TITLE:

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

AUTHOR(S):

Kambayashi, Chiaki; Kurabayashi, Atsushi; Nakano, Takafumi

CITATION:

Kambayashi, Chiaki ...[et al]. Evaluating the ontogenetic external morphology of an ectoparasitic *Torix tukubana* (Hirudinida: Glossiphoniidae), with records of its new host amphibian species. *Parasitology Research* 2019, 118(2): 663-666

ISSUE DATE:

2019-02

URL:

<http://hdl.handle.net/2433/236132>

RIGHT:

This is a post-peer-review, pre-copyedit version of an article published in 'Parasitology Research'. The final authenticated version is available online at: <https://doi.org/10.1007/s00436-018-6141-y>.; The full-text file will be made open to the public on 13 November 2019 in accordance with publisher's 'Terms and Conditions for Self-Archiving'.; この論文は出版社版ではありません。引用の際には出版社版をご確認ご利用ください。; This is not the published version. Please cite only the published version.

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 Kambayashi¹ • Atsushi Kurabayashi^{2,3} • Takafumi Nakano⁴

6

7 ¹ Amphibian Research Center, Hiroshima University, Hiroshima, Japan

8 ² Department of Bio-Science, Nagahama Institute of Bio-Science and Technology, Shiga,

9 Japan

10 ³ Unit for Environmental Sciences and Management, North-West University, Potchefstroom,

11 South Africa

12 ⁴ Department of Zoology, Graduate School of Science, Kyoto University, Kyoto, Japan

13

14 Corresponding author: Chiaki Kambayashi

15 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 proboscideate 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

35 **Acknowledgments** We are grateful to Junichi Naito and Norio Shimizu for their kindness in
36 conducting the amphibian investigation. We also appreciate Nobuaki Furuno and Ryosuke

37   Takehashi for providing valuable advice on this study.

38

39   **Funding information**   This study was financially supported by the Japan Society for the

40   Promotion of Science (JSPS) KAKENHI Grant numbers JP15J00720, JP17K20064,

41   JP26291080 and JP18H02497.

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

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 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

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 (Katoh 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

146 **References**

147

148 Boulenger GA (1879) Étude sur les grenouilles rousses *Ranae temporariae* et description

149 d'espèces nouvelles ou méconnues. Bull Soc Zool France 4:158–193

150 Dasch GA, Weiss E (1992) The genera *Rickettsia*, *Rochalimaea*, *Ehlichia*, *Cowdaria*, and

151 *Neorickettsia*. In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer K-H (eds)

152 The Prokaryotes, vol 3. Springer-Verlag, New York, pp 2407–2470

153 de Carle D, Ocegüera-Figueroa A, Tessler M, Siddall ME, Kvist S (2017) Phylogenetic

154 analysis of *Placobdella* (Hirudinea: Rhynchobdellida: Glossiphoniidae) with

155 consideration of COI variation. Mol Phylogenet Evol 114:234–248.

156 <https://doi.org/10.1016/j.ympev.2017.06.017>

157 Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of

158 mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. Mol

159 Mar Biol Biotechnol 3:294–299

160 Houttuyn M (1782) Het onderscheidt der Salamanderen van de Haagdissen in't algemeen, en

161 van de Gekkoos in't byzone aangetoond. Verh Zeeuwsch Genootsch Wetensch

- 162 Vlissingen 9:305–336
- 163 Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7:
164 improvements in performance and usability. Mol Biol Evol 30:772–780.
165 [https://doi.org/ 10.1093/molbev/mst010](https://doi.org/10.1093/molbev/mst010)
- 166 Kikuchi Y, Fukatsu T (2005) *Rickettsia* infection in natural leech populations. Microb Ecol
167 49:265–271.
168 <https://doi.org/10.1007/s00248-004-0140-5>
- 169 Kikuchi Y, Sameshima S, Kitade O, Kojima J, Fukatsu T (2002) Novel clade of *Rickettsia*
170 spp. from leeches. Appl Environ Microb 68:999–1004.
171 <https://doi.org/10.1128/aem.68.2.999-1004.2002>
- 172 Nakano T (2012) A new species of *Orobdella* (Hirudinida, Arhynchobdellida,
173 Gastrostomobdellidae) and redescription of *O. kawakatsuorum* from Hokkaido, Japan
174 with the phylogenetic position of the new species. ZooKeys 169:9–30.
175 <https://doi.org/10.3897/zookeys.169.2425>
- 176 Oka A (1925a) Notices sur les Hirudinées d’Extreme Orient, I–IV. Annot Zool Jpn 10:311–
177 326
- 178 Oka A (1925b) Notices sur les Hirudinées d’Extrême Orient, V–VII. Annot Zool Jpn 10:327–

- 179 335
- 180 Oka A (1935) Description d'un nouveau genre d'Hirudinée de la famille des Glossiphonides,
- 181 *Oligoclepsis tukubana* n. g. n. sp. Proc Imp Acad 11:66–68
- 182 Okada Y (1928) Notes on Japanese frogs. Annot Zool Jpn 11:269–277
- 183 Sasaki A (2015) First record of *Torix tagoi* (Oka, 1925) in Yamanashi Prefecture, and
- 184 Shizuoka Prefecture. Nat Hist Tokai Distr 8:5–8
- 185 Sawyer RT (1986) Leech Biology and Behaviour. Clarendon Press, Oxford
- 186 Siddall ME, Burreson EM (1998) Phylogeny of leeches (Hirudinea) based on mitochondria
- 187 cytochrome *c* oxidase subunit. Mol Phylogenet Evol 9:156–162.
- 188 <https://doi.org/10.1006/mpev.1997.0455>
- 189 Tamura K, Stecher G, Peterson D, Filipowski A, Kumar S (2013) MEGA6: Molecular
- 190 Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30:2725–2729.
- 191 <https://doi.org/10.1093/molbev/mst197>
- 192 Tessler M, de Carle D, Voiklis ML, Gresham OA, Neumann JS, Cios S, Siddall ME (2018)
- 193 Worms that suck: Phylogenetic analysis of Hirudinea solidifies the position of
- 194 Acanthobdellida and necessitates the dissolution of Rhynchobdellida. Mol Phylogenet
- 195 Evol 127:129–134.

- 196   <https://doi.org/10.1016/j.ympev.2018.05.001>
- 197   Werner F (1903) Über Reptilien und Batrachier aus Guatemala und China in der
- 198   zoologischen Staats-Sammlung in München nebst einem Abhang über seltene Formen
- 199   aus Anderer gebieten. Abh Math-Phys Kl K Bayer Akad Wiss 22:342–384
- 200   Yoshida K (2009) [Occurrence record of *Torix tukubana* (Annelida: Hirudinea:
- 201   Rhynchobdellida) collected from Mt. Tenzan (Kyuragi-machi, Karatsu-shi, Saga
- 202   Prefecture)]. Saga Nat Stud 15:47

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

Voucher	Collection Date	Host	Stage	Ventral Annulation	CoxI 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

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