubana* (KUZ Z2017, Z2019, and  
106   Z2020) (Table 2), and thus we concluded that the small leeches from *R. tagoi* and *R.*  
107   *ornativentris* were the juveniles of *T. tukubana*. Although few *cox1* divergences (*p*-distance:  
108   0.1–0.9%) were observed among other specimens (including both juveniles and large  
109   individuals), the values fall in the range of intraspecific *cox1* divergences for other leeches  
110   (e.g. de Carle et al. 2017).

111 It is known that *T. tukubana* is distinguishable from other congenic species by the  
112 possession of numerous papillae on each annulus and a unique mid-body annulation pattern  
113 (dorsally biannulate and ventrally triannulate: Fig. 1). In addition, one pair of eyes and a  
114 small caudal sucker (with a diameter less than half the body width) are recognized as the  
115 diagnostic characteristics of this species (Sawyer 1986). Some of these characteristics were  
116 commonly observed in both the large and small individuals collected here. However, dorsal  
117 papillae, which are quite prominent in large individuals, were not observed in juveniles.  
118 Furthermore, there were some variations in the ventral annulus structures of their mid-body  
119 segments (uniannulate: KUZ Z2016; biannulate: Z2025 and Z2028; triannulate: Z2026)  
120 (Table 1). These morphological characters are assumed to be ontogenetic traits, and the  
121 morphologies will be developed along with the growth. In addition, the characteristics of no  
122 papillae on the back and mid-body segments biannulate dorsally and ventrally are consistent  
123 with the description of *T. tagoi* in Sawyer (1986). As a result, it is possible that the juveniles  
124 of *T. tukubana* have been confused with *T. tagoi*. Further taxonomic studies will be needed to  
125 clarify whether there is a genetic or morphological difference between the two.

126 Although only *R. tagoi* and *R. japonica* are known hosts of *T. tukubana* so far, our  
127 observations added new host records for this leech. Because host *Onychodactylus*

128 salamanders and *Rana* frogs belong to distinct amphibian orders, the host specificity of *T.*  
129 *tukubana* seems to be low. Moreover, the leech was also found on a larva of *O. japonicus*,  
130 suggesting that all amphibians that use stream environments, regardless of their growth stage,  
131 can be potential hosts of *T. tukubana*.

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

145

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203 Table 1. Specimens collected and used in this study.

Voucher	Collection Date	Host	Stage	Ventral Annulation	<i>Cox1</i> INSDC Accession #
KUZ Z2016	23 Mar 2016	<i>Rana tagoi</i> , <i>Rana ornativentris</i>	Juvenile	uniannulate	LC413905
KUZ Z2017	18 Jun 2017	<i>Onychodactylus japonicus</i>	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	<i>Rana tagoi</i>	Juvenile	biannulate	LC413909
KUZ Z2026	19 May 2018	<i>Rana tagoi</i>	Juvenile	triannulate	LC413910
KUZ Z2028	26 May 2018	<i>Onychodactylus japonicus</i> (Larvae)	Juvenile	biannulate	LC413911
KUZ Z2056	02 Jun 2018	Free-living	Large	triannulate	LC413912

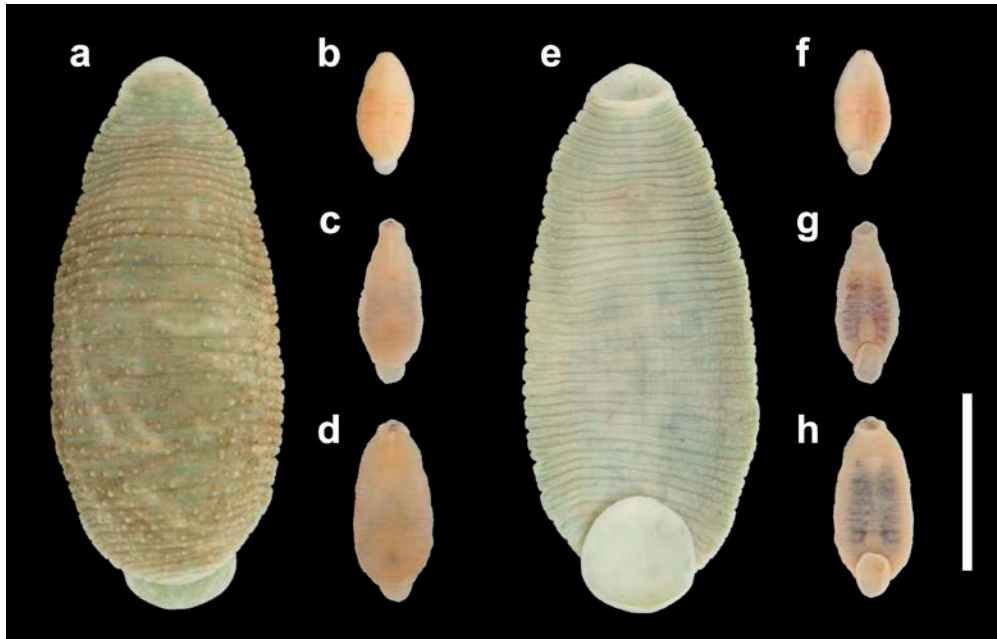
204



205 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	

206



207

208 **Fig. 1** Dorsal (a-d) and ventral (e-h) views of *Torix tukubana* collected in this study. Large

209 individual (KUZ Z2020: a, e) and juveniles; ventrally uniannulate (KUZ Z2016: b, f),

210 biannulate (KUZ Z2025: c, g) and triannulate (KUZ Z2026: d, h). Scale bar: 5 mm

211