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ORIGINAL ARTICLE

Discovery of two specialist insects that feed on immature acorns of *Castanopsis cuspidata* (Thunb.) Schottky (Fagaceae) and their importance in acorn production

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Abstract

The acorns of fagaceous species contain considerable amounts of nutrients, especially within the cotyledons. Seed predation that damages cotyledons, primarily caused by *Curculio* weevils, has been considered to be an important factor affecting acorn production. We investigated acorn falling patterns and predispersal insect damage in *Castanopsis cuspidata* (Fagaceae), whose cotyledons began to develop late in the growing season and acorns were covered by a cupule until maturity, from an initial stage of acorn development in two well-developed evergreen forests in Japan: Higashiyama in Kyoto Prefecture and Aya in Miyazaki Prefecture. Based on the identification using appearance and genetic analyses of insects damaging acorns, undescribed *Heliozela* sp. were dominant, followed by undescribed *Eurytoma* sp. in immature-sized acorns in both Higashiyama and Aya, whereas *Curculio* weevils were predominant in mature-sized acorns. Seasonal sampling of acorns from the crowns indicated that larvae of *Heliozela* sp. and *Eurytoma* sp. feed on the pericarp of the acorn during early stages of acorn development and that these insects have a multivoltine life cycle. In both Higashiyama and Aya, the total number of acorns damaged by *Heliozela* sp. and *Eurytoma* sp. amounted to approximately half of the total number of acorns produced in 1 year, when initial acorn production was considerably less. Our results suggest that a variety of seed-feeding insects can evolve to adjust to acorn growth patterns, and that such insects, in turn, have a large impact on acorn production, even prior to cotyledon development.

Key words: *Eurytoma*, *Heliozela*, plant–insect interaction, predispersal stage, seed predation.

INTRODUCTION

Fagaceae consists of seven genera, including *Fagus*, *Quercus* and *Castanopsis*. It comprises approximately 900–1,000 species that are distributed in temperate and subtropical forests worldwide and are often the dominant species within these forests (Iokawa 2016). The seeds of fagaceous species are called "acorns" or "nuts"; they become large-sized when mature and contain considerable quantities of nutrients, especially within the cotyledons, to support seedling growth and attract animals for seed dispersal (Vander Wall 2001; García-Cebrían *et al.* 2003; Yang *et al.* 2023). High nutrient content within the acorns is also assumed to attract seed predators during the predispersal stage.

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Previous studies have reported that *Curculio* weevils and moths belonging to the Tortricidae, whose larvae feed mainly on cotyledons, caused heavy losses to acorn crops prior to dispersal (Maeto & Ozaki 2003; Bonal *et al.* 2007; Xiao *et al.* 2007; Teruya *et al.* 2010; Espelta *et al.* 2017; Bogdziewicz *et al.* 2018).

Seed predation directly reduces seed production by the host species, which affects host recruitment and fitness (Crawley 1992; Hulme & Benkman 2002). Accordingly, acorns also have multiple mechanisms of defense against high predation pressure. Previous studies have shown that acorns often contain high levels of tannins or total phenols (Shimada & Saitoh 2006; Xiao *et al.* 2007; Yang *et al.* 2023), have protective cupules (Fukumoto & Kajimura 1999; Yang *et al.* 2023) and/or delay nutrient accumulation (Yang *et al.* 2023). In addition, fagaceous species often exhibit masting or mast seeding, which is a highly variable yet synchronous seed production over the years within a population (Kelly & Sork 2002). It has been hypothesized in previous studies that this strategy allow fagaceous species to escape seed predation, mainly by *Curculio* weevils, by alternately satiating and starving them through variable acorn production (the predator satiation hypothesis; Maeto & Ozaki 2003; Bonal *et al.* 2007; Bogdziewicz *et al.* 2021).

However, seed predation in fagaceous species occurs not only after cotyledon development but also soon after flowering (Igarashi & Kamata 1997; Fukumoto & Kajimura 1999). Fukumoto and Kajimura (1999) found that a cynipid wasp that laid eggs on the pistillate flowers of *Quercus serrata* Murray predominantly exploited *Q. serrata* acorns. In a different study, Hirayama *et al.* (2022) indicated that annual variability in acorn production of *Q. serrata* was directly regulated by annual variability in reproductive loss caused by predispersal seed predation by *Cyllorhynchites ursulus* (Roelofs), adults of which preyed on immature acorns immediately after emergence; emergence mainly occurred from late June to early July when the acorns were very small and cotyledons were underdeveloped. Therefore, to clearly identify and elucidate seed predation patterns in fagaceous species, which affect not only acorn production patterns but also the recruitment and fitness of the species, we need to identify the seed-feeding insects that damage the acorns, as well as the timing and mechanisms of damage, from an initial stage of acorn development.

Castanopsis cuspidata (Thunb.) Schottky is an evergreen fagaceous species distributed in warm temperate regions of western Japan and on Jeju Island in Korea (Iokawa 2016), and is one of the most important trees there (Iokawa 2016). In western Japan, the trees usually flower from April to early May (K. Hirayama, pers. obs., 2014–2022). Flowers are arranged in a unisexual inflorescence, comprising one to more than 20 female flowers (Fig. 1A), and require 2 years to produce mature acorns. While female flowers show little growth in the flowering year, the acorn cupules and pericarps start to develop from late June in the next year after flowering (K. Hirayama, unpubl. data, 2014; Fig. S1). Then, the acorns, enclosed by cupules until maturity, gradually develop until mid-September and grow rapidly in size from late September to October. Cotyledon development begins in October. When acorns become mature, their cupules come off and the acorns fall off to the ground mainly in November. Only *Curculio hilgendorfi* (Harold), whose larvae feed mainly on acorn cotyledons of the genus *Castanopsis*, is a known specialist seed-feeding insect (Ueda 2002). *Curculio sikkimensis* (Heller), which feeds on the acorns of several fagaceous species, has also been found on *Castanopsis* acorns (Aoki *et al.* 2008). However, it remains unclear whether the cupule of *C. cuspidata* plays a protective role against seed predation during the early stages of acorn development, and whether insects can damage acorns during these early developmental stages.

In this study, we investigated the acorn falling patterns and predispersal insect damage, from an initial stage of acorn development in *C. cuspidata* in two well-developed warm-temperate evergreen forests in western Japan: Higashiyama in Kyoto Prefecture and Aya in Miyazaki Prefecture. *Castanopsis cuspidata* has been dominant for more than 100 years in these forests (Association to make Aya Forest World Heritage Site 2005; Okuda *et al.* 2007), where the original insect–plant interactions would have been maintained for a long time. We also conducted seasonal sampling of *C. cuspidata* acorns from the crown and identified insects that damaged the acorns in the early developmental stages based on their appearance and genetic analyses. Finally, we discussed the effects of feeding insects in the early stages on acorn production in *C. cuspidata*.

MATERIALS AND METHODS

Study sites

We conducted this study in two warm temperate, well-developed evergreen broadleaved forests, western Japan: Higashiyama in Kyoto Prefecture (34.9981°N, 135.7883°E, 200–220 m a.s.l.) and Aya in Miyazaki Prefecture (32.0241°N, 131.1912°E, 160–200 m a.s.l.). The mean annual temperature and precipitation were

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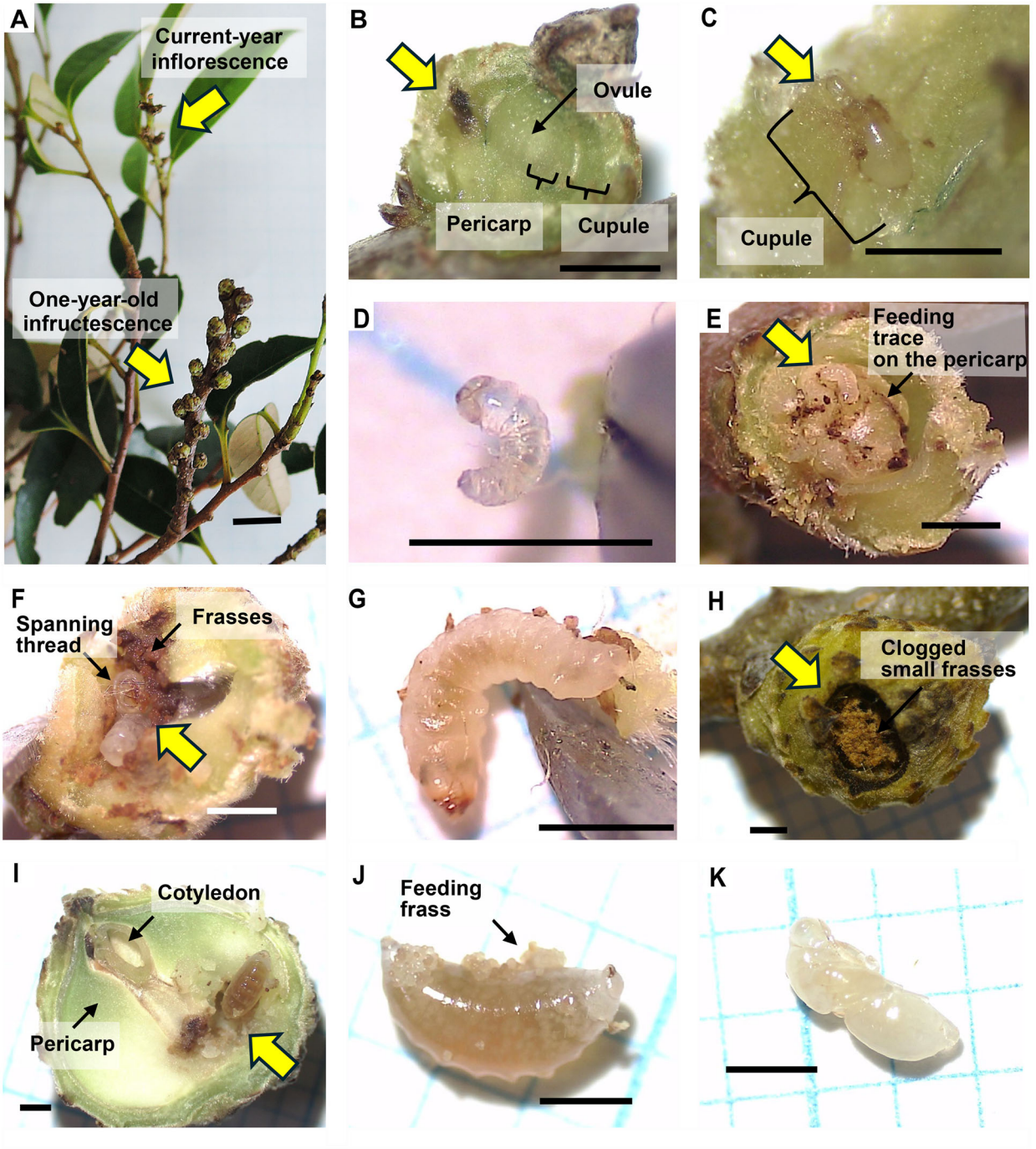


Figure 1 Immature acorns of *Castanopsis cuspidata*. (A) Current-year inflorescence and 1-year-old infructescence on a twig of *C. cuspidata* in early July. (B) Scar (yellow arrow) created by the ovipositor of the insect considered as *Heliozela* sp. in early July. (C) Egg (yellow arrow) inside the scar within the cupule, in the same acorn shown in (B). (D) First instar larva of the insect considered as *Heliozela* sp. (E) Second instar larva (yellow arrow) of the insect considered as *Heliozela* sp. with its feeding trace in the pericarp. (F) Fourth (final) instar larva (yellow arrow) of the insect considered as *Heliozela* sp. spinning thread to make a cocoon in the acorn. (G) Dorsal view of the fourth instar larva of the insect considered as *Heliozela* sp. (H) Scar (yellow arrow) dropping an oval case from the acorn clogged small frasses. (I) Larva (yellow arrow) of the insect considered as *Eurytoma* sp., feeding on the pericarp. (J) Larva, with feeding frasses, as shown in (I). (K) Pupa of the insect considered as *Eurytoma* sp. Scale bars: 1 cm (A); 0.5 mm (C,D); 1 mm (others).

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ca. 16.2°C and 1,522.9 mm in Higashiyama, based on data collected between 1991 and 2020 by the Kyoto local meteorological observatory (35.9154°N, 135.7328°E, 41 m a.s.l.), and ca. 17.7°C and 2,625.5 mm in Aya, based on the data from the Miyazaki local meteorological observatory (31.9387°N, 131.4140°E, 9 m a.s.l.).

We established study plots, 20 × 40 m in Higashiyama (Hirayama *et al.* 2016), and 40 × 40 m in Aya. We recorded all tree stems with diameter at breast height ≥5 cm in these plots (Table S1). *Castanopsis cuspidata* was the dominant species, accounting for ca. 70% of total basal area in Higashiyama, and ca. 30% in Aya. For fagaceous species, *Quercus gilva* Blume and *Quercus salicina* Blume f. *angustata* (Nakai) H. Ohba occurred to a lesser extent in Aya, whereas no fagaceuous species were found in Higashiyama.

Collecting acorns of *C. cuspidata* using seed traps at stand level

In each study plot, we set seed traps (0.5 m^2 surface area with a circular mouth) made of polyethylene cloth (mesh size, 1 mm) in the center of a 10 × 10 m quadrat (8 traps in Higashiyama, and 16 traps in Aya). We collected the contents of the traps once a month, sorted and counted female reproductive organs of fagaceous species. Among them, in this study, we focused on *C. cuspidata* acorns, whose cupules and pericarps started to develop, and dealt with the acorns, which fell into the seed traps from mid-April 2016 to early April 2018 in Higashiyama and Aya.

We first classified *C. cuspidata* acorns into immature-sized (ca. <9 mm in width and ca. <11 mm in length) and mature-sized (ca. ≥9 mm in width and ca. ≥11 mm in length) based on their shape and size. Then, we dissected all acorns and carefully observed whether there were frasses, feeding traces, penetration and oviposition scars or insects inside using a stereomicroscope (NSFNS; Nikon Corporation, Tokyo, Japan or S9D; Leica Microsystems, Wetzlar, Germany). We classified the acorns in accordance with Hirayama *et al.* (2017):

1 Sound acorns (attaining mature size with sound cotyledons).
2 Aborted acorns (failing to attain mature size and/or with undeveloped cotyledons without any damage).
3 Broken acorns, caused mainly by vertebrate predation.
4 Acorns suffering sap suction by adult weevils.
5 Acorns damaged by lepidopteran larvae.
6 Acorns damaged by larvae of *Kobuzo rectirostris* (Roelofs).
7 Acorns damaged by larvae of *Curculio* weevils, which were either *C. hilgendorfi* or *C. sikkimensis*.

In addition, for a lot of immature-sized *C. cuspidata* acorns in the study sites, we found insect damage that had not been observed in the previous study (Hirayama *et al.* 2017). We classified those as follows:

8 Acorns suffering type A damage: with clogged small frasses and sometimes with a large oval hole (ca. 3 mm in length and 1.5 mm in width) on the lower side of the acorn, or with larva within the acorn without visible entry holes (Fig. S2A).
9 Acorns suffering type B damage: with small light brown frasses and black meconium, and sometimes with chalcid wasps without visible entry holes, or with a small circular exit hole (ca. 0.6 mm in diameter) on the acorn (Fig. S2B).

Rearing and identification of insect adults causing type A acorn damage

For acorns with type A damage, we found no living organisms within the acorns, each of which had an oval hole. However, in the seed traps set in Higashiyama and Aya, we found a number of oval cases, each of which had been cut out from a *C. cuspidata* cupule and had an attached single cocoon covered in frasses (Fig. S3A,B). Within the oval case, there was one living organism that appeared to be a lepidopteran prepupa (Fig. S3C,D). In 2019 and 2020, we periodically gathered the oval cases from seed traps set in and around the study plots in Higashiyama and Aya in order to rear the prepupae. We reared them to adult emergence within plastic Petri dishes (9 cm diameter, 2 cm height) containing moistened wiping paper in a non-air-conditioned room, where the temperature change was similar to field conditions in Higashiyama. The emerged adults were considered as *Heliozela* sp. (Heliozelide), and identified based on morphological observations using the following publications: Lee *et al.* (2006) and Lee and Hirowatari (2013). We recorded the emergence dates of the adults, and the emerged adults were stored as dried specimens. Several adults were used for sequencing analyses, described below.

Rearing and identification of insect adults causing type B acorn damage

As for acorns of *C. cuspidata* with type B damage, chalcid wasps were sometimes found within the acorns (ca. 5–10%), and they were considered as *Eurytoma* sp. (Eurytomidae). We identified them based on

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morphological observation using Burks (1971). We stored the wasps in plastic tubes with 99.5% ethanol, and samples from both study sites, Higashiyama and Aya, were used in sequencing analyses, described below.

To confirm whether the wasps would really emerge from *C. cuspidata* acorns, we sampled a number of immature acorns from tree crowns in Higashiyama on 18 September 2018 and kept them in the non-air-conditioned room. We recorded the emergence dates of the adult wasps and identified them.

Seasonal sampling of acorns from the crown

To observe the acorn utilization patterns of the insects, we sampled twigs with acorns seasonally, especially during the earlier stages of acorn development in the crown, from 2018 to 2019 in Higashiyama, and from 2019 to 2021 in Aya (Table S2). We sampled them using a sickle attached to a 15 m carbon pole in Higashiyama and a pruning hook deployed from the canopy trail in Aya. Because the number of acorns in the crowns fluctuated greatly between years and sampling acorns from the crown was difficult, we were unable to sample them regularly. However, we sampled the acorns intermittently to observe the seasonal utilization by insects. We dissected the sampled acorns and attempted to identify the living organisms and their developmental stage (eggs, larvae or pupae of insects) and their feeding traces within the acorns using a stereomicroscope (S9D; Leica Microsystems). We first classified the organisms based on their morphology and behavior within the acorns. When a larva was assumed to be an insect, a *Heliozela* sp., we measured its head capsule width to determine its instar (Fig. S4). Our classification system for organisms found in acorns was as follows:

(a) Eggs of the insect considered as *Heliozela* sp. (Fig. 1B,C).
(b) First instar larvae of the insect considered as *Heliozela* sp. (Fig. 1D).
(c) Second instar larvae of the insect considered as *Heliozela* sp. (Fig. 1E).
(d) Third instar larvae of the insect considered as *Heliozela* sp.
(e) Fourth (final) instar larvae of the insect considered as *Heliozela* sp. (Fig. 1F,G).
(f) Larvae of the insect considered as *Eurytoma* sp. (Fig. 1I,J).
(g) Pupae of the insect considered as *Eurytoma* sp. (Fig. 1K).
(h) Other than the insect considered as *Heliozela* sp. or *Eurytoma* sp.

All organisms were photographed and stored in plastic tubes containing 99.5% ethanol. We also identified scars from dropping oval cases, which were assumed to be cut by *Heliozela* sp., in the sampled acorns (Fig. 1H).

Sequencing analyses for insects causing type A and B damage

For the insects considered as *Heliozela* sp. or *Eurytoma* sp., we confirmed the species identification of immature organisms using sequence similarity of the mitochondrial gene cytochrome *c* oxidase subunit I (*COI*) to sequences from the adult samples. For this analysis, we extracted total DNA from the abovementioned adults of each species and from one to several samples of each of the immature stages (a) through (g) using the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol. We first amplified 701 bp fragments of *COI* using the primer set COI_pF2 (Simon *et al.* 1994) and COI_2437d (Kaartinen *et al.* 2010) for both species. The reaction cycle was 94°C for 2 min followed by four cycles of 94°C for 30 s, 45°C for 1 min and 72°C for 1 min, followed by 34 cycles of 94°C for 30 s, 50°C for 1 min and 72°C for 1 min, and then 72°C for 5 min. For the insect considered as *Heliozela* sp., although we could not amplify the 658 bp *COI* barcoding region using the primer set Lep-F1 (Hebert *et al.* 2004) and Lep-R1 (Hebert *et al.* 2004), we could amplify 388 bp fragments of the *COI* barcoding region using Lep-F1 (Hebert *et al.* 2004) and the newly designed primer COIBar_362-382R (5′-GATCTACAGATGATCCTGC ATGAGC-3′). By combining the 701 bp fragments amplified using COI_pF2 + COI_2437d and the 388 bp fragments amplified using Lep-F1 + COIBar_362-382R, we obtained in total an 893 bp partial *COI* sequence including the 658 bp *COI* barcoding region for the insect considered as *Heliozela* sp. (Fig. S5). The reaction cycle for Lep-F1 + COIBar_362-382R was 94°C for 3 min followed by 40 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 45 s, followed by 72°C for 5 min. Purification and sequencing were performed as described by Nakabayashi and Ohshima (2024). The obtained sequences were deposited in the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp) under the accession numbers listed in Tables S3 and S4. Based on the Basic Local Alignment Search Tool (BLAST; Camacho *et al.* 2009), we did not find sequences that showed more than 90% identity to the sequences of the insect considered as *Heliozela* sp. For the insect considered as

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Eurytoma sp., we found a sequence that showed 91% identity to their sequences, which was *Eurytoma bigeloviae* Ashmead.

For the insect considered as *Heliozela* sp., the obtained 893 bp partial *COI* sequences were used for calculating uncorrected pairwise genetic distances among the sequences of all intraspecific samples including adults and immatures using MEGA X (Kumar *et al.* 2018). In addition, the 658 bp *COI* barcoding region was used for genetic distance and phylogenetic analyses for the insect considered as *Heliozela* sp. In the analyses, sequences of *Heliozela castaneella* Kuroko (Genbank accession number: MF118310.1, available at http://ncbi.nlm.nih.gov/genbank) and *Heliozela sericiella* Haworth (Genbank accession number: HM875024.1, available at http://ncbi.nlm.nih.gov/genbank), both of which are leaf miners of fagaceous species, were used for sequence comparison and as outgroups for the maximum likelihood (ML) analysis of the phylogenetic tree by MEGA X (Kumar *et al.* 2018).

For the insect considered as *Eurytoma* sp., the obtained 701 bp partial *COI* sequences were used for calculating intraspecific uncorrected pairwise genetic distances. In addition, *E. bigeloviae* (Genbank accession number: OR336224.1, available at http://ncbi.nlm.nih.gov/genbank), *Eurytoma amygdali* Enderlein (Genbank accession number: EU017316.1, available at http://ncbi.nlm.nih.gov/genbank) and *Eurytoma samsonowi* Vassiliev (Genbank accession number: MZ571204.1, available at http://ncbi.nlm.nih.gov/genbank), all of which are seed-feeding wasps (Plaut & Mansour 1973; Wannassi *et al.* 2023; Smithsonian Institution 2024), were used for genetic distance and phylogenetic analyses as outgroup species. We used the overlapping portion of the available sequence for each of these *Eurytoma* species in the analyses (655 bp for *E. bigeloviae*, 274 bp for *E. amygdali* and 470 bp for *E. samsonowi*).

The Hasegawa–Kishino–Yano model with gamma distribution (HKY+G) was selected for the insect considered as *Heliozela* sp. and the Tamura 3-parameter model (T92) was selected for the insect considered as *Eurytoma* sp. based on the Bayesian information criterion for the datasets. The ML trees were searched with Sub-tree Pruning & Re-grafting (SPR) level-5 branch swapping using a neighbor-joining tree as the starting point. A nonparametric bootstrap test (Felsenstein 1985) was performed using 1,000 replicates to assess the confidence level for each cluster.

Statistical analyses

To determine the difference in the proportion of acorns damaged by insects considered as *Heliozela* sp. or *Eurytoma* sp. among sites and years, we conducted a Tukey's multiple comparison test following a generalized linear mixed model (GLMM) analysis with a log link and negative binomial error using seed trap data at the stand level. In the GLMM, the number of acorns damaged by the insects considered as *Heliozela* sp. or *Eurytoma* sp. in each trap and year (mid-April to next early April) was designated as the response variable, whereas annual total number of falling acorns, excluding the number of broken acorns, in each trap and year (mid-April to next early April) was the offset variable. The explanatory variables were the study site and year (Higashiyama 2016/17, Higashiyama 2017/18, Aya 2016/17 and Aya 2017/18) and each seed trap was treated as a random factor. The GLMMs were fitted using the lme4 package of R 4.2 (R Core Team 2022), and multiple comparison tests were carried out using the mulcomp package of R 4.2 (R Core Team 2022).

RESULTS

Adult emergence of insects causing type A and B damage to *C. cuspidata* acorns and their species identification

Oval cases of insects causing type A damage were found within the seed traps from July to October in both Higashiyama and Aya (Fig. S6A–D), although the number of oval cases collected from Aya was low until August in both 2019 and 2020 (Fig. S6C,D). Adult emergence from oval cases collected after mid-September from both Higashiyama and Aya occurred mainly in June of the following year (Fig. S7B–D). In the oval cases collected from Higashiyama on 27 July, 4 August and 3 September in 2019, adults emerged from mid-August to mid-September of the same year (Fig. S7A). All of the emerged adults were previously undescribed as *Heliozela* sp., based on morphological observations by Lee *et al.* (2006) and Lee and Hirowatari (2013) (Fig. 2). Similarly, all of the chalcid wasps found within *C. cuspidata* acorns with type B damage were previously undescribed as *Eurytoma* sp., based on morphological observations by Burks (1971) (Fig. 3). From the sampled acorns collected on 18 September 2018, the same species of wasps emerged from late September to early October of the same year (Fig. S8).

Sequencing analyses of sampled insects considered as *Heliozela* sp. and *Eurytoma* sp.

Among the adults and immature samples considered as *Heliozela* sp., the maximum pairwise genetic distances were 1.12% based on the sequences of the 893 bp partial *COI* fragment, and 1.22% based on the sequences

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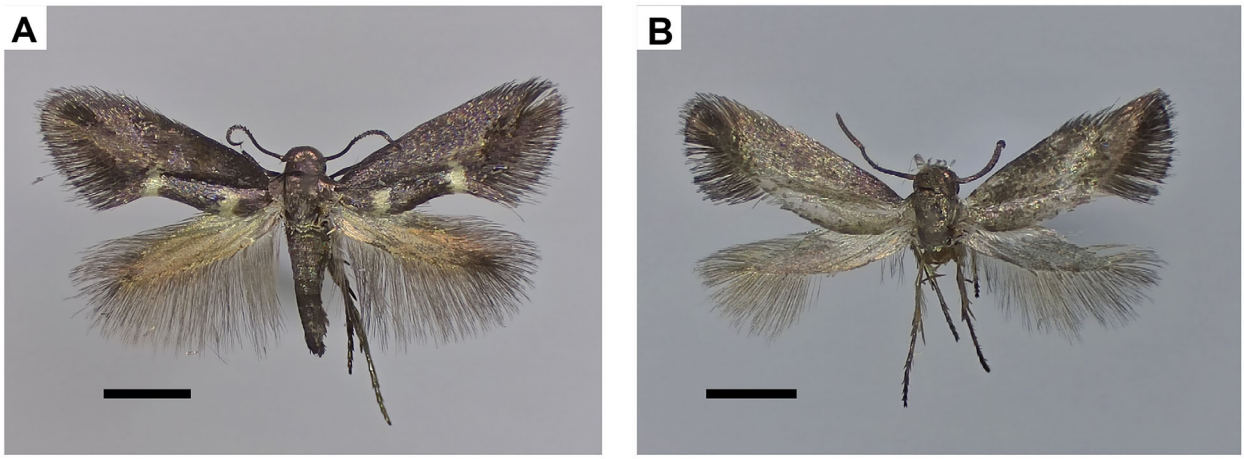


Figure 2 Emerged adults of our *Heliozela* sp. (A) Whole image. (B) Specimen used for the sequencing analysis (KT126). Scale bars: 1 mm.

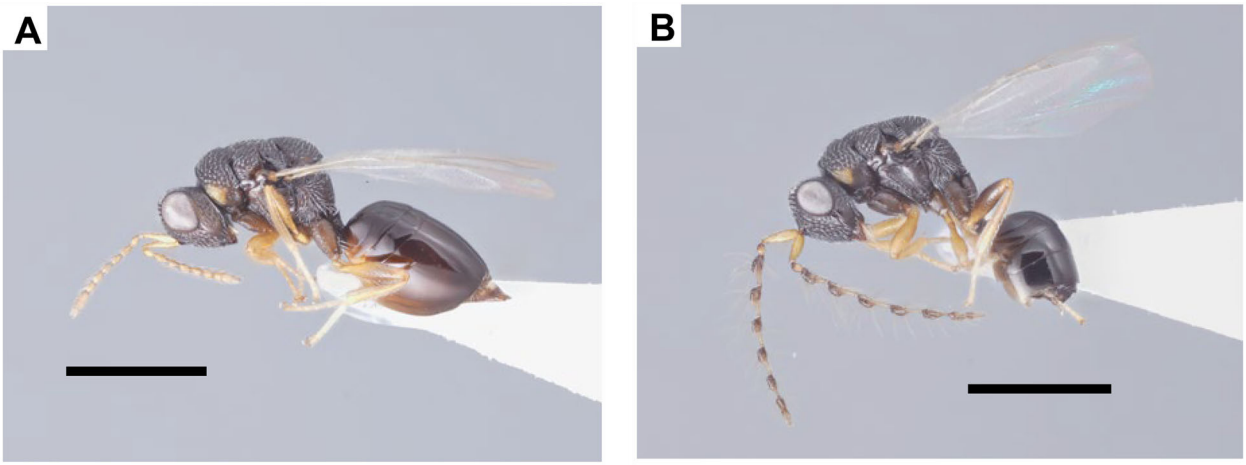


Figure 3 Emerged adults of our *Eurytoma* sp. (A) Adult female. (B) Adult male. Scale bars: 1 mm.

of the 658 bp *COI* barcoding region, indicating that the samples belonged to the same single species. Based on sequences of the 658 bp *COI* barcoding region, the minimum pairwise genetic distances were 13.1% between our sampled *Heliozela* sp. and *H. castaneella*, and 18.4% between our sampled *Heliozela* sp. and *H. sericiella*, and the sequences of our samples comprised a monophyletic group (bootstrap value = 100%) in the inferred ML tree (Fig. S9). This indicated that our sampled *Heliozela* sp. was clearly a different species than *H. castaneella* and *H. sericiella*. There was no clear geographic or seasonal structure in *Heliozela* sp. in the ML tree.

Among the adult and immature samples considered as *Eurytoma* sp., maximum pairwise genetic distances were 0.29% based on sequences of the 701 bp partial *COI* fragment, indicating that the samples belonged to the same single species. Based on the sequences of the common parts of *COI* fragments, the minimum pairwise distances were 9.3% between our sampled *Eurytoma* sp. and *E. bigeloviae* (655 bp), 12.0% between our sampled *Eurytoma* sp. and *E. amygdali* (274 bp), and 13.2% between our sampled *Eurytoma* sp. and *E. samsonowi* (470 bp). These distances indicated that our sampled *Eurytoma* sp. was clearly a different species from the three seed-feeding *Eurytoma* wasps. There was no geographic structure in *Eurytoma* sp. in the ML trees (Fig. S10).

Seasonal sampling of acorns from the crown

Acorns with insects identified as *Heliozela* sp. were found dominantly soon after acorns began to develop, from the first half of July until early September in both

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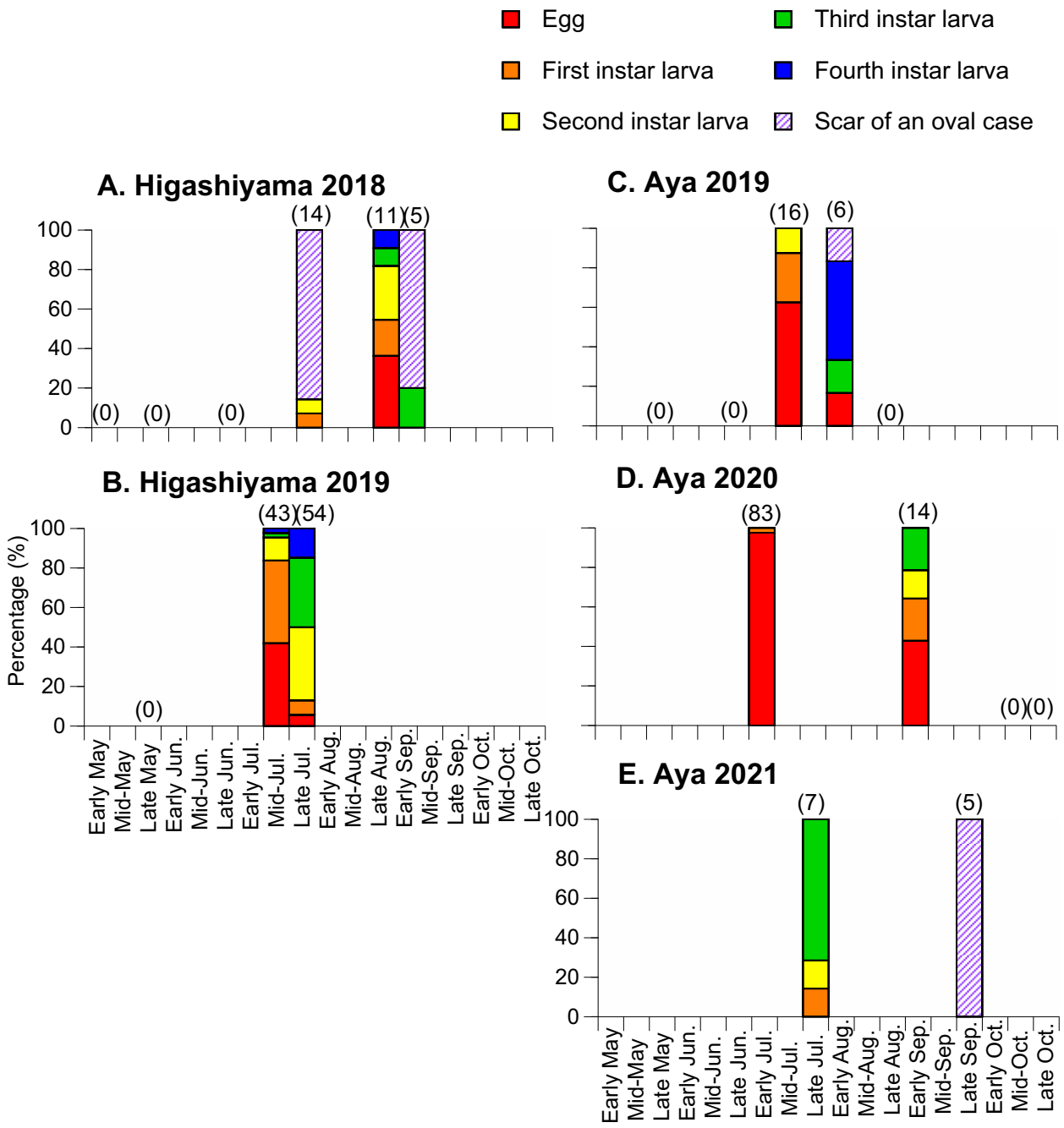


Figure 4 Seasonal changes in composition of developmental stages of *Heliozela* sp. within the sampled acorns of *Castanopsis cuspidata* from the crowns for each sampling site and year. Numbers in parentheses indicate the total number of *Heliozela* sp. observed in each duration.

Higashiyama and Aya (Fig. S11). Eggs and first instar larvae were abundant in the first half of July in Higashiyama 2019 and Aya 2020 (Fig. 4B,D), and also found in early August in Aya 2019 (Fig. 4C), in late August in Higashiyama 2018 (Fig. 4A) and in early September in Aya 2020 (Fig. 4D). Within each acorn, we found one egg or larva of *Heliozela* sp., except in one case. The larvae ate the green immature acorn pericarps (Fig. 1E). A fourth (final) instar larva formed a cocoon and cut out the oval piece of the cupule attached to the cocoon (Fig. 1F,H). Acorns with scars of dropping oval cases were found in late July and

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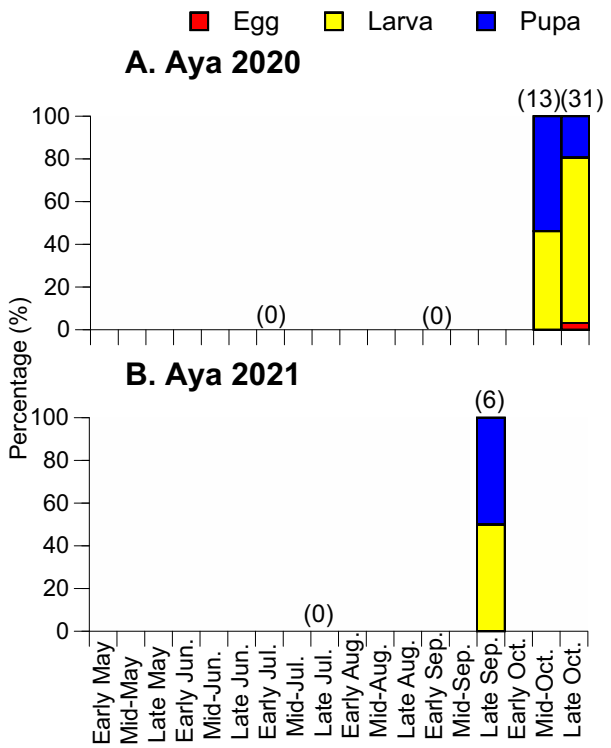


Figure 5 Seasonal changes in composition of developmental stages of *Eurytoma* sp. within the sampled acorns of *Castanopsis cuspidata* from the crowns for each sampling site and year. Numbers in parentheses indicate the total number of *Eurytoma* sp. observed in each duration.

early September in Higashiyama 2018 (Fig. 4A), in early August in Aya 2019 (Fig. 4C) and in late September in Aya 2021 (Fig. 4E).

We began to find acorns with insects identified as *Eurytoma* sp. from late September in Aya 2021 (Fig. S11E). In October, we mainly found acorns with *Curculio* weevils or *Eurytoma* sp. among the insect-damaged acorns in Aya 2020 (Fig. S11D). Although the larvae of *Curculio* weevils were feeding on cotyledons, the larvae and pupae of *Eurytoma* sp. were found within their feeding holes, with their frasses in the pericarps of the acorns (Figs 1I,5). Within each acorn, we found one to five larvae and pupae of *the Eurytoma* sp. We also found one egg of the *Eurytoma* sp. within the acorn during late October (Fig. 5A).

Collecting acorns using seed traps at the stand level

In seed traps, both acorns damaged by *Heliozela* sp. and acorns damaged by *Eurytoma* sp. were found from July, with a peak from August to September, irrespective of the sites and years (Fig. S12). Although the total number of acorns differed between years and sites, the acorns damaged by *Heliozela* sp. were dominant in immature-sized damaged acorns and the acorns damaged by *Curculio* weevils were dominant in mature-sized damaged acorns (Fig. 6). Among the immature-sized damaged acorns, those damaged by *Eurytoma* sp. showed the second highest number, except in Aya during 2017/18.

Tukey's multiple comparison test showed that the proportion of acorns damaged by *Heliozela* sp. to the total number of acorns was significantly higher in 2016/17 than in 2017/18 both in Higashiyama and Aya (Fig. 7A), following the GLMM (Table S5). The proportion of acorns damaged by *Eurytoma* sp. to the total number of acorns was also significantly higher in 2016/17 than in 2017/18 in Aya, but not in Higashiyama in Tukey's multiple comparison test (Fig. 7B), following the GLMM (Table S6). In 2016/17, the sum of the proportion of acorns damaged by *Heliozela* sp. and *Eurytoma* sp. amounted to approximately 0.45 in both Higashiyama and Aya. In 2017/18, when the number of acorns produced increased in both Higashiyama and Aya, the proportion of acorns damaged by these insects decreased dramatically, especially in Aya (Fig. 7).

DISCUSSION

The acorns of *C. cuspidata* were intensively damaged by insects even before the cotyledon development. The insect fauna that damaged the acorns clearly differed between the immature-sized and mature-sized stages. During 2 years of observations, acorns damaged by *Curculio* weevils were predominant among mature-sized acorns; acorns damaged by *Heliozela* sp. were dominant, followed by those damaged by *Eurytoma* sp. in immature-sized acorns. Both *Heliozela* sp. and *Eurytoma* sp. are undescribed species, and neither they nor their relatives have been found to be seed predators of other fagaceous species in Japan (e.g. Igarashi & Kamata 1997; Fukumoto & Kajimura 1999; Ueda 2002; Hirayama *et al.* 2017). In addition, we did not find these insects within the acorns of other fagaceous species that coexisted at the study sites (K. Hirayama, unpubl. data, 2014–2022).

Species belonging to the genus *Heliozela* are usually known as leaf miners, which cut distinctive oval- or shield-shaped cases from the leaf surface and descend to the ground to pupate (Mutanen *et al.* 2007; Milla *et al.* 2018). *Heliozela sericiella* and *H. castaneella* are known to mine fagaceous leaves (Emmet 1976; Kuroko 1982). However, genetic analyses revealed that

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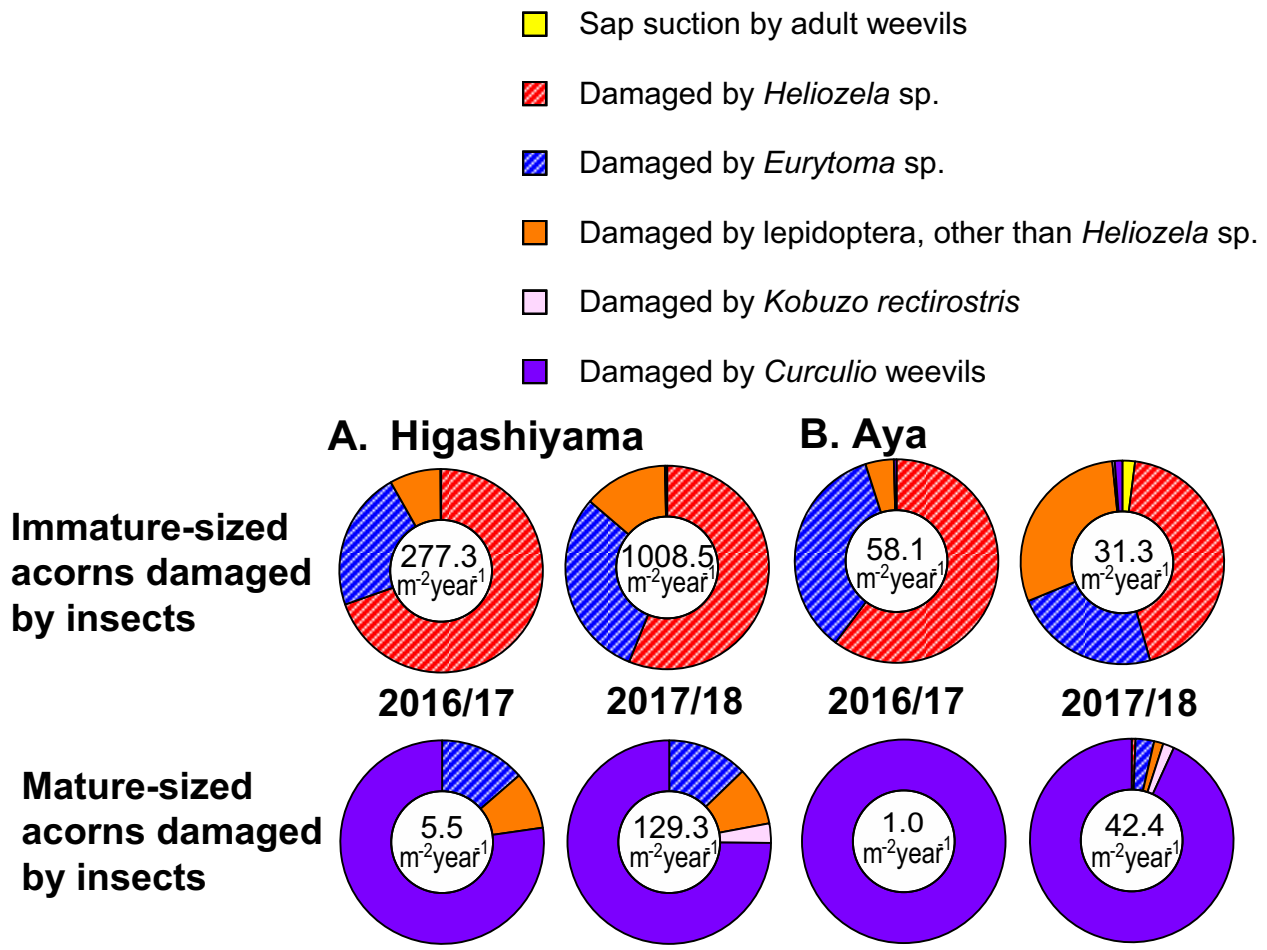


Figure 6 Percentage of insect damage in immature-sized and mature-sized *Castanopsis cuspidata* acorns within the seed traps during 1 year.

the sequences from our *Heliozela* sp. samples were clearly distinguishable from those of *H. sericiella* and *H. castaneella*. For our *Heliozela* sp., which ate the green pericarps of *C. cuspidata* acorns, eggs within cupules and first instar larvae feeding on acorn pericarps began to be found in the first half of July, when acorn cupules and pericarps began to develop. Later, we found the fourth (final) instar larvae and/or scars dropping oval cases on the acorns from the second half of July. Additionally, eggs and first instar larvae were found in August and early September. When we kept oval cases that dropped into the seed traps from late July to early September, adults emerged from mid-August to mid-September of the same year; the adult emergence occurred in June next year from oval cases dropped from late September. These observations suggest that *Heliozela* sp. has a bivoltine or partially bivoltine life cycle. Such repeated acorn utilization by *Heliozela* sp. could be linked to their dominance in insect damaged acorns during the early stages of development.

Eurytoma is the largest genus of Eurytomidae, constituting approximately 700 species, and is known for its diverse natural history (Noyes 2019). Most *Eurytoma* species are parasitoids or hyperparasitoids of insect larvae, whereas others are phytophagous on the seeds or fleshy fruits of host plants (Park & Lee 2021). Based on our genetic analyses, samples from *Eurytoma* sp., which ate the green pericarps of *C. cuspidata* acorns, (similar to that of *Heliozela* sp.), were clearly distinguishable from published sequences of other seed-feeding *Eurytoma* species, such as *E. amygdali*, *E. samsonowi* and *E. bigeloiviae*.

While we found live larvae and pupae of *Eurytoma* sp. within *C. cuspidata* sampled acorns from the crown from late September, acorns damaged by *Eurytoma* sp. were collected from July in seed traps, similar to those damaged by *Heliozela* sp. in both Higashiyama

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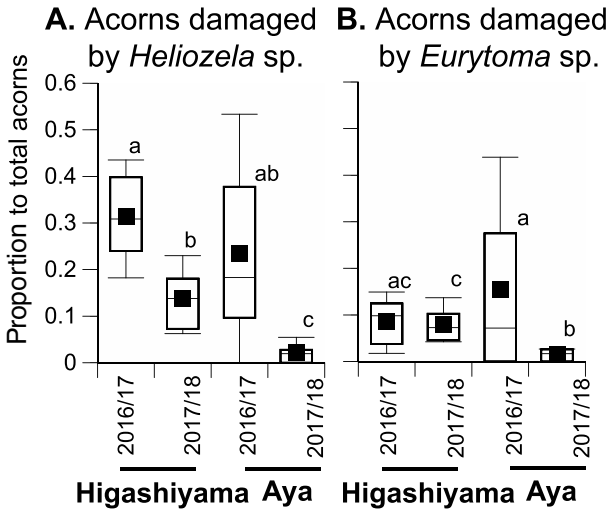


Figure 7 Boxplots of the proportion of the acorns damaged by (A) *Heliozela* sp. or (B) *Eurytoma* sp. to the total number of acorns within the seed traps in each site and year. Different letters above the boxes indicate significant differences in the proportion among sites and years within each species, based on the multiple comparison test ($P < 0.05$).

and Aya. In the seed traps at both sites, dead adults of *Eurytoma* sp. were found within acorns of various sizes from August to November (Y. Fukuhama, F. Hosobuchi, I. Tanaka, unpubl. data, 2016–2017). When we kept *C. cuspidata* acorns that were sampled from the crown in mid-September, the emergence of *Eurytoma* sp. adults occurred between late September and early October of the same year. In addition, an egg and a number of live larvae were found within acorns sampled from the crown in October. These observations suggest that our *Eurytoma* sp. have multivoltine life cycles. During winter, we found no live larvae, pupae or adults of *Eurytoma* sp. within the acorns falling into seed traps (F. Hosobuchi, S. Okubo, unpubl. data, 2017–2020). In our rearing conditions, where the temperature had a range similar to that of the field conditions, some adults emerged in late September and lived until the following April (S. Ohkubo, unpubl. data, 2018–2019). This implies that *Eurytoma* sp. would overwinter as adults outside the acorns, which is similar to the multivoltine seed-feeding wasp *Macrodasyceras japonicum* (Ashmead), associated with temperate *Ilex* species (Nishimon *et al.* 2024). Because we sometimes found multiple larvae and pupae within one acorn, the population size of *Eurytoma* sp. could have increased rapidly through multivoltinism, even if it became smaller due to overwintering.

In both Higashiyama and Aya, the total number of acorns damaged by *Heliozela* sp. and *Eurytoma* sp. amounted to approximately half of the total number of acorns produced in 2016/17, when initial acorn production was considerably less. In 2017/18, at both sites, the number of produced acorns increased, the proportion of acorns damaged by these insects drastically decreased and the number of sound acorns increased compared to 2016/17. The predator satiation hypothesis states that variable seed production would have an advantage for the loss of seeds to predators; years of high seed production should satiate predators, whereas predators should starve in low-seed years (Silvertown 1980; Bogdziewicz *et al.* 2021). High acorn production in 2017/18 seemed to satiate acorn predation by *Heliozela* sp. and *Eurytoma* sp., which would lead to increased sound acorn production.

In fagaceous species, *Curculio* weevils, whose larvae fed on developing acorn cotyledons, are considered to be an important seed-feeding insect and are thought to contribute to variable acorn production processes, such as masting (Maeto & Ozaki 2003; Bogdziewicz *et al.* 2021). At both the study sites, acorns damaged by *Curculio* weevils were dominant at maturity. Cotyledons of *C. cuspidata* acorns began to develop late in the growing season, and acorns were covered by cupules until they were mature. This has been considered a defensive mechanism against seed predation by *Curculio* weevils (Fukumoto & Kajimura 1999; Yang *et al.* 2023). However, the present results clearly demonstrate that *Heliozela* sp. and *Eurytoma* sp. are specialized to feed on the pericarp of *C. cuspidata* acorns prior to cotyledon development. During acorn growth, the insects that feed at earlier stages of acorn development would have an advantage for acorn utilization over those feeding at later stages (Fukumoto & Kajimura 2001; Hirayama *et al.* 2017). Moreover, *Heliozela* sp. and *Eurytoma* sp. showed multivoltinism, suggesting that these two species have a larger impact on acorn production than univoltine *Curculio* weevils. This study suggests that a variety of seed-feeding insects have evolved to adjust their seed growth patterns, which can significantly affect seed production patterns.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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

Table S1. Tree species composition of each of the study sites, Higashiyama and Aya.
Table S2. Number of observed acorns, which were collected from the crown of *Castanopsis cuspidata* in each site and year.
Table S3. Detailed lists of *Heliozela* sp. samples used for genetic analyses.
Table S4. Detailed lists of *Eurytoma* sp. samples used for genetic analyses.
Table S5. Results of the generalized linear mixed model testing the difference in the proportion of acorns damaged by insects considered as *Heliozela* sp. among sites and years.
Table S6. Results of the generalized linear mixed model testing the difference in the proportion of acorns damaged by insects considered as *Eurytoma* sp. among sites and years.
Figure S1. Seasonal changes in the length of female reproductive organs (flowers, acorns enclosed by cupules or mature acorns dropping off cupules in mid-October) for *Castanopsis cuspidata* in Higashiyama, 2014. The female reproductive organs on current-year and on 1-year-old shoots are shown. Mean ± SE of 5–30 fruits from three reproductive trees, whose diameter at breast height were 63.2, 47.0 and 38.5 cm, are indicated.
Figure S2. Type A and B damage to acorns. (A) Type A damage: with clogged small frasses and sometimes with a large oval hole (ca. 3 mm in length and 1.5 mm in width) on the lower side of the acorn, or with a larva inside the acorn without visible entry holes. (B) Type B damage: with small, light brown frasses and black meconium, and sometimes with chalcid wasps without visible entry holes, or with a small circular exit hole (ca. 0.6 mm in diameter) on the acorn.
Figure S3. Oval case created by cutting the cupule of *Castanopsis cuspidata*. (A) Oval case: cut from one side of the cupule of *C. cuspidata*. (B) Oval case: cocoon is covered by frasses on the other side. (C) Prepupa within an oval case. (D) Ventral view of a prepupa.
Figure S4. Frequency distribution of head capsule width of living larvae for insects considered as *Heliozela* sp. within the acorns sampled from the crown. Based on the distribution, larvae are classified into four instars.
Figure S5. Diagram of amplified sequences using primers, the 893 bp obtained sequence and the 658 bp *COI* barcoding region for *Heliozela* sp.
Figure S6. Seasonal changes in the number of oval cases collected from the seed traps in (A,B) Higashiyama and (C,D) Aya.
Figure S7. Seasonal changes in number of emerged adults from oval cases collected from seed traps in each study site. They were reared in a non-air-conditioned room.
Figure S8. Seasonal changes in number of emerged adults from immature acorns collected from the crown of *Castanopsis cuspidata* on 18 Sep 2018. Acorns were reared in a non-air-conditioned room.
Figure S9. Phylogenetic tree estimated using maximum-likelihood (ML) analysis for *Heliozela* sp. for the emerged adults and the organisms at each life stage within the acorns of *Castanopsis cuspidata*. Prepupae were derived from oval cases. DNA extraction number, sampling locality and the life stage are indicated. The tree was constructed with the sequences of *Heliozela castaneella* (Genbank accession no. MF118310.1) and *Heliozela sericiella* (Genbank accession no. HM875 024.1), based on 658 bp, which was the mitochondrial *COI* barcoding region. Bootstrap values are indicated on the nodes.
Figure S10. Phylogenetic trees estimated using maximum likelihood (ML) analyses for *Eurytoma* sp. for the identified adults, and pupae and larvae within the acorns of *Castanopsis cuspidata*. The trees constructed with (A) the sequences of *Eurytoma bigeloviae* (Genbank accession no. OR3362224.1) based on 655 bp, (B) the sequences of *Eurytoma amygdali* (Genbank accession no. EU017316.1) based on 274 bp, and (C) the sequences of *Eurytoma samsonowi* (Genbank accession no. MZ571204.1) based on 470 bp. In each sample, DNA extraction number, sampling locality and the life stage are indicated. Bootstrap values are indicated on the nodes.
Figure S11. Seasonal changes in composition of insects within the sampled acorns of *Castanopsis cuspidata* in the crown for each sampling site and year. Numbers in parentheses indicate the total number of acorns damaged by insects during the observation.

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Figure S12. Seasonal falling patterns of each type of the *Castanopsis cuspidata* acorns within seed traps in each study site in each year. Broken acorns, which had no relationship with the acorns damaged by the insects, amounted to 1.5 m^{-2} $year^{-1}$ in Higashiyama 2016/17, 1.1 m^{-2} $year^{-1}$ in Aya 2016/17, 13.5 m^{-2} $year^{-1}$ in Higashiyama 2017/18 and 13.6 m^{-2} $year^{-1}$ in Aya 2017/18.

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