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[1] • Atsushi Kurabayashi[2,3] • Takafumi Nakano[4]

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 proboscidate 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

37   Kakehashi for providing valuable advice on this study.

38

42

Introduction

43   **Introduction**

44

45   *Torix* is a leech genus classified within the proboscidate 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 (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*

9

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, Oceguera-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

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 mitochobdrial

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, Filipski 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	*Cox1* INSDC Accession #
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

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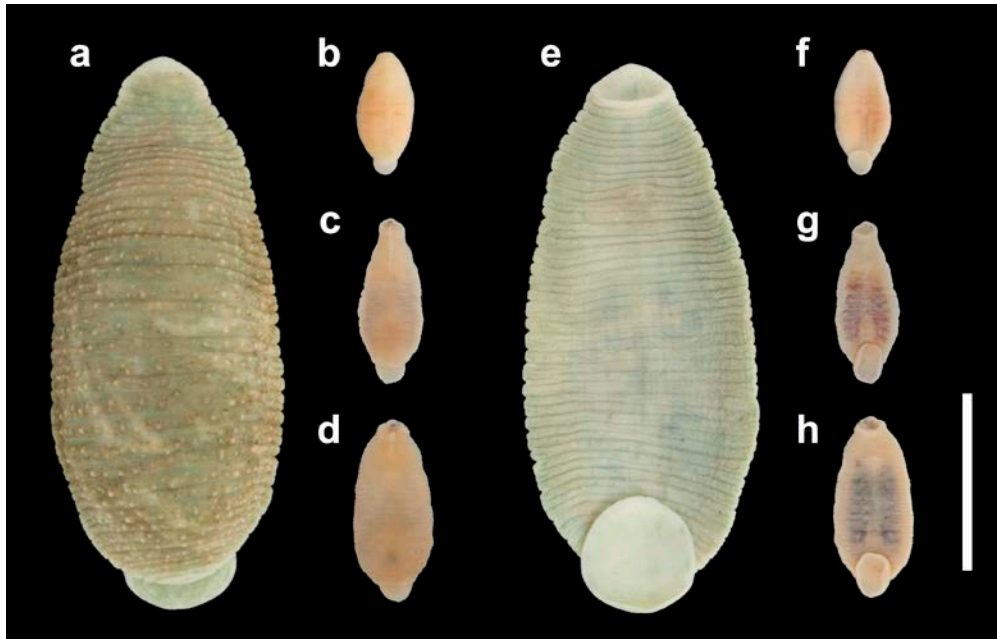
Fig. 1 Dorsal (a-d) and ventral (e-h) views of *Torix tukubana* collected in this study. Large

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