Heterogony in *Cycloneuroterus* (Hymenoptera: Cynipidae: Cynipini) From Rearing Experiments and DNA Barcoding

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Abstract

Heterogony was confirmed in the cynipid genus *Cycloneuroterus* Melika and Tang in rearing experiments with DNA barcoding. These experiments involved *Cycloneuroterus gilvus* Tang and Melika, which was previously only described from the sexual generation adult. The first rearing experiment was conducted using unidentified asexual generation females collected from *Quercus gilva* Blume, and gall formation by the sexual generation offspring was confirmed on folded or unfolded young leaves of *Q. gilva*. The second experiment was conducted using sexual generation males and females reared from the leaf galls collected from *Q. gilva*, and gall formation by the asexual generation offspring was observed on leaves of *Q. gilva*. Based on the morphological features of the sexual generation adults and galls, this species was identified as *C. gilvus*. The species identity of wasp specimens of sexual and asexual generations used in the rearing experiments was cross-checked using DNA barcoding with the partial sequences of the cytochrome c oxidase subunit I (COI) region (658 bp). The asexual generation adult and gall of *C. gilvus* are described based on these results. The importance of ‘closing the life cycle,’ in this case a demonstration of heterogony, in oak gall wasps (Cynipini) is discussed.

Key words: alternation of generations, *Cycloneuroterus gilvus*, oak gall wasp, *Quercus gilva*

The cynipid gall wasps comprise approximately 1,400 species worldwide (Ronquist et al. 2015, Pénzes et al. 2018). Most of them (>1,000 species) belong to the tribe Cynipini (oak gall wasps), which induces galls on trees of the family Fagaceae, principally *Quercus* L. (Liljeblad et al. 2008, Ronquist et al. 2015, Pénzes et al. 2018). Alternation of generations (heterogony) occurs in this tribe. In general, the sexual generation galls and adults appear in spring or early summer, the asexual generation galls develop slowly, and the asexual generation adults emerge from late fall to the following spring (Yukawa and Masuda 1996, Stone et al. 2002). The gall and adult morphologies differ considerably between sexual and asexual generations even within the same species (Askew 1984, Pujade-Villar et al. 2001, Stone et al. 2002). Nevertheless, only adults of either the sexual or asexual generation have been described in many species of Cynipini. Therefore, the occurrence of heterogony is likely underestimated (Hood et al. 2018).

*Cycloneuroterus* Melika and Tang is a genus of Cynipini that contains 17 species, known from Taiwan, Japan, and mainland China (Tang et al. 2011, 2016; Ide et al. 2012; Abe et al. 2014). All but one species are described based only on the sexual generation adult. Although the asexual generation gall is known in *C. arakashipagus* Ide, Wachi, and Abe and *C. lohsei* Melika and Tang, their asexual generation adults have not yet been described (Ide et al. 2012, Tang et al. 2016). The adult female of *C. uangi* Abe, Ide, and Odagiri examined by Abe et al. (2014) was considered to be the asexual generation because of its emergence period, but the sexual generation wasp remains unknown (Abe et al. 2014). Therefore, there are no *Cycloneuroterus* species for which the morphology of both sexual and asexual generation adults is known.

*Cycloneuroterus gilvus* Melika and Tang is only known from Taiwan. According to Tang et al. (2016), the sexual generation gall is blister-like in shape and are induced on folded or unfolded young leaves of *Quercus gilva* Blume in mid-March in Taiwan. In addition, the sexual generation adult emerge from the gall from late March to early April under laboratory conditions (Tang et al. 2016). Although this species is expected to have an alternation of generations like other species of Cynipini, the asexual generation adult and gall are not yet known similar to most members of *Cycloneuroterus*. Our field observations of oviposition by the sexual generation female of *C. gilvus* enabled us to describe the asexual generation gall and adult, which had not yet been reported.

Because *Q. gilva* is also distributed in Japan and mainland China (Govaerts and Frodin 1998), *C. gilvus* can potentially be found in these countries. Here, we report new observations of *C. gilvus* in

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Japan. The alternation of generations in this species was confirmed in rearing experiments and with DNA barcoding. These results complete the heterogonic life cycle of *C. gilvus* and we describe the asexual generation adult and gall of this gall wasp species. This is the first *Cycloneuroterus* species in which a heterogonic life cycle and the morphology of both sexual and asexual generation adults are confirmed. Our results emphasize the biological significance of "closing the life cycle" in oak gall wasps.

**Materials and Methods**

**Field Observations and Identification of the Sexual Generation**

Unidentified galls induced on folded young leaves of *Q. gilva* were collected by T.L. in Nishinokorakata and its vicinity, Miyazaki Prefecture, Japan every March or April from 2010 to 2013. Male and female wasps that emerged from the galls were considered to be sexual generation individuals. Later, these wasps and galls were identified as the sexual generation of *C. gilvus* based on their morphological features based on Tang et al. (2016).

**Rearing Experiments to Reveal a Heterogonic Life Cycle**

**Experiment 1 (Alternation From the Asexual Generation to the Sexual Generation)**

On 25 February 2012, 11 unidentified adult female gall wasps staying on the buds of a *Q. gilva* tree were collected in Nishinokorakata, where the sexual generation galls of *C. gilvus* were observed from 2010 to 2013. Because no males were observed and the emergence period was almost 2 mo earlier than that of the sexual generation females of *C. gilvus*, these wasps were considered to be the asexual generation females. The females were brought to the laboratory (Ito Campus, Kyushu University, Fukuoka Prefecture, Japan) and kept alive by feeding them honey for later use in rearing experiments. On 27 February 2012, four branches of a planted *Q. gilva* tree growing on the Ito Campus, where no cynipid galls had been observed on *Q. gilva* since April 2009, were randomly selected, and each branch was covered with a nonwoven polyester bag. Five females were released in each of two bags from 27 February 2012 to 5 March 2012, and were then immersed in 99.5% (v/v) ethanol after the experiment. Wasps were not released in the remaining two bags. Each branch was observed once a week to assess gall induction until the end of July 2012.

**Experiment 2 (Alternation From the Sexual Generation to the Asexual Generation)**

On 14 April 2013, galls on folded young leaves of *Q. gilva* were collected in Kobayashi, Miyazaki Prefecture, and brought to the laboratory. Four female and nine male gall wasps emerged from those galls on the same day. On 15 April 2013, two branches of the tree were selected randomly, and each branch was covered with a nonwoven polyester bag. Two pairs of the gall wasps were released in one of the two bags from 15 to 22 April 2013, and were then immersed in 99.5% (v/v) ethanol. No wasps were released in the other bag. Subsequently, each branch was observed once a week to determine gall induction until the end of September 2014.

**DNA Barcoding for Cross-Checking the Results of the Rearing Experiments**

DNA was extracted from three sexual generation wasps and one asexual generation wasp that had been used in the above rearing experiments using a DNeasy Blood and Tissue Kit (QIAGEN KK, Tokyo, Japan). To amplify the mitochondrial cytochrome c oxidase subunit I (COI) region, which has species-specific sequences in most animals (Hebert et al. 2003), polymerase chain reaction (PCR) was conducted using the primers LCO1490 (5′-GGTCAACAATTATAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al. 1994). The PCR thermal protocol followed Hebert et al. (2003): 94°C for 60 s; five cycles at 94°C for 60 s, 45°C for 9 s, and 72°C for 90 s; 35 cycles at 94°C for 60 s, 50°C for 90 s, and 72°C for 60 s; and a final extension at 72°C for 5 min. The PCR products were purified using a QIAquick PCR Purification Kit (QIAGEN KK). The sequences of both strands of the PCR products were verified using a commercial sequencing service (Eurofins Genomics KK, Tokyo, Japan). The complementary forward and reverse sequences were assembled in Mega 6.06 (Tamura et al. 2013) using ClustalW and checked by eye. Voucher specimens were deposited in the Tsukuba Research Department, National Museum of Nature and Science, Tsukuba, Japan (NSM), under accession numbers NSMT-I-Hym 75438-75441.

**Morphological Examination for Description and Identification**

Adults were killed in 99.5% (v/v) ethanol and dried following the t-butyl-alcohol freeze-drying method (originally developed by Inoué and Osataka 1988) using a freeze-drying device (JFD-320, JEOL, Tokyo, Japan) following the manufacturer’s protocol. The specimens examined were deposited in the NSMT.

The external structures of dry-mounted wasps were examined using binocular stereomicroscopes (S8APO and MZ APO, Leica Microsystems KK, Tokyo, Japan) fitted with digital single-lens reflex cameras (E-30, Olympus, Tokyo, Japan) and a scanning electron microscope (JSM-6380LV, JEOL, Tokyo, Japan) operating at 1.5 kV. The length of each body part was measured using an ocular micrometer.

The following morphological abbreviations were used: POL, postocellar line (the distance between the inner edges of the two lateral ocelli); OOL, ocellar-ocellar line (the distance from the outer edge of a lateral ocellus to the compound eye); and LOL, lateral-ocellar line (the distance between the median and lateral ocelli). The morphological terminology follows Richards (1977), Ronquist and Nordlander (1989), and Liljeblad et al. (2008); the description of surface sculptures follows Harris (1979).

**Results**

**Rearing Experiment**

**Experiment 1**

On 4 April 2012, 10 galls appeared on young leaves of one of the two branches, as a result of exposure to the five unidentified asexual generation females. By 10 April 2012, 10 males emerged from the galls. These galls and adults were identified as the sexual generation of *C. gilvus* based on Tang et al. (2016). No galls were observed on the other three branches covered with bags by the end of July 2012.

**Experiment 2**

On 20 May 2013, 31 galls appeared on a leaf of a branch that had been exposed to two sexual generation males and two females of *C. gilvus*. These galls fell onto the bottom of the bag from the leaf after maturity until early June. Subsequently, they were kept in a plastic cup in the shade under field conditions until late May 2014, but no wasps emerged. No galls were observed in the other bag by the end of September 2014.
DNA Barcoding

The wasps used for the two rearing experiments had identical COI sequences. All of the determined sequences were deposited in the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) under accession numbers LC456876-456879. These accession numbers are also available in GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

Taxonomy

*Cycloneuroterus gilvus* Tang and Melika, 2016

*Cycloneuroterus gilvus* Tang and Melika: Tang et al. 2016: 266 (sexual generation male, female, and gall).

Material Examined

Asexual generation (Figs. 1–3): 11 females, Kamamuta, Takaharu, Nishimorokata, Miyazaki Prefecture, Kyushu, Japan, 25-II-2012, T. Ide leg.; seven females, Higashikata, Kobayashi, Miyazaki Prefecture, Kyushu, Japan, 17-III-2012, T. Ide leg.; one female, Oho, Ogori, Fukuoka Prefecture, Kyushu, Japan, gall 25-VI-2017, em. 4-III-2018, K. Matsunaga leg. Sexual generation (Fig. 4): nine females, Kitakawachi, Kitago, Nichinan, Miyazaki Prefecture, Kyushu, Japan, gall (collection of gall) 10-IV-2011, emergence of adult 18–21-IV-2011, T. Ide leg.; five females and four males, Kamamuta, Takahara, Nishimorokata, Miyazaki Prefecture, Kyushu, Japan, gall 21-IV-2012, em. 22-IV-2012, T. Ide leg.; four females and nine males, Higashikata, Kobayashi, Miyazaki Prefecture, Kyushu, Japan, gall 14-IV-2013, em. 14–15-IV-2013, T. Ide leg.

Asexual Generation Female

Head black to dark brown, gradually lighter toward lower face; antenna and palpi uniformly yellow; mandible yellow, except for darker margin. Mesosoma brown; tegula yellow; legs yellow, except for darker tarsal claws. Metasoma dark brown.

Head slightly broader than mesosoma in dorsal view. Vertex and frons coriarius; median ocellus transversely oblong; lateral ocellus round; POL:OOL:LOL = 17:16:8. Diameter of antennal rim 1.17 times as broad as distance between inner margins of rims, 0.70 times as broad as distance between lateral margin of antennal rim and inner margin of compound eye. Inner margins of compound eye almost parallel; distance between inner margins of compound eyes 1.27 times as broad as height of compound eye. Lower face smooth, covered with sparse setae; facial strigae absent; malar area coriarius; subocular impression absent. Clypeus smooth; ventral clypeal margin rounded without median incision. Gena coriarius, not broadened behind compound eye. Antenna 14-segmented; scape except for base as long as pedicel; relative lengths of flagellomeres 1–12: 11, 11, 11, 11, 11, 11, 10, 10, 10, 10, 10, and 13.

Mesosoma longer than height in lateral view. Pronotum smooth with sparse setae posteriorly; submarginal pronotal impression narrow, sharply widening posteriorly, marked by ledge posteriorly; pronotal plate set off, with anterolateral corners. Mesoscutum smooth, almost as long as width in dorsal view; impression mesad parascutal carina anteriorly continuing to anterior end of notaulus; notaulus present only anteriorly; row of sparse setae indicating remaining part of notaulus; anteroadmedian and parapsidal signa, and median mesoscutal impression absent; middle part of posterior mesoscutal margin curved anteriorly. Mesoscutum and
mesoscutellum fused; transscutal fissure absent. Mesoscutellum smooth; sparse setae present near lateral margin; lateral and posterior scutellar margin rugose; scutellar fovea present as broad transverse furrow, smooth; indistinct foveal septum present posteriorly, or absent; posterior margin of fovea closed. Mesopleuron smooth, without longitudinal striae medially; mesopleural triangle smooth, without setae; ventral border of mesopleural triangle marked throughout by distinct ledge. Metascutellum subrectangular; metanotal trough smooth without setae. Metepimeron marked by distinct ledge posteriorly, without setae. Propodeum smooth, with sparse setae on lateral propodeal area; lateral propodeal carina reaching above dorsal propodeal margin, slightly diverging ventrad.

Fig. 2. Asexual generation female of *Cycloneuroterus gilvus*. (A) Head, anterior view (scale bar = 100 μm). (B) Head and mesosoma, lateral view (scale bar = 100 μm). (C) Head and mesosoma, dorsal view (scale bar = 100 μm). (D) Antenna, lateral view (scale bar = 100 μm). (E) Propodeum and metascutellum, dorsal view (scale bar = 100 μm).

Fig. 3. Asexual generation female of *Cycloneuroterus gilvus*. (A) Metasoma, lateral view (scale bar = 100 μm). (B) Hypopygial spine, ventral view (scale bar = 50 μm).
Marginal cell of forewing 5.43 times as long as width. Apex of metatarsal claw distinctly bent; base not expanded to lobe. Lengths of forewing (mean ± SD, n = 11) 1.81–2.20 (2.00 ± 0.13) mm, and of hind tibia 0.44–0.52 (0.48 ± 0.03) mm.

Metasoma smooth, not distinctly compressed laterally in dorsal view. Metasomal tergum II without setae. Lateral flap of hypopygium rounded in lateral view; projecting part of hypopygial spine only slightly protruded in lateral view, tapered to apex in ventral view, 1.21 times as long as height in lateral view, 1.60 times as long as width in ventral view; subapical setae sparse, reaching beyond apex of spine.

Asexual Generation Gall
Ten or more galls usually induced on a leaf blade. Each gall about 1.0–1.4 mm in diameter, spherical with flat upper and lower sides; immature ones pale yellow or yellowish green, mature ones brownish yellow or brown; lower side covered with minute white setae; outer wall thin; containing one larva.

Sexual Generation Adult and Gall
Female, male, and gall are shown in Fig. 4. Main differences between asexual and sexual generation females are as follows: 1) the head is broader than the mesosoma in the former and slightly narrower in the latter; 2) the relative lengths of flagellomeres 1–11 are nearly equal in the former, while F1 is 1.2 times as long as F2, F2 is as long as F3, and F4–F11 are progressively shorter in the latter; 3) the mesosoma is longer than high in the lateral view in the former, but higher than long in the latter; 4) the submarginal impression of the pronotum is marked by a ledge in the former and is not marked in the latter; 5) the pronotal plate is marked only anterolaterally in the former and is distinctly set off with ventral and lateral margins in the latter; 6) the scutellar fovea often has an indistinct foveal septum posteriorly in the former, but is always absent in the latter; and 7) the central propodeal area does not have any wrinkles in the former and has strong rugae radiating from the nucha in the latter. Tang et al. (2016) clearly illustrated the sexual generation male, female, and gall. No morphological differences were observed between the Japanese and Taiwanese populations.

Host Plant
Quercus gilva (family Fagaceae: genus Quercus: subgenus Cerris Oerst.: section Cyclobalanopsis [Oerst.]).

Geographic Distribution
Japan: Kyushu (new record); Taiwan: Hsinchu and Taoyuan (Tang et al. 2016).
T.I. collected old asexual generation galls of C. gilvus from a planted Q. gilva tree in Todori, Hachioji, Tokyo Prefecture, Honshu, Japan, on 28 April 2016. Old asexual generation galls were also collected by A. Nagai and T. Komoda in Higashikata, Kobayashi, Miyazaki Prefecture, Japan, on 1 July 2009, and Takaharu, near Lake Miike, Nishimorokata, Miyazaki Prefecture, Japan, on 7 January 2010, respectively. These galls were sent to Y.A. However, no wasps emerged from these galls.

Biology
The biology of C. gilvus is shown in Figs. 5–7. According to K. Matsunaga (personal communication), she collected five asexual generation galls of C. gilvus on 25 June 2017 in Oho, Ogori, Fukuoka Prefecture, Kyushu, Japan, and maintained them under field conditions. An asexual generation female emerged from one of
Based on her observation and our rearing experiments, field observations, and collection data, the life cycle of *C. gilvus* can be summarized as follows. The sexual generation gall appears on the leaf of *Q. gilva* in early April. The sexual generation adult emerges from the gall from mid to late April (Fig. 5A). The sexual generation female lays her eggs in young leaves from late-flushing buds. (C) Asexual generation galls begin to appear on leaves from mid to late May, and fall to the ground after maturity; traces of galls are found on leaves. (D) Asexual generation females emerge from the galls on the ground from late February to early March, and lay their eggs in buds.

**Discussion**

‘Closing the Life Cycle’ of Species and Genera in Cynipini

Here we report for the first time ‘closing the life cycle’ in the Asian endemic genus *Cycloneuroterus* using a combination of rearing experiments and DNA barcoding of *C. gilvus*. Even in the species-rich western Palearctic and Nearctic regions, only the sexual or asexual generation is known for many species, or even genera, of Cynipini (Pujade-Villar et al. 2001). The situation is similar in the eastern Palearctic and Oriental regions (Abe et al. 2007). The life cycles of most known species have not yet been closed, and the genera *Belizinella* Kovalev and *Cyclocynips* Melika, Tang and Sinclair are also only known from the asexual generation (Pénzes et al. 2018). As noted by Hood et al. (2018), ‘closing the life cycle’ via a multidisciplinary approach is necessary to understand the true species-diversity, phylogeny, and evolution of Cynipini. Because cynipid fauna have not yet been resolved well in the Palearctic and Oriental regions, undescribed species will continue to be discovered in these areas (Abe et al. 2007). Therefore, ‘closing the life cycle’ of species and genera in Cynipini is an important endeavor in these regions.

In recent years, three oak gall wasp species with unique life cycles have been found in Asia. *Dryocosmus zhuili* Liu and Zhu induces galls on *Castanea* Mill. (Zhu et al. 2015). Although the gall and adult morphology of *D. zhuili* are similar to those of the chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu, which reproduces asexually without males, *D. zhuili* reproduces bisexually and, unlike all other species of Cynipini, is considered to have one sexual generation in 1 yr (Zhu et al. 2015, Pang et al. 2018). In addition, *Dryocosmus nanlingensis* Abe, Ide, and Odagiri was described based only on one male from China, indicating that it is the sexual generation (Abe et al. 2014). However, this male was collected in winter (December), which is typically the emergence period of the asexual generation in most species of Cynipini (Abe et al. 2014). Moreover, the sexual generation adults in most species of Cynipini emerge in spring and their offspring emerge as the asexual generation late in the fall or winter of the same year (Yukawa and Masuda 1996, Stone et al. 2002). However, the heterogonic life cycle of *Plagiotrochus*...
masudai Ide, Wachi, and Abe requires 3 yr to complete, and the asexual generation adults emerge in May (Ide et al. 2010). The presence of these unique gall wasps in Asia implies that the life cycle patterns of Cynipini are more diverse than expected from hitherto known species in the well-studied western Palearctic and Nearctic regions. Because Cycloneuroterus is the second-most species-rich genus in the eastern Palearctic and Oriental regions (Pénzes et al. 2018), our demonstration of closing the life cycle of Cycloneuroterus will serve as a stepping stone for future studies of the evolution and diversity of life cycle patterns of Cynipini.

Although galls of Cynipini usually have closed communities including gall inducers, inquilines, and parasitoids (Askew 1984, Stone et al. 2002), such communities associated with galls of C. gilvus have not yet been resolved. Interaction within these communities can influence the evolution of heterogenic life cycles in Cynipini (Forbes et al. 2015, Hood and Ott 2017, Hood et al. 2018). Because heterogeneity as an adaptation for avoidance of natural enemies is of broad interest, further study is needed to reveal the inquilines and parasitoids associated with sexual and asexual generations of heterogenic species of Cynipini as well as their life cycles.

Validity of Diagnostic Features of Cycloneuroterus in the Asexual Generation

Several diagnostic features of a genus in Cynipini often cannot be applied to both the sexual and asexual generations of the genus because morphological features often differ remarkably between these two generations, even within a genus (Askew 1984, Pujadé-Villar et al. 2001, Stone et al. 2002). For example, the trifurcate hypopygial spine of females is a main diagnostic feature of the genus Lataspinia, which was established based only on the sexual generation (Monzen 1954, Melika et al. 2010); however the asexual generation females possess a needle-shaped hypopygial spine, as in both sexual and asexual generations of adult females in almost all other genera of Cynipini (Ide and Abe 2016).

Diagnostic features of Cycloneuroterus are also based on the sexual generation (Tang et al. 2011, 2016; Ide et al. 2012) and can be summarized as follows: 1) most body parts are not covered with dense setae; 2) the malar sulcus is absent; 3) the dorsal surface of the mesosoma and mesoscutellum is smooth; 4) the notaulus is absent or present only anteriorly; 5) the transscutal fissure is absent; 6) the scutellar fovea is present as a transverse furrow; 7) the lateral propodeal carina is complete, and bent outward; and 8) the projecting part of the hypopygial spine lacks lateral latches and dense setae. The asexual generation female of C. gilvus exhibits all eight of these diagnostic features of the genus (present study), as in C. wangi (Abe et al. 2014).

This finding suggests that these diagnostic features are applicable to both generations in Cycloneuroterus.

Potential Diversity of Cycloneuroterus

The genus Quercus was recently reorganized into two subgenera: Quercus and Cerris by Denk et al. (2017). The latter subgenus contains the section Cyclobalanopsis (previously known as a subgenus of Quercus), which is distributed only in the eastern Palearctic and Oriental regions. This section is the main host of Cycloneuroterus because 15 of the 17 known species of this genus are associated with this section (Pénzes et al. 2018). Although C. gilvus and Cycloneuroterus fortitutus Tang and Melika were recorded from both Japan and Taiwan (Ide et al. 2012, present study), other species of Cycloneuroterus are endemic to Japan, Taiwan, or mainland China (Tang et al. 2011, 2016; Ide et al. 2012). Because the hosts of the known species of Cycloneuroterus include widely distributed species of Cyclobalanopsis, e.g., Quercus glauca Thumb., which is distributed from Japan to the Himalayan region (Govaerts and Frodin 1998), further studies may reveal additional Cycloneuroterus species that are widely distributed in Asia.

In addition, the genera Castanopsis (D. Don) Spach and Lithocarpus Blume (Fagaceae) have been recorded as hosts of Cycloneuroterus (Tang et al. 2011, 2016). These two plant genera are also endemic to the Palearctic and Oriental regions (Govaerts and Frodin 1998) and possibly support a number of Cycloneuroterus species throughout these areas. Although 12 Cynipini species are associated with these plant genera, their life cycles have not yet been closed (Pénzes et al. 2018). The patterns of their life cycles may differ from those of other known Cycloneuroterus or Cynipini species, as in some Asian species already mentioned above. Given these issues, future studies must focus not only on the diversity of species, but also on life cycles.

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