

1 **Host-parasite relationships between seabirds and the haemadipsid leech**
2 *Chtonobdella palmyrae* (Annelida: Clitellata) inhabiting oceanic islands in the
3 **Pacific Ocean**
4
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17 **Running title:** Parasitic *Chtonobdella* leeches in seabirds
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22

23 **Abstract**

24 The duognathous haemadipsid leeches of the genus *Chtonobdella* show a trans-oceanic
25 distribution throughout the Indo-Pacific region. Although passive long-distance
26 dispersal of *Chtonobdella* leeches by birds has been suggested, little is known about the
27 host-parasite relationships between avian hosts and *Chtonobdella* leeches. In the present
28 study, we investigated *Chtonobdella* leech infestations of the eyes and other mucus
29 membranes of migratory procellariiform seabirds, *Pterodroma hypoleuca* and
30 *Oceanodroma tristrami*, captured at six locations in the Bonin Islands, Honshu and
31 Okinawa Island, Japan. Analyses of the partial sequences of 18S rRNA, 28S rRNA, and
32 mitochondrial cytochrome *c* oxidase subunit I (COI) and morphological examination of
33 the specimens demonstrated that the *Chtonobdella* leeches belonged to *Chtonobdella*
34 *palmyrae*, which is indigenous to Palmyra Atoll in the Northern Line Islands. A
35 dominant COI sequence type was observed in samples from all six sites; therefore, *C.*
36 *palmyrae* almost surely dispersed approximately 1000 km by infesting the eyes and
37 mucus membranes of procellariiform seabirds. The host-parasite relationships between
38 procellariiform seabirds and *C. palmyrae* provide explicit evidence of the long-distance
39 dispersal of duognathous haemadipsid leeches. The taxonomic status of *Haemadipsa*
40 *zeylanica ivosimae* from the Volcano Islands is also briefly discussed.

41

42 **Key words:**

43 Hirudiniformes; Haemadipsidae; blood-feeding; avian host; migration; overseas

44 dispersal

45

46 **Key Findings**

47 • Terrestrial *Chtonobdella* leeches were found infesting membranes of procellariiform
48 seabirds.

49 • Host-parasite relationships may lead to trans-oceanic distribution of *Chtonobdella*
50 *palmyrae*.

51 • *Chtonobdella palmyrae* populations may be harboured in the Bonin and/or Volcano
52 Islands, Japan.

53

54 **Introduction**

55

56 Studying a host-parasite relationship can lead to a better understanding of how present
57 parasite distributions have formed and in turn shed light on the biogeography and
58 evolutionary history of the parasite (Hoberg and Brooks, 2010). Migratory vertebrates,
59 especially birds, have been deemed to be major promoters of long-distance dispersal
60 (LDD) events for parasites (Dietrich *et al.*, 2011; Gillespie *et al.*, 2012; Viana *et al.*,
61 2016), and birds provide many habitats for diverse ecto- and endo-parasitic metazoans
62 (Crompton, 1997). Leeches, which are blood-feeding/predatory annelid worms, include
63 several bird-parasitic species (Sawyer, 1986; Davies *et al.*, 2008). The latest systematic

64 framework has been used to classify leeches into five suborders (Tessler *et al.*, 2018a):
65 the proboscidate Glossiphoniiformes and Oceanobdelliformes, jawed Hirudiniformes,
66 and jawless Americobdelliformes and Erpobdelliformes. Members of
67 Glossiphoniiformes and Hirudiniformes are known to parasitize birds. Glossiphoniiform
68 leeches, belonging to the genus *Theromyzon*, infest the nasopharynges and eyes of
69 various waterfowls (see Davies *et al.*, 2008) and are believed to use their avian hosts as
70 transport for circumboreal distribution (Sawyer, 1986). A hirudiniform *Parapraobdella*
71 *lineata* (Sciacchitano, 1959), which inhabits southern Africa and is classified within the
72 family Praobdellidae (Phillips *et al.*, 2011), was found infesting an orbital cavity of a
73 migratory bird, *Phoenicurus phoenicurus* (Linnaeus, 1758) (Siddall *et al.*, 2013); this
74 highlighted the possibility that praobdellid leeches promote and shape their distribution
75 via bird migrations. The hirudiniform species of Ornithobdellidae are also considered to
76 feed on birds' blood (Benham, 1909).

77 Blood-sucking terrestrial leeches belonging to the hirudiniform family
78 Haemadipsidae are widely distributed throughout Indo-Pacific forests (Borda and
79 Siddall, 2011) and have been observed to feed on avian species (Sawyer, 1986; Janovy,
80 1997; Davies *et al.*, 2008). Recent molecular phylogenetic studies revealed that
81 haemadipsids are composed of three phylogroups (Borda and Siddall, 2011; Tessler *et*

82 *al.*, 2016; Huang *et al.*, 2019): (1) the trignathous (three-jawed) genus *Haemadipsa*, (2)
83 the trignathous *Sinospelaeobdella* and *Tritetrabdella*, and (3) the duognathous (two-
84 jawed) *Chtonobdella*. In contrast to the trignathous haemadipsid leeches, whose
85 distribution is restricted to Oriental and Sino-Japanese regions, the duognathous
86 haemadipsids are widely distributed throughout the Indo-Pacific area (Borda and
87 Siddall, 2011), ranging from Madagascar in the southwestern Indian Ocean (Borda,
88 2006) to the Juan Fernandez Islands in the southeastern Pacific (Johansson, 1924).
89 Therefore, the passive LDD of *Chtonobdella* leeches by birds has been suggested, given
90 the leeches' wide distribution and endemities to Indo-Pacific oceanic islands (Lande,
91 1994; Borda *et al.*, 2008; Borda and Siddall, 2011). However, little is known about the
92 host-parasite relationships between birds and leeches. Migratory seabirds belonging to
93 Procellariiformes appear to be potential hosts for *Chtonobdella skottsbergi* (Johansson,
94 1924) inhabiting the Juan Fernandez Islands (Ringuelet, 1955). To our knowledge,
95 however, almost all birds recorded as being infested by *Chtonobdella* leeches have been
96 non-migratory passerines (Passeriformes) and flightless cassowaries (Casuariiformes)
97 (Richardson, 1975). Although invertebrate-derived DNA (iDNA) analyses have
98 accelerated our understanding of the hidden host-parasite relationships among
99 vertebrate hosts and haemadipsid leeches (Schnell *et al.*, 2015; Tessler *et al.*, 2018b),

100 the avian host species detected by previous iDNA studies (Schnell *et al.*, 2018; Fahmy
101 *et al.*, 2019) were also sedentary or flightless.

102 Migratory procellariiform seabirds with eyes heavily infested by segmented
103 worms have been found at several localities in the Bonin Islands, Honshu Island, and
104 Okinawa Island of Japan. Because these parasites possessed obvious caudal suckers,
105 five pairs of eyes forming a parabolic arc in the head region and laterally situated
106 nephridiopores, they were definitively identified as haemadipsid species. *Haemadipsa*
107 *zeylanica ivosimae* Oka, 1930 was described from Kita-Ioto Island in the Volcano
108 Islands (Oka, 1930), located more than 150 km southwest of the Bonin Islands (Fig. 1);
109 however, its systematic status remains uncertain (Nakano, 2017). The present study
110 provides a taxonomic account of newly collected haemadipsids for both molecular
111 phylogenetic analyses and morphological examination. The host-parasite relationships
112 between the migratory seabirds and the haemadipsids presented in this study will help
113 us to elucidate features of the passive LDD of these terrestrial blood-suckers.

114

115 **Materials and methods**

116

117 *Sampling and morphological examination*

118

119 Haemadipsid leeches were obtained from the eyes or other mucous membranes of
120 procellariiform seabirds captured at six locations around Japan from August 2000 to
121 October 2016. The captured host seabirds had been weakened by leech infestations.
122 Most of the leech specimens were directly fixed in 70–90% ethanol. For DNA
123 extraction, botryoidal tissue was removed from around the anterior oral sucker or
124 posterior caudal sucker, and preserved in absolute ethanol. The remainder of the body
125 was re-fixed in 10% formalin and preserved in 70% ethanol.

126 Four measurements were taken: body length (BL) from the anterior margin of
127 the oral sucker to the posterior margin of the caudal sucker, maximum body width
128 (BW), caudal sucker length (CL) from the anterior to the posterior margin of the caudal
129 sucker, and caudal sucker width (CW) from the right to the left margin of the caudal
130 sucker. Examination, dissection, and drawing of the specimens were conducted using a
131 stereoscopic microscope with a drawing tube (Leica M125). The leech specimens and a
132 seabird host examined in this study were deposited in the Zoological Collection of
133 Kyoto University (KUZ).

134 The type series of *H. z. ivosimae* was not found in Oka's small leech collection
135 kept at The University Museum, The University of Tokyo (Nakano and Itoh, 2011). The

136 reminder of the collection housed at the National Museum of Nature and Science,
137 Tsukuba, Japan (NSMT) (see Nakano, 2010) was also searched to locate the type series,
138 however, it is believed that the series may have been lost or destroyed in the past.

139 The numbering convention is based on Moore (1927): body somites were
140 denoted by Roman numerals, and the annuli in each somite were given alphanumeric
141 designations.

142

143 *PCR and DNA sequencing*

144

145 Genomic DNA was extracted from the botryoidal tissues preserved in absolute ethanol
146 following the methods described by Nakano (2012a). The primer sets for the PCR and
147 cycle sequencing reactions for the nuclear 18S rRNA (18S), 28S rRNA (28S) and
148 histone H3 (H3), and mitochondrial cytochrome *c* oxidase subunit I (COI) used in this
149 study were taken from previous studies (Nakano, 2016; Nakano *et al.*, 2016). PCR and
150 DNA sequencing were performed using a modified version of a method mentioned in
151 Nakano (2012b), and detailed conditions were identical to those in previous studies
152 (Nakano, 2016; Nakano *et al.*, 2016). In total, 27 new sequences were obtained from the
153 haemadipsid leeches and deposited in the International Nucleotide Sequence Database

154 Collaboration (INSDC) through the DNA Data Bank of Japan with the following
155 INSDC accession numbers: 18S sequence LC414431, 28S sequence LC414432, H3
156 sequence LC414433, and 24 COI sequences LC414407–LC414430.

157

158 *Molecular phylogenetic and network analyses*

159

160 The phylogenetic position of the newly collected haemadipsid leeches within the genus
161 *Chtonobdella* was estimated based on the 18S, 28S, and COI sequences; the H3
162 sequence of the present specimen was not included due to the lack of those of other in-
163 group taxa. The in-group taxa were selected from the datasets analysed in previous
164 molecular phylogenetic studies of Haemadipsidae (Borda and Siddall, 2011; Tessler *et*
165 *al.*, 2016) (Supplementary Material Table S1). According to the previous results (Borda
166 and Siddall, 2011; Tessler *et al.*, 2016), four *Tritetrabdella* species and
167 *Sinospelaeobdella cavatuses* (Yang, Mo, and Wang, 2009) were used as the outgroup.

168 The 18S, 28S, and COI sequences were aligned using MAFFT version 7.402 L-INS-i
169 (Kato and Standley, 2013). The lengths of the 18S, 28S, and COI sequences were
170 1833, 2093, and 1246 bp, respectively. The concatenated sequences yielded 5172 bp of
171 aligned positions.

172 Phylogenetic trees were inferred using maximum likelihood (ML) and
173 Bayesian inference (BI). The best-fit partition scheme and substitution models were
174 identified with the Bayesian information criterion using PartitionFinder version 2.1.1
175 (Lanfear *et al.*, 2017) with the “greedy” algorithm (Lanfear *et al.*, 2012): for 18S,
176 SYM+I+G; for 28S, GTR+I+G; for COI 1st position, TIM+I+G (GTR+I+G for BI); for
177 COI 2nd position, F81+I; and for COI 3rd position, TRN+G (GTR+G for BI). The ML
178 phylogeny was inferred using IQ-TREE version 2.0.5 (Minh *et al.*, 2020) with non-
179 parametric bootstrapping (BS) conducted with 1000 replicates. BI and Bayesian
180 posterior probabilities (PPs) were estimated using MrBayes version 3.2.7a (Ronquist *et*
181 *al.*, 2012). Two independent runs of four Markov chains were conducted for 20 million
182 generations, and the tree was sampled every 100 generations. The parameter estimates
183 and convergence were checked using Tracer version 1.7.1 (Rambaut *et al.*, 2018), and
184 the first 50001 trees were discarded based on the results.

185 Relationships among the COI haplotypes (1271 bp) obtained from the present
186 leech specimens were estimated by a statistical parsimony network using PopART
187 version 1.7 (Leigh and Bryant, 2015). According to the results of our molecular
188 phylogenetic analyses, further relationships between the Japanese COI haplotypes and
189 the previously published COI sequence (HQ203158) of *Chtonobdella palmyrae*

190 (Richardson, 1975) were calculated based on 985 bp alignment positions by statistical
191 parsimony network using PopART.

192

193 **Results**

194

195 *Leech infestation records*

196

197 In total, 25 haemadipsid leeches were collected from their attachment sites on the eyes
198 or in the throat of procelariid or hydrobatid seabirds (Table 1) from six localities in
199 Japan. Among the specimens, 16 leeches were collected from seabirds captures in the
200 Bonin Islands (Fig. 1). One specimen (KUZ Z1648) was obtained from the eye of a
201 Bonin petrel, *Pterodroma hypoleuca* (Salvin, 1888), captured on Chichijima Island on
202 16 August 2012; and the other three individuals (KUZ Z2044–Z2046) were obtained
203 from the eyes of a Bonin petrel from Hahajima Island on 25 September 2015. A heavy
204 infestation of leeches in the eyes of a Tristram’s storm petrel, *Oceanodroma tristrami*
205 (Salvin, 1896), was observed on Anijima Island on 17 November 2013 (Fig. 2); in total,
206 12 leeches (KUZ Z1636–1647) were removed from the eyes of this petrel. When the
207 leeches were collected from the petrel’s eyes by the second and third authors, they were

208 not sensitive to the exhaled human breath. The bird was already debilitated and died
209 soon after the leeches were removed; therefore, this specimen was preserved in KUZ
210 (KUZ B491).

211 Leeches were also collected on Honshu Island of the Japanese Archipelago.
212 Two individuals (KUZ Z2047, Z2048) were collected from eyes of a *P. hypoleuca* petrel
213 captured in Kuroto, Kisarazu, Chiba Prefecture, on 11 August 2000. Additionally, five
214 individuals (KUZ Z2049–Z2053) were extracted from the eyes of a single *P. hypoleuca*
215 captured in Shinagawa, Tokyo, on 10 August 2002. The other two individuals (KUZ
216 Z2557, Z2558) were found in an oral cavity or the throat of a young bird identified as *P.*
217 *hypoleuca* caught in Kunigami, Okinawa Prefecture (Okinawa Island, Ryukyu Islands),
218 on 23 October 2016; these two leeches were found loose from the petrel’s mouth and
219 collected.

220

221 *Molecular phylogenies and COI networks*

222

223 The BI tree (mean ln likelihood [L] = -20241.493; Fig. 3A) for estimating the
224 phylogenetic position of the present specimens had an identical topology to that of the
225 ML tree (ln L = -20222.492; not shown). The topology of the present phylogenies was

226 almost concordant with those described in previous reports (Borda and Siddall, 2011;
227 Tessler *et al.*, 2016). The haemadipsid leech (KUZ Z1636) formed a fully supported
228 clade with *C. palmyrae*, which was collected from Palmyra Atoll (BS = 100%, PP =
229 1.0).

230 The COI data of the 985 bp alignment positions showed there was a low
231 genetic diversity between the Japanese individuals ($N = 24$) and the *C. palmyrae* from
232 the atoll ($N = 1$) (Fig. 3B); 3/985 (0.30%) nucleotides were polymorphic. There was no
233 variation among the Japanese specimens based on the COI sequences with lengths of
234 985 bp. The COI sequences of 1271 bp alignment positions also highlighted the low
235 genetic diversity within the Japanese *Chtonobdella* leeches; 2/1271 (0.16%) nucleotides
236 were polymorphic. Three sequence types were present in the Japanese populations, and
237 the number of nucleotide differences between any two types ranged from one to two
238 substitutions (Fig. 3C). The dominant sequence type (“a”) was observed in 22 samples
239 from six localities. The other two sequence types were observed in samples from
240 Hahajima Island in the Bonin Islands (“b”: KUZ Z2046) and from Tokyo (“c”: KUZ
241 Z2053). These two localities were also where the dominant sequence type leeches,
242 along with the unique haplotype sample from a single host seabird, were collected. The
243 substitution detected in the sample from Tokyo (KUZ Z2053) was nonsynonymous

244 (leucine in the dominant haplotype to isoleucine in the minor haplotype).

245

246 *Morphological characteristics*

247

248 The 25 haemadipsid specimens (KUZ Z1636–Z1648, Z2044–Z2055), with BLs ranging
249 from 5.1–20.5 mm, BWs ranging from 1.7–6.0 mm, CLs ranging from 0.8–3.0 mm, and
250 CWs ranging from 1.0–2.7 mm without obvious clitellum, possessed the following
251 characteristics (Figs 4 and 5).

252 *External morphology:* Somites I completely merged with prostomium. Somites
253 II (= peristomium) to IV uniannulate. Somite V biannulate, $(a1 + a2) > a3$; each of
254 interocular plates of V ($a1 + a2$) subdivided. Somite VI dorsally triannulate, $a1 = a2 =$
255 $a3$, ventrally biannulate, $(a1 + a2) > a3$; somite V to somite VI ($a1 + a2$) forming
256 posterior margin of oral sucker. Somite VII triannulate, $a1 = a2 < a3$ (rarely with slight
257 dorsal furrow, $b5 = b6$), or quadrannulate, $a1 = a2 = b5 = b6$. Somite VIII
258 quadrannulate, $a1$ (occasionally with slight furrow, $b1 = b2$) $> a2 = b5 = b6$. Somites
259 IX–XXII quinquannulate, $b1 = b2 = a2 = b5 = b6$. Somite XXIII generally
260 quadrannulate, $b1 = b2 = a2 < a3$ (occasionally with slight furrow, $b5 = b6$). Somite
261 XXIV triannulate, $a1 = a2 = a3$. Somite XXV–XXVII uniannulate; ambilateral margins

262 of each somites XXV–XXVII forming lobes of one pair of respiratory auricles. Anus at
263 posterior margin of somite XXVII. Caudal sucker ventral, elliptical; prehensile lobe
264 developed, sharply hooked; with 74–82 friction rays. Male gonopore in somite XI b5/b6
265 or rarely in middle of somite XI b6. Female gonopore in somite XII a2/b5. Gonopores
266 separated by 4, or rarely 1/2 + 3 annuli. Eyes in 5 pairs, in parabolic arc: 1st pair on
267 somite II, 2nd pair on III, 3rd pair on IV, 4th pair on V (a1 + a2), and 5th pair on VI a2.
268 Nephridiopores, when detectable, in 13–15 pairs, one each situated laterally at posterior
269 margin of a1 of each somite in IX–XXIII. Nephridiopores of somite XXIV under
270 respiratory auricles.

271 *Internal morphology [based on KUZ Z1636, BL 9.3 mm]:* Jaws duognathous.

272 Genital organs undeveloped; testisacs undetectable; atrium small, globular; vaginal sac
273 undeveloped tubular; vaginal duct short.

274 *Colouration:* In life, uniformly dark brown. Colour faded in preservative.

275

276 **Discussion**

277

278 *Taxonomic account*

279

280 The leeches in this study, which were obtained from the procellariiform seabirds *P.*
281 *hypoleuca* and *O. tristrami*, unquestionably belonged to a *Chtonobdella* species, based
282 on the presence of two jaws within the oral cavity. Additionally, molecular phylogenetic
283 and network analyses identified the leeches as *C. palmyrae*, which was originally
284 described based on an individual collected from Palmyra Atoll in the Northern Line
285 Islands (Richardson, 1975; Fig. 1). Therefore, this study provides additional distribution
286 records of this species well beyond its original known range; only trignathous
287 *Haemadipsa* leeches have previously been recorded in the Far East (Lai *et al.*, 2011;
288 Seo *et al.*, 2013; Morishima and Aizawa, 2019).

289 The present specimens revealed the intraspecific variation of the body somite
290 annulation and male gonopore position in *C. palmyrae*. While the precedence
291 descriptions of the material from Palmyra Atoll stated that almost all of them had a
292 quadrannulate somite VII (Richardson, 1975, 1981), the Japanese specimens generally
293 possessed the triannulate somite VII with a slight secondary furrow on its last annulus.
294 Although most Japanese specimens had its male gonopore in the annular furrow of
295 somite XI b5/b6 as with the individuals from Palmyra Atoll (Richardson, 1975, 1981),
296 some of them possessed the male gonopore opening in the middle of somite XII b6. The
297 previous study also stated that the colouration of the specimens from Palmyra Atoll was

298 variable (Richardson, 1981): the holotype and paratype of *C. palmyrae* had slight
299 longitudinal stripes with/without oval patches on the dorsal surface, but the small
300 specimens were uniformly darkish brown without any stripes or markings. The
301 colouration of the Japanese specimens is similar to that of the Richardson's (1981)
302 small leeches.

303 The molecular analyses support that the Japanese specimens are clearly
304 conspecific with *C. palmyrae*, but nonetheless, their characteristics of the
305 nephridiopores, respiratory auricles, and friction rays of the caudal sucker are different
306 from those described by Richardson (1981). In all Japanese specimens, nephridiopores
307 opening between somites IV/V were not observed, contrary to the specimens from
308 Palmyra Atoll that possessed the first nephridiopores in somites IV/V. In addition, the
309 present specimens possessed the observable unlobate respiratory auricles (vs. no
310 developed respiratory auricles in the specimens from Palmyra Atoll), and 74–82 friction
311 rays on the ventral surface of caudal sucker (vs. 90 friction rays in the holotype of *C.*
312 *palmyrae*). The continuation of faunal surveys on islands in the Pacific Ocean and
313 morphological and genetic studies based on the evaluation of additional specimens are
314 necessary to investigate the morphological discordance between the specimens from
315 Japan and those from Palmyra Atoll.

316 The original description of *H. z. ivosimae* stated that the leeches on Kita-Ioto
317 Island sucked the blood of birds' eyes and, moreover, rendered their hosts blind (Oka,
318 1930). The colouration of *H. z. ivosimae* was also reported to be uniform reddish or
319 greenish (Oka, 1930). However, *H. z. ivosimae* was unquestionably described as a
320 trignathous species clearly excluding it from the duognathous *Chtonobdella* (Oka,
321 1930). The original description of *H. z. ivosimae* also mentioned that this species
322 possesses five annuli between the gonopores, and the original drawing depicted trilobate
323 respiratory auricles. These characteristics are also inconsistent with those of *C.*
324 *palmyrae* including the present material, which generally possess four annuli between
325 the gonopores as well as unlobate or undeveloped respiratory auricles (Richardson,
326 1975; Richardson, 1981). The systematic account of *H. z. ivosimae* should be clarified
327 in a future study. Because the type series of this taxon is deemed to be lost, newly
328 collected haemadipsid specimens from Kita-Ioto Island are essential to unveil the true
329 diversity of haemadipsid species of the Far East.

330

331 *LDD of leeches via seabirds*

332

333 The present results revealed that the duognathous haemadipsid *C. palmyrae* is an avian-

334 specific blood-sucking parasite that infests the eyes and other mucus membranes of
335 procellariiform migratory seabirds, and they corroborated the passive LDD of
336 duognathous *Chtonobdella* leeches suggested by previous studies (Lande, 1994; Borda
337 *et al.*, 2008; Borda and Siddall, 2011). The following avian host-*Chtonobdella* parasite
338 relationships were recorded by Richardson (1975): the leg of the passeriform *Eopsaltria*
339 *australis* (Shaw, 1790) was parasitized by *Chtonobdella limbata* Grube, 1866; the head
340 of the passeriform *Gymnorhina tibicen* G. R. Gray, 1840 was parasitized by
341 *Chtonobdella bilineata* (Richardson, 1975); and the nasal chambers of the casuariiform
342 *Casuarius* species were infested with an unidentified haemadipsid species and
343 *Chtonobdella novabritanniae* (Richardson, 1975). In addition to the flightless
344 *Casuarius* birds, both of the recorded passeriforms are known to be non-migratory birds
345 (Boles, 2020; Russell *et al.*, 2020).

346 A precedence iDNA study using *Haemadipsa* leeches collected from Southeast
347 Asia and *Chtonobdella* individuals from Australia and Madagascar could detect several
348 avian species from the leeches' bloodmeals (Schnell *et al.*, 2018). From the Australian
349 *Chtonobdella* leeches, the flightless emu *Dromaius novaehollandiae* (Latham, 1790)
350 (Casuariiformes) (Folch *et al.*, 2020) and sedentary lyrebirds of *Menura* (Lill and
351 Boesman, 2020*a, b*) were identified (Schnell *et al.*, 2018). Two avian genera

352 *Dryolimnas* (Rallidae) and *Mystacornis* (Vangidae) were identified from the Malagasy
353 *Chtonobdella* samples (Schnell *et al.*, 2018); but birds belonging to the genera are
354 deemed to be sedentary (Collar *et al.*, 2020; Taylor, 2020). Schnell *et al.* (2018) detected
355 the other three genera (*Bycanistes*, *Chalcophaps*, and *Gallirallus*) from bloodmeals of
356 the Malagasy *Chtonobdella* leeches; but species of these genera are flightless or
357 basically sedentary, and moreover not indigenous to Madagascar (Lepage, 2020a).
358 Another iDNA analysis utilizing Malagasy *Chtonobdella* species identified the avian
359 brachypteraciid species *Atelornis crossleyi* Sharpe, 1875 and *Atelornis pittoides*
360 Lafresnaye, 1834, and the muscicapid *Copsychus albospecularis* (Eydoux and Gervais,
361 1836) (Fahmy *et al.*, 2019); however, all of birds species were also determined to be
362 sedentary (Collar *et al.*, 2020; Langrand, 2020). Although Fahmy *et al.* (2019) detected
363 five additional avian species, they were either domesticated species, or species not
364 indigenous to Madagascar (Lepage, 2020a). Therefore, the present findings finally
365 provide insights into how *Chtonobdella* leeches have achieved a widespread distribution
366 throughout the Indo-Pacific region, including on oceanic islands.

367 The present results suggest the possibility that at least one population of *C.*
368 *palmyrae* is maintained somewhere in the Bonin Islands and Volcano Islands
369 (hereinafter Bonin-Volcano Islands), particularly given the fact that (1) breeding sites of

370 *P. hypoleuca* and those of *O. tristrami* overlap in the Bonin and Volcano Islands
371 (Momiya, 1930; Chiba *et al.*, 2007; Chiba *et al.*, 2012) and (2) the present samples
372 had unique COI sequences compared with the sequence of *C. palmyrae* collected in
373 Palmyra Atoll (Borda and Siddall, 2011). Although both *P. hypoleuca* and *O. tristrami*
374 are known to breed in a western part of the Hawaii Islands (Seto and O’Daniel, 2020;
375 Slotterback, 2020), haemadipsids have not been recorded in the Hawaiian Islands
376 (Eldredge and Miller, 1997).

377 Both *C. palmyrae* leeches obtained from *P. hypoleuca* at Chiba (locality
378 number 4 in Fig. 1) and Tokyo (locality 5) in Honshu, and an individual from *P.*
379 *hypoleuca* at Okinawa Island (locality 6) share a COI sequence type (“a” in Fig. 3C)
380 that was dominant among the obtained sequences. Their host *P. hypoleuca* is a migrant
381 seabird that breeds in the Bonin-Volcano Islands and has been known to visit Honshu
382 and Okinawa irregularly (The Ornithological Society of Japan, 2012). Moreover,
383 *Chtonobdella* leeches are not considered to be endemic to either Honshu or Okinawa
384 islands (Itoh, 2003; Aizawa and Morishima, 2018). Because the Bonin-Volcano Islands
385 are respectively located approximately 1000 km and 1500 km from Chiba and Tokyo
386 and from Okinawa Island, *C. palmyrae* may be able to disperse around 1000 km
387 overseas by infesting the eyes and mucus membranes of *P. hypoleuca*.

388 The COI sequences revealed there was low genetic diversity between the
389 Japanese individual leeches and *C. palmyrae* from Palmyra Atoll; nonetheless, they did
390 not share the same sequence type, suggesting that the two populations may be
391 genetically distinct. Neither *P. hypoleuca* nor *O. tristrami* have ever been recorded in
392 Palmyra Atoll (Lepage, 2020b); therefore, it is unknown how the trans-oceanic *C.*
393 *palmyrae* populations were established in the Bonin-Volcano Islands and Palmyra Atoll.
394 Its trans-oceanic distribution, in which the Bonin-Volcano Islands population is
395 approximately 6300 km from the Palmyra Atoll population, can be explained by either
396 the direct or stepping-stone dispersal (see Saura *et al.*, 2014). If the leeches dispersed
397 directly between these two habitats along with the migration of their avian hosts, *C.*
398 *palmyrae* may also parasitize other procellariiform seabirds, e.g., *Bulweria bulwerii*
399 (Jardine and Selby, 1828), which are known to occur in both the Bonin-Volcano Islands
400 and Palmyra Atoll (Chiba *et al.*, 2007; Lepage, 2020b). It is also feasible that there are
401 additional undiscovered habitats of *C. palmyrae*, where migratory birds occurring in
402 Bonin-Volcano Islands or Palmyra Atoll are known to visit, that may act as stepping
403 stones for the trans-oceanic distribution of this leech species. The western Hawaii
404 Islands are potential candidates for undiscovered habitats of *C. palmyrae*, as they
405 contain breeding grounds for both *P. hypoleuca* and *O. tristrami*.

406 Our knowledge of how duognathous leeches colonize remote areas is hampered
407 by the lack of a detailed understanding of the natural history for *C. palmyrae*. All
408 specimens obtained from *P. hypoleuca* and *O. tristrami* were deemed to be immature
409 individuals because they bore detectable clitella, and the dissected specimen, which had
410 a body length of ca. 1 cm, lacked developed genital organs. Meanwhile, the free-living
411 individuals, whose body lengths exceeded 1.5 cm, collected in Palmyra Atoll were
412 deemed to be almost mature individuals, as they possessed developed female organs
413 (Richardson, 1981). The bat-specific haemadipsid *Sinopelaebdella* species and, in
414 particular, avian-specific glossiphoniid *Theromyzon* leeches are known to detach from
415 their hosts to copulate and deposit a cocoon (Wilkialis and Davies, 1980; Huang *et al.*,
416 2019). Therefore, individuals of *C. palmyrae* may detach from their avian hosts to
417 undergo reproductive behaviours after they have fed on sufficient bird blood.

418 Haemadipsids are known to be vectors of several pathogens, including
419 *Bartonella* (Kang *et al.*, 2016) and *Trypanosoma* (see Sawyer, 1986). Although no
420 pathogens have ever been recorded in *C. palmyrae*, trypanosome blood-parasites have
421 been detected in other *Chtonobdella* species (Richardson and Hunt, 1968; Ewers, 1974;
422 Richardson, 1974). Our knowledge of the pathogens of procellariform seabirds is
423 severely limited; nonetheless, coccidia have been known to infest procellariform species

424 (Yabsley, 2008) and to be infectious in leeches (O'Donoghue, 2017). Therefore, the
425 host-parasite relationships between procellariform seabirds and *Chtonobdella* leeches
426 described in this study highlight the possibility that haemadipsids may act as vectors of
427 procellariform pathogens.

428

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445

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447

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449

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709

710 **Table 1.** Samples of *Chtonobdella palmyrae* (Richardson) infesting seabirds in Japan.

711 Locality numbers (see Fig. 1) are accompanied by collection locality, geographical

712 coordinates, individual counts, voucher, INSDC accession numbers for cytochrome *c*

713 oxidase subunit I (COI), and COI haplotype. Acronym: KUZ, Zoological Collection of

714 Kyoto University.

No.	Locality	Host	Voucher	COI INSDC	
				numbers	Haplotype
1	Anijima, Bonin Islands (27.117°N,	<i>Oceanodroma</i>	KUZ Z1636–	LC414407–	a (<i>N</i> = 12)
	142.207°E)	<i>tristrami</i>	Z1647 (<i>N</i> = 12)	LC414418	
2	Chichijima, Bonin Islands (~27.06°N,	<i>Pterodroma hypoleuca</i>	KUZ Z1648 (<i>N</i> =	LC414419	a (<i>N</i> = 1)
	~142.20°E)		1)		
3	Hahajima, Bonin Islands (~26.65°N,	<i>Pterodroma hypoleuca</i>	KUZ Z2044–	LC414420–	a (<i>N</i> = 2) and
	~142.15°E)		Z2046 (<i>N</i> = 3)	LC414422	b (<i>N</i> = 1)
4	Kisarazu, Chiba, Honshu (35.4111°N,	<i>Pterodroma hypoleuca</i>	KUZ Z2047 and	LC414423,	a (<i>N</i> = 2)
	139.9003°E)		Z2048 (<i>N</i> = 2)	LC414424	
5	Shinagawa, Tokyo, Honshu	<i>Pterodroma hypoleuca</i>	KUZ Z2049–	LC414425–	a (<i>N</i> = 4) and
	(35.6272°N, 139.7363°E)		Z2053 (<i>N</i> = 5)	LC414429	c (<i>N</i> = 1)

6	Kunigami, Okinawa, Ryukyu Islands	<i>Pterodroma hypoleuca</i>	KUZ Z2054 and	LC414430	a (N = 1)
	(26.731095°N, 128.169827°E)		Z2055 (N = 2)		

715

716

717 **Figure legends**

718

719 **Fig. 1.** Map showing sampling localities for *Chtonobdella palmyrae* (Richardson)
720 infesting seabirds from Japan; the white star denotes the type locality of the species.

721

722 **Fig. 2.** Heavy infestation of *Chtonobdella palmyrae* (Richardson) in the eyes of a petrel,
723 *Oceanodroma tristrami* Salvin (KUZ B491), from Anijima Island, Bonin Islands; arrow
724 heads indicate respective leeches.

725

726 **Fig. 3.** Molecular phylogenetic position and haplotype networks of *Chtonobdella*
727 *palmyrae* (Richardson) infesting seabirds in Japan. (A) Bayesian inference tree for 5172
728 bp alignment positions of nuclear 18S rRNA, 28S rRNA and mitochondrial cytochrome
729 *c* oxidase subunit I (COI) markers; numbers on nodes indicate bootstrap values for
730 maximum likelihood and Bayesian posterior probabilities. Statistical parsimony
731 networks of (B) the 985 bp COI haplotypes of Japanese (Jpn) leeches and *C. palmyrae*
732 from Palmyra Atoll, and (C) the 1271 bp COI haplotypes only between the Japanese
733 leeches; filled circles indicate missing haplotypes; each numeral in parentheses denotes
734 the sample size of the respective haplotype.

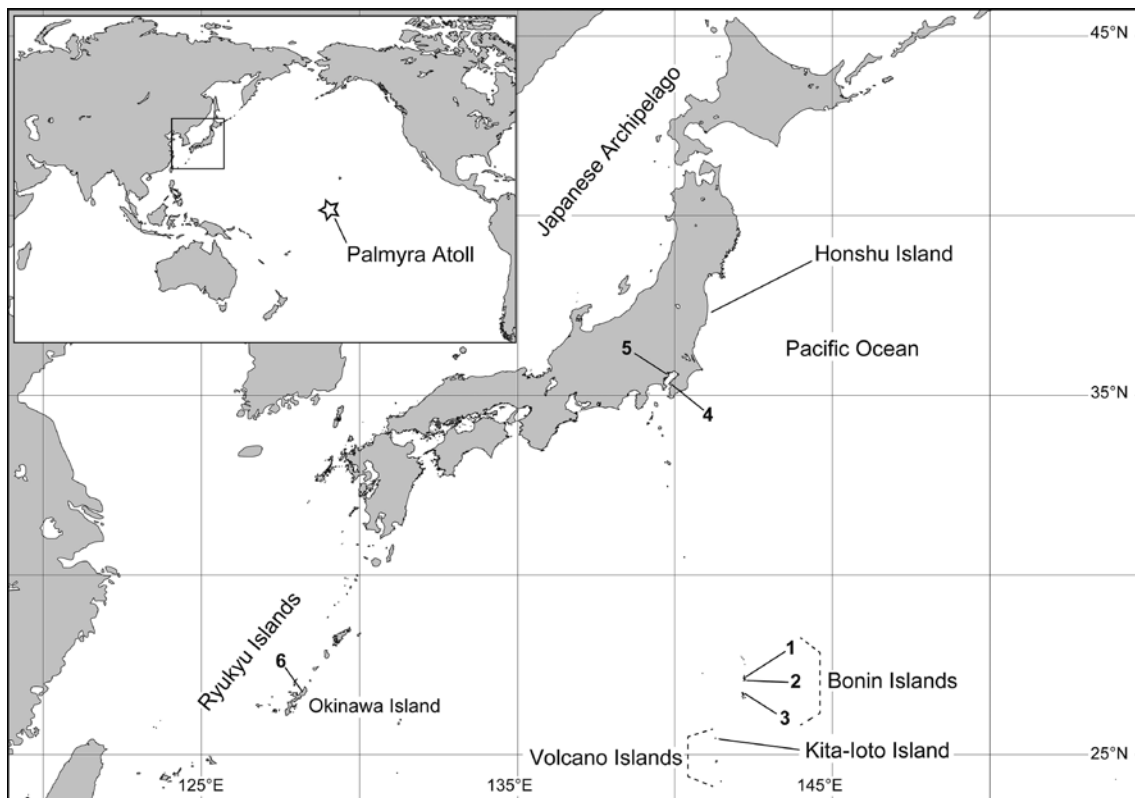
735

736 **Fig. 4.** Preserved specimen of *Chtonobdella palmyrae* (Richardson) (KUZ Z1636)
737 infesting *Oceanodroma tristrami* Salvin (KUZ B491) from Anijima Island, Bonin
738 Islands. (A) Dorsal and (B) ventral views. Scale bar: 2 mm.

739

740 **Fig. 5.** Drawings of *Chtonobdella palmyrae* (Richardson) (KUZ Z1636) infesting
741 *Oceanodroma tristrami* Salvin (KUZ B491) from Anijima Island, Bonin Islands. (A)
742 Dorsal, (B) lateral and (C) ventral views of somites I–X. (D) Ventral view of somites XI
743 and XII. (E) Dorsal, (F) lateral and (G) ventral views of somites XXI–XXVII and
744 caudal sucker. (H) Dorsal view of median reproductive systems, including positions of
745 ganglia XI–XIII. Abbreviations: at, atrium; fp, female gonopore; fr, friction ray; mp,
746 male gonopore; np, nephridiopore; phl, prehensile lobe; ra, respiratory auricle; vd,
747 vaginal duct; and vs, vaginal sac. Scale bars: (A–G) 0.5 mm and (H) 0.1 mm.

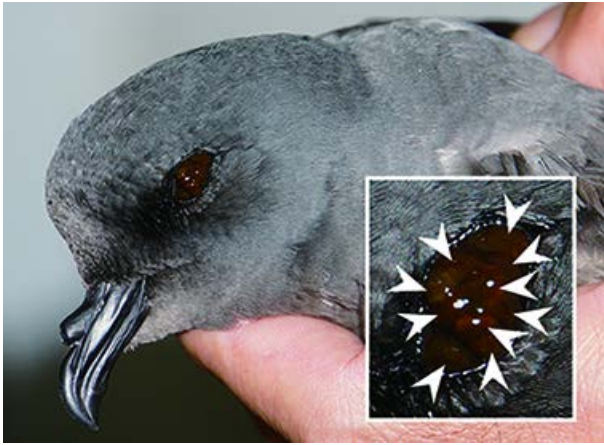
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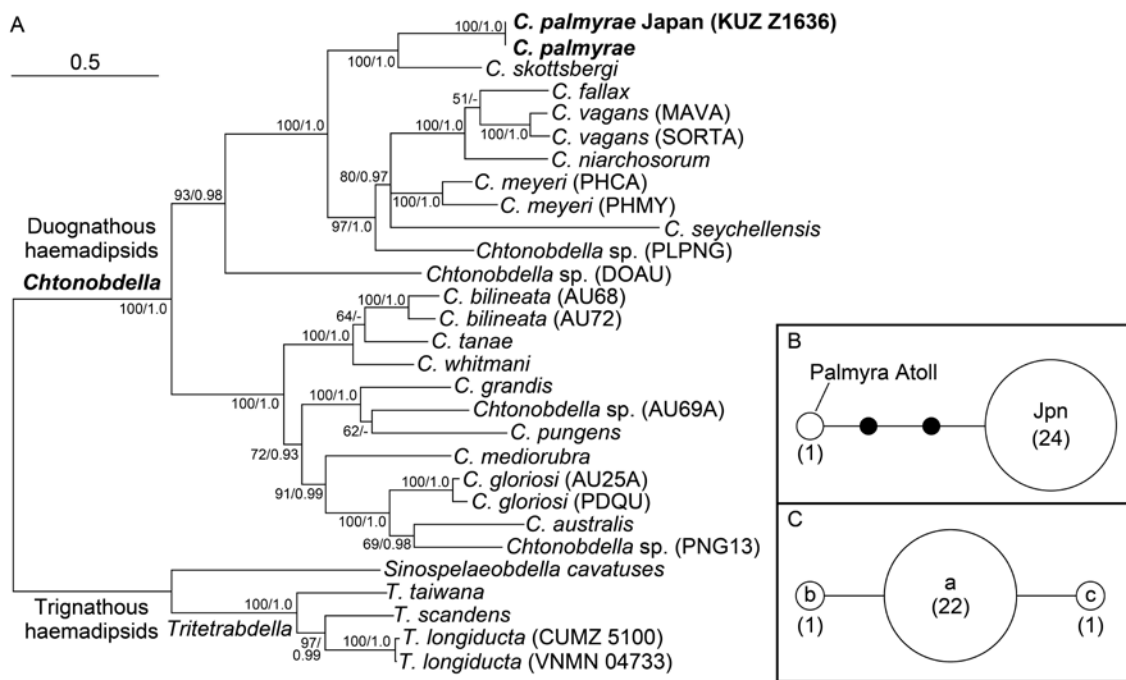
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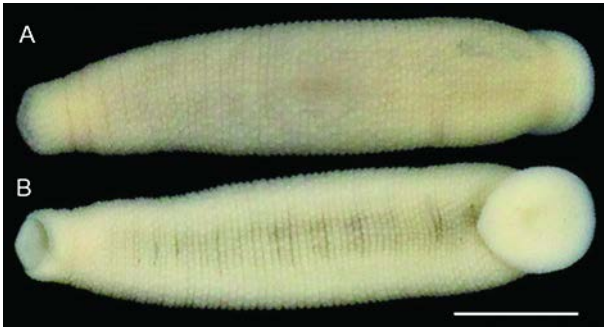
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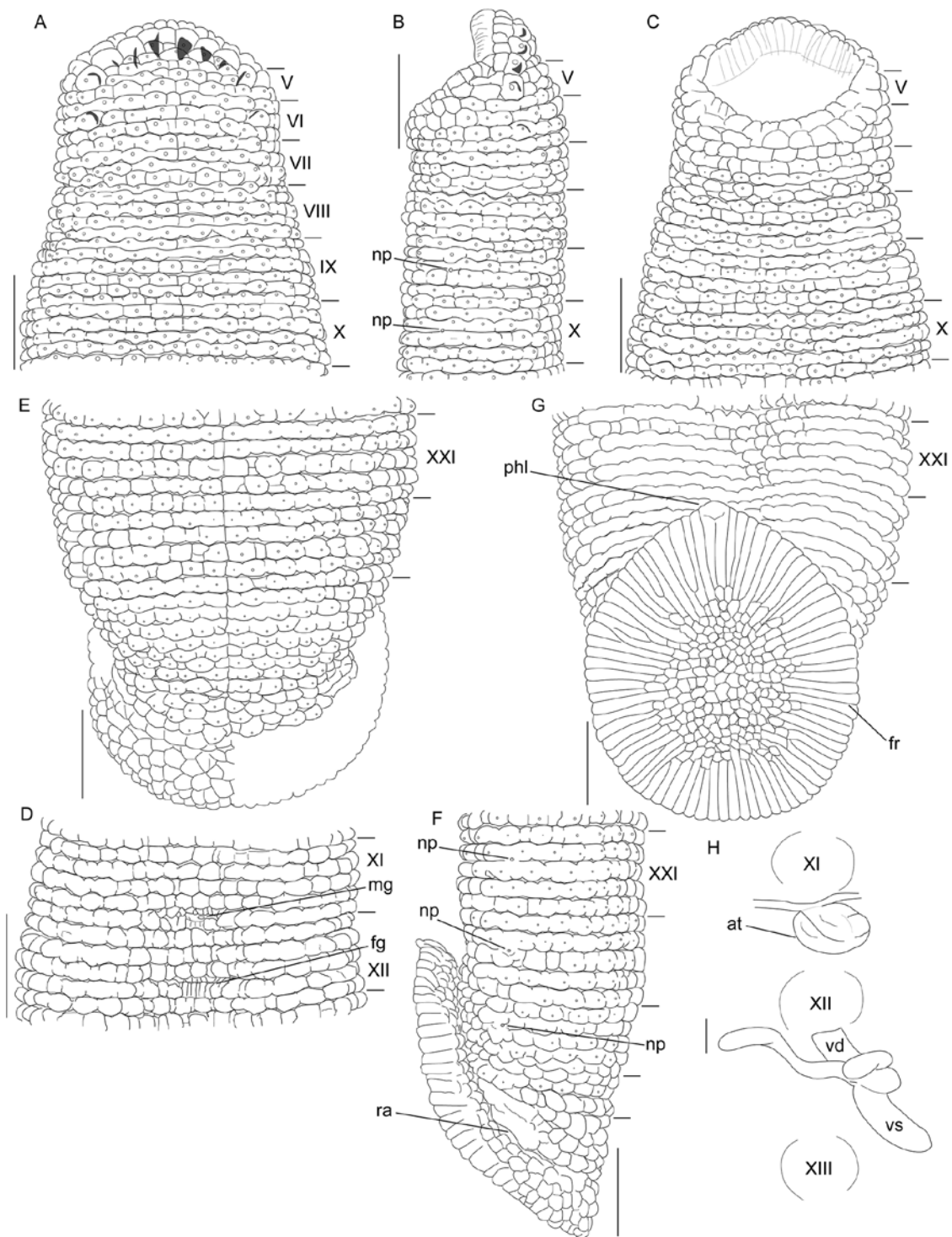
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