1 **Defensive nymphs in the water-repellent gall of the social aphid *Colophina***

2 ***monstrifica* (Hemiptera: Aphididae: Eriosomatinae)**

3

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15

16 **Abstract**

17 The aphid *Colophina monstifica* forms woolly colonies with sterile soldiers on the
 18 secondary host *Clematis uncinata* in Taiwan. However, the gall or primary-host
 19 generation of *C. monstifica* has not been found to date. We successfully induced galls
 20 of the species on trees of *Zelkova serrata* through attaching its eggs onto the trees, and
 21 also found a few naturally formed galls on another *Z. serrata* tree. The identity of the
 22 aphids was confirmed by examining their morphology and mitochondrial DNA
 23 sequences. First- and second-instar nymphs in the galls exhibited attacking behavior
 24 toward artificially introduced moth larvae. Observations with a scanning electron
 25 microscope revealed that the gall inner surface was densely covered with minute
 26 trichomes. This indicates the water repellency of the inner surface, and strongly
 27 suggests that young nymphs of *C. monstifica* dispose of honeydew globules outside the
 28 gall, as known in the congener *C. clematis*.

29

30

31 **Key words:** *Clematis uncinata*, life cycle, molecular phylogeny, trichome, *Zelkova*
 32 *serrata*.

33

34 INTRODUCTION

35

36 Species of the aphid genus *Colophina* (Eriosomatinae; Eriosomatini) exhibit facultative
 37 host-alternation between the primary and secondary hosts. On the primary host plant,
 38 *Zelkova serrata* (Ulmaceae), a foundress (or fundatrix) forms a gall on the leaf, in
 39 which she produces offspring by parthenogenesis and young nymphs perform colony
 40 defense against intruding predators (Aoki 1980). The gall inner surface of *C. clematis* is
 41 densely covered with trichomes, which enables young nymphs to perform gall cleaning
 42 by pushing globules of honeydew out of the gall (Uematsu *et al.* 2018). This
 43 water-repellent structure can be regarded as an “extended phenotype” of the aphids in
 44 the gall (Uematsu *et al.* 2018; see also Stone & Schönrogge 2003; Kutsukake *et al.*
 45 2019). On the secondary host plants, *Clematis* spp. (Ranunculaceae), they form dense,
 46 woolly colonies, where sterile first-instar nymphs called “soldiers” perform colony
 47 defense. Sterile soldier nymphs have been found in *C. clematis* (Aoki 1977a), *C. arma*
 48 (Aoki 1977b), *C. monstifica* (Aoki 1983) and *C. clematicola* (Akimoto 1998). Soldiers
 49 of the first three species are characterized by their enlarged fore and mid legs, with
 50 which they cling tightly to a predator and sting it with their stylets (Ijichi *et al.* 2005).

51 The gall or primary-host generation of *Colophina* has been recorded in three
 52 Japanese species, *C. clematis*, *C. arma* and *C. clematicola* (Aoki 1980; Aoki & Kurosu
 53 2000). However, galls of *Colophina* (*C. arma* and *C. clematicola* in particular) are
 54 rarely found under natural conditions, despite the fact that trees of their primary host, *Z.*
 55 *serrata*, are common in Japan (Kurosu & Aoki 1991; Aoki & Kurosu 2000). Since some
 56 young nymphs produced on the secondary host plant overwinter on buds near the
 57 ground (Aoki *et al.* 1997) or in crevices of the bark of lignified stems of *Clematis* (Aoki
 58 1977a, 1980; Blackman & Eastop 2021), the aphids can continue their life cycles
 59 without returning to the primary host. By collecting its sexuparae from the secondary
 60 host *Clematis terniflora* and transferring them to a potted tree of *Z. serrata*, Aoki and

61 Kurosu (2000) succeeded in inducing a few galls of *C. clematicola*, which had been
62 unknown before.

63 *Colophina monstifrica* is known from mountainous areas of Taiwan (Aoki
64 1983). The species forms dense, woolly colonies on stems of the secondary host
65 *Clematis uncinata* (reported as “*C. floribunda*” in Aoki (1983)), an evergreen vine, and
66 produces winged sexuparae in autumn. *Zelkova serrata* is widely distributed in
67 mountainous areas of Taiwan, where galls of *C. clematis* are rather commonly found
68 (Bo-Fei Chen 2007, unpublished Master Thesis, National Chung Hsing University).
69 However, galls of *C. monstifrica* have not been found to date. Here, we report that *C.*
70 *monstifrica* also forms galls on *Z. serrata* in Taiwan. We succeeded in inducing galls of
71 the species on trees of *Z. serrata*, and also found a few galls under natural conditions.
72 As in *C. clematis*, the inner surface of its galls was covered by dense trichomes. In
73 addition, we show that first- and second-instar nymphs of *C. monstifrica* perform
74 altruistic colony defense against intruding predators.

75

76 MATERIALS AND METHODS

77

78 Collection of galls

79 Large colonies of *Colophina monstifrica* were found on *Clematis uncinata* at Huisun
80 Experimental Forest Station (24°05'17"N, 121°02'08"E), Nantou County, Taiwan, in
81 November 2013. We collected many winged adults (sexuparae) from the colonies, and
82 confined approximately 100 sexuparae in each of three clear plastic containers with
83 bundles of bark pieces of *Z. serrata*, expecting that the sexuparae would produce
84 sexuals and that the sexual females would lay eggs between the bark pieces. (Sexual
85 nymphs of Eriosomatinae mature into adults without taking food.) The containers were
86 kept outdoors. On 23 December 2013, these bark bundles were attached to three trees of
87 *Z. serrata* at the coffee plantation of Huisun Experimental Forest Station (hereafter
88 “Coffee Plantation”). In the following spring, on 18 and 28 April 2014, we found 12

galls of *C. monstifca* formed on leaves of two of the three *Zelkova* trees (Fig. 1). We also found seven galls of *C. monstifca* on leaves of another tree of *Z. serrata*, to which we had not attached the bark bundles, near the lodges of Huisun Experimental Forest Station (24°05'22"N, 121°02'05"E, hereafter "Lodge"). These galls were brought to the laboratory at Chung Hsing University, Taichung, and subjected to the following behavioral experiments. Three galls were preserved in 70 or 85% ethanol together with galled leaves to investigate the gall inner structure.

Attacking behavior against predators

To test attacking behavior of aphids inside the galls, we used young caterpillars of *Assara formosana* (Pylalidae) found in a gall of the aphid *Ceratoglyphina styracicola* as a model predator, because caterpillars of some *Assara* species are known to prey on aphids in galls (Aoki & Kurosu 2010). One caterpillar was introduced to each of four galls of *C. monstifca*. Ten minutes later, the galls were cut open with a razor and the caterpillars were taken out of the galls, and aphids attacking the caterpillars were observed under a dissecting microscope. We also tapped a few young nymphs in a cut gall with a fine brush, and video-recorded their reaction.

Morphological examination

Collected aphids, including those used in the experiment mentioned above, were preserved in 70 or 85% ethanol. Some of them were heated in 10% KOH solution, stained with acidic fuchsine or Evan's blue, dehydrated through an ethanol-xylene series, and mounted on microscope slides in balsam or Mount-Quick (Daido Sangyo). The mounted individuals were photographed using a digital camera (Nikon 1) attached to a light microscope. Some slide-mounted aphids of *C. monstifca* used in this study are deposited as voucher specimens in the collections of Department of Entomology, National Chung Hsing University, Taichung, Taiwan.

117 **Gall inner structure**

118 After removing all aphids from their galls, the three gall-harboring *Z. serrata* leaves
 119 kept in ethanol were first transferred to 50% ethanol, then to FAA (formaldehyde 3.7%
 120 and acetic acid 5% in 50% ethanol), dehydrated through an ethanol series and dried.
 121 The dried samples were observed with a scanning electron microscope (SEM) and
 122 photographed. Density and length of trichomes in a 0.5 x 0.5 mm square area of the
 123 surface were measured based on the photographs using ImageJ
 124 (<https://imagej.nih.gov/ij/>). Statistical significance between the gall inner surface and
 125 the underside of the same leaf was analyzed using the linear mixed model (*lmer*
 126 function in the *lme4* package in R v. 3.3.3 (R Core Team 2017)) with gall identity
 127 treated as a random factor.

128

129 **Molecular phylogenetic analysis**

130 Total DNA was extracted from three aphids of *C. monstifica* fixed in ethanol: one
 131 collected from an artificially induced gall, another from a natural gall, and the other
 132 from a colony on *C. uncinata*. A mitochondrial DNA fragment (ca. 1.6 kb) containing
 133 small subunit rRNA, tRNA-Val, and large subunit rRNA genes was amplified by PCR,
 134 as described in Aoki *et al.* (2018), and sequenced. The DNA sequences are deposited in
 135 the DNA Data Bank of Japan (DDBJ) (accession no. LC626871). These DNA
 136 sequences and those of *C. clematis* (DDBJ/EMBL/GenBank accession no. AF275224.1)
 137 and *Eriosoma lanigerum* (accession no. NC_033352.1) were subjected to molecular
 138 phylogenetic analyses. *Ceratovacuna nekoashi* (Hormaphidinae, Cerataphidini) was
 139 used as an outgroup (accession no. AB035879.1). Multiple alignment of the nucleotide
 140 sequences was generated using MAFFT (Katoh & Standley 2013). The GTR+I+G
 141 model was selected as the nucleotide substitution model using the program jModelTest2
 142 (Darriba *et al.* 2012) based on AIC. A maximum likelihood phylogenetic tree was
 143 generated using RAxML (Stamatakis 2014). Bootstrap tests were performed with 1,000
 144 replications.

145

146 **RESULTS**

147

148 **Galls of *C. monstifica***

149 Twelve globular galls (Fig. 1) were formed on the leaf of two *Z. serrata* trees, to which
150 the bark bundles with eggs of *C. monstifica* had been tied in the previous December.
151 No galls were found on other *Z. serrata* trees, to which the bark bundles had not been
152 tied, in Coffee Plantation.

153 A few galls were also found on a tree of *Z. serrata* in Lodge, near the
154 collection site of the free-living colonies on *C. uncinata*. There was no difference in
155 shape between the experimentally induced galls and the natural galls. The long distance
156 from the tree to Coffee Plantation (about 2.2 km away in a straight line) and its close
157 proximity to the colonies on *C. uncinata* (about 180 m away) suggest that these galls
158 were formed naturally, possibly by grandoffspring of the sexuparae which had flown
159 from colonies on the nearby secondary host plants.

160

161 **Colony composition**

162 Table 1 shows the composition of inhabitants for nine galls of *C. monstifica*. The
163 collected galls contained a high proportion of first- and second-instar young nymphs,
164 and a small number of nymphs with wing buds, but no winged adults. All foundresses
165 survived and still contained embryos inside.

166

167 **Attacking behavior**

168 When tapped with a fine brush, young nymphs in the gall exhibited an aggressive
169 response by clutching the brush using their forelegs (Movie S1). To test their attacking
170 response to predators, a caterpillar of *A. formosana* was introduced into four galls (Galls
171 #5, 6, 7, 9 in Table 1). One to five (average = 2.25) first-instar nymphs and one to six
172 (average = 2.7) second-instar nymphs clung onto the caterpillar and stung it with their

173 stylets (Fig. 2). We confirmed under a dissecting microscope that their stylets were
174 inserted in the body of the caterpillar, and their claws penetrated the skin. Thirty
175 minutes later, all four caterpillars were completely immobilized.

176

177 **Morphology of gall inhabitants**

178 Among the gall inhabitants of *C. monstifrica*, the first- and second-instar nymphs (Fig.
179 3) had well-developed fore and mid legs with large, strongly curved claws. In particular,
180 fore and mid legs of the first-instar nymphs were distinctly thickened (Fig. 3a). As in *C.*
181 *clematis* and *C. arma* (Aoki 1980), the first-instar nymphs (Fig. 3a) were discriminated
182 from the second-instar nymphs (Fig. 3b) by the long, usually capitate dorsoapical setae
183 on the second segment of each tarsus, and by the lack of short, spine-like seta (sense
184 peg) on the first tarsal segment. No remarkable morphological differences were
185 observed between attacking and non-attacking individuals, indicating that the first- and
186 second-instar nymphs are monomorphic defenders.

187 These gall inhabitants were morphologically distinguishable from the primary
188 host generation of *C. clematis*, which has been the only known *Colophina* species that
189 forms a globular gall on the leaf of *Z. serrata* in Taiwan. The first-instar nymphs of *C.*
190 *monstifrica* had a pair of small, half ring-like cornicles on the flat tergite (Fig. 4c),
191 whereas the first instar nymphs of *C. clematis* have distinctly protruded cornicles (Fig.
192 4d). In addition, the apex of each antenna was rounded in the first instar nymphs of *C.*
193 *monstifrica* (Fig. 4a), while the apex is conical in those of *C. clematis* (Fig. 4b).

194

195 **Molecular phylogenetic analysis**

196 The DNA sequences (1,579 bp) of the three individuals of *C. monstifrica* (collected
197 from an artificially induced gall, from a natural gall, and from a colony on *C. uncinata*)
198 were completely identical with each other. The molecular phylogenetic analysis
199 including other eriosomatine aphids also showed that the three samples were clearly
200 distinct from *C. clematis* (Fig. 5), confirming our identification of *C. monstifrica*.

201

202 **Trichomes on the inner surface of galls**

203 The observations using an SEM revealed that the inner surface of the galls of *C.*
 204 *monstrifica* was densely covered with tiny trichomes (Fig. 6). The trichome density was
 205 $298.3 \pm 44.7 / \text{mm}^2$ ($n = 12$), which was significantly higher than the trichome density
 206 on the underside of the same leaf ($16.7 \pm 3.7 / \text{mm}^2$) ($n = 12$, $\chi^2 = 74.7$, $df = 1$, $P <$
 207 0.001). On the other hand, trichomes on the inner surface of the galls were 82.2 ± 30.0
 208 μm ($n = 30$) in length and shorter than those on the underside of the leaf, which were
 209 $105.0 \pm 45.3 \mu\text{m}$ ($n = 30$, $\chi^2 = 4.97$, $df = 1$, $P = 0.026$). The high trichome density on the
 210 gall inner surface was comparable to that of *C. clematis* (221.7 trichomes / mm^2 on
 211 average) found in a previous study (Uematsu *et al.* 2018).

212

213 **DISCUSSION**

214

215 We collected naturally occurring and artificially induced galls of *C. monstrifica* on
 216 *Zelkova serrata* in Taiwan, and confirmed the identity of aphids in the galls by
 217 subsequent morphological and molecular analyses. The aphids were distinct in
 218 morphology from *C. clematis*, the only congener known in Taiwan. The molecular
 219 analyses also revealed the identity of the mitochondrial DNA sequences between the
 220 aphids in these galls on *Z. serrata* and those collected from the secondary host *Clematis*
 221 *uncinata*. Although migration of winged adults from the gall to *C. uncinata* has not
 222 been observed, these results indicate that, like other *Colophina* species, *C. monstrifica*
 223 has a life cycle with host alternation between *Zelkova* and *Clematis*. We found colonies
 224 of *C. monstrifica* on *C. uncinata* at Huisun Experimental Forest Station also on 28
 225 March 2011. This indicates that the host alternation of *C. monstrifica* is facultative, or
 226 that its colonies can persist on the clematis throughout the year.

227 We confirmed that first- and second-instar nymphs of *C. monstrifica* attack
 228 predators introduced in their gall. Their attack using the claws and stylets can

229 immobilize and kill such potential predators as pyralid larvae. In addition, we showed
 230 that the inner surface of galls of *C. monstiflora* was densely covered with minute
 231 trichomes. The galls of *C. clematis* and *C. arva* also have dense trichomes on the inner
 232 surface. It is known that these trichomes collect wax particles produced by aphids, make
 233 the inner surface water repellent and facilitate the formation of small globules of
 234 honeydew covered with the wax. Young nymphs of *C. clematis* actively dispose of
 235 honeydew globules outside their gall through a small opening (Uematsu *et al.* 2018).
 236 Although we did not directly observe honeydew-disposing behavior in this species, the
 237 microstructure of the inner surface of the gall strongly suggests that young nymphs of *C.*
 238 *monstiflora* also dispose of honeydew globules.

239 There are many species of Eriosomatinae and Hormaphidinae whose gall or
 240 primary-host generations are unknown (Blackman & Eastop 2021). It is not clear
 241 whether these species lost their primary-host generations irreversibly or still retain the
 242 ability to induce galls on the primary host. In this study, we obtained eggs of *C.*
 243 *monstiflora* by collecting sexuparae from the secondary host, attached them to its
 244 presumed primary host, and successfully induced galls of the species, which had been
 245 unknown before. This method may be applied to other species for elucidating their life
 246 cycles.

247

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256

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310

311 SUPPORTING INFORMATION

312

313 Additional Supporting Information may be found online in the Supporting Information
314 section at the end of the article.

315 **Movie S1.** Attacking behavior of young nymphs of *Colophina monstifica* toward a fine
316 brush in a cut gall.

317

318

319

320 **Figure legends**

321

322 **Figure 1** A gall of *Colophina monstifica* formed on a leaf of *Zelkova serrata*.

323

324 **Figure 2** Young nymphs of *Colophina monstifica* attacking a larva of the moth *Assara*
325 *formosana* introduced into their gall.

326

327 **Figure 3** Gall generation of *Colophina monstifica*: (a) first-instar nymph (from a gall
328 collected on 18 April 2014); (b) second-instar nymph (from a gall collected on 28 April
329 2014). Scale bars represent 100 μ m.

330

331 **Figure 4** First-instar nymphs of *Colophina monstifica* and *C. clematis* produced in the
332 galls on *Zelkova serrata*: (a) apical antennal segments of *C. monstifica*; (b) apical
333 antennal segments of *C. clematis*; (c) posterior abdominal tergites of *C. monstifica*; (d)
334 posterior abdominal tergites of *C. clematis*. The right cornicle is indicated by an arrow
335 in (c) and (d). The photographed nymphs (a, c) of *C. monstifica* were collected on 18
336 April 2014, and the photographed nymph (b, d) of *C. clematis* was collected in Miaoli,
337 Taiwan, on 15 May 2002. Scale bars represent 100 μ m.

338

339 **Figure 5** Molecular phylogenetic analysis of eriosomatine aphid species. The maximum
340 likelihood phylogeny inferred from aligned 1,572 nucleotide sites of the mitochondrial
341 rRNA gene is shown. Bootstrap probability in percentage is shown at the nodes. The
342 DDBJ/EMBL/GenBank accession number for each DNA sequence is indicated in
343 square brackets.

344

345 **Figure 6** Trichomes on the inner surface of a gall of *Colophina monstifica*. Scale bar
346 represents 200 μ m.

347

Fig. 1

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Fig. 2

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(a)

(b)

Fig. 4

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Fig. 6

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