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Discovery of cryptic diversity in phytophagous gall midges (Diptera: Cecidomyiidae) associated with different ecotypes of the perennial herb *Cimicifuga simplex*

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ABSTRACT

Studying the diversification patterns of species-rich phytophagous insect taxa can help us understand the factors that cause species diversification. We conducted a molecular phylogenetic analysis of the mitochondrial *COI* gene of larvae of gall midges (Diptera: Cecidomyiidae) using three genetically differentiated morphs of *Cimicifuga simplex* plants and found that the gall midges could be divided into five major clades. Gall midges collected from morph I of *C. simplex* belonged to four *Schizomyia* clades. Gall midges collected from morph II of *C. simplex* belonged to one of the four *Schizomyia* clades collected from morph I. Gall midges collected from morph III belonged one *Contarinia* clade. On morphs I and II of *C. simplex*, the *Schizomyia* species induced galls on the flower bud, whereas on morph III of *C. simplex*, the *Contarinia* species was collected from normal fruits (not gall inducer); thus, morph III plants were used differently by gall midges than plants of morphs I and II. These results indicate that the cryptic diversity of these phytophagous insects correspond to that of plant ecotypes, and suggests that the diversification of the host plant contributed to parallel diversification of the phytophagous gall midges.

Introduction

Phytophagous insects often specialize to their host plants, and they are thought to have diversified through ecological speciation as a result of that specialization (Weiblen, 2002; Kawakita et al., 2004). It is widely accepted that, throughout their evolutionary history, the switching of host plants by phytophagous insects has contributed to their diversification, and this idea is well-supported by molecular and phylogenetic evidence (Funk et al., 1995a; Peccoud et al., 2009; Yamamoto et al., 2020). Recent advances in genetic analyses have revealed that plants have genetically and ecologically different ecotypes that are ecologically adapted to various environments (e.g. Mitsui et al., 2011; Hirao et al., 2019; Sakaguchi et al., 2019; Wang et al., 2020). However, only a few studies have examined whether phytophagous insects similarly differentiated when plants speciated or differentiated into ecotypes (e.g. Becerra and Venable, 1999; Jousselin and Elias, 2019). In this study, we examined whether diversification of phytophagous insects occurred as a result of the intraspecific diversification of plants into ecotypes.

In this study, we treated gall midges (Diptera: Cecidomyiidae) belonging to *Contarinia* and *Schizomyia*. Many species of gall inducers and a taxonomic group that has diversified in close association with its host plants (Karban and Agrawal, 2002; Harris et al., 2003). Many gall midges have a characteristic ability form or induce galls for their own reproduction in various plant organs (Skuhravá, 1986; Yukawa and Rohfritsch, 2005). Most gall midges are specific to their host plants on the species or genus level (e.g. Gagné, 1994; Yukawa & Masuda 1996; Tokuda et al., 2008). This strong host-plant dependence means that gall midges are a good model for clarifying links between plant ecotypes and the diversification of phytophagous insects.

We focused on undescribed species of gall midges collected from the perennial herb *Cimicifuga simplex*. Within *C. simplex*, there are at least three genetically and ecologically distinct ecotypes (Pellmyr, 1986; Kuzume and Itino, 2013; Toji and Itino, 2020). On this plant, we collected gall midges induced in the plant buds (*Schizomyia* species) and gall midges within the fruit (*Contarinia* species, not galler). This suggests that several species of gall midge may utilize *C. simplex*. To investigate the diversification of phytophagous insects that use this ecotypically diversified host plant, we conducted a molecular phylogenetic analysis.

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Materials and methods

Host plant Cimicifuga simplex

Cimicifuga simplex (Ranunculaceae) is a perennial herb distributed in eastern and northeastern Asia (Fig. 1A; Emura, 1970; Nakai, 1916). It is a common species in the mountains of central Japan. They have at least three genetically and ecologically distinct morphs (Pellmyr, 1986; Kuzume and Itino, 2013; Toji et al., 2018). The DNA bases positions at 50, 418, and 565 in the ITS1-2 region are GTC, AGC, and GKC (K indicates heterozygous G and T bases) in Morph I, II, and III in that order. Morph I and III are relatively closely related in that they can be distinguished only by heterozygous differences in one position (Yamaji et al., 2005; Kuzume and Itino, 2013). Morph I grows at high altitudes in sunny location and blooms from late July to early September. Morph II grows in the sun along forest edges at middle altitudes and blooms strongly scented flowers from early September to early October. Morph III grows in shady forest floor locations in lowland areas and blooms from early October to early November.

Gall midges on C. Simplex

A gall is induced on the flower buds of *C. simplex* by an undescribed gall midge (*Schizomyia* species) (Fig. 1B, Yukawa and Masuda, 1996). Each induced gall contains multiple gall midge larvae, which apparently emerge from the gall when the *C. simplex* seeds mature, after which they burrow into the ground (Fig. 1C). In previous studies (Toji et al., 2020), we observed larvae that had emerged from galls on *C. simplex* inflorescences that had been covered with nylon mesh bags (Fig. 1C). Thus, the gall midges that use *C. simplex*, like other common Cecidomyiidae insects, probably overwinter in the ground as larvae (Gagné 1994). We also found gall midges growing inside the fruit in this study, which to our knowledge have not been described in the past literature (Fig. 1D).

Sampling

We sampled gall midges from *C. simplex* populations in Matsumoto, Nagano, Japan. Gall midges of morph I were collected from scattered groups of plants growing between 2050 and 2340 m a.s.l. on Mt. Norikura (Norikura 2050–2340 m; hereafter, populations are identified by

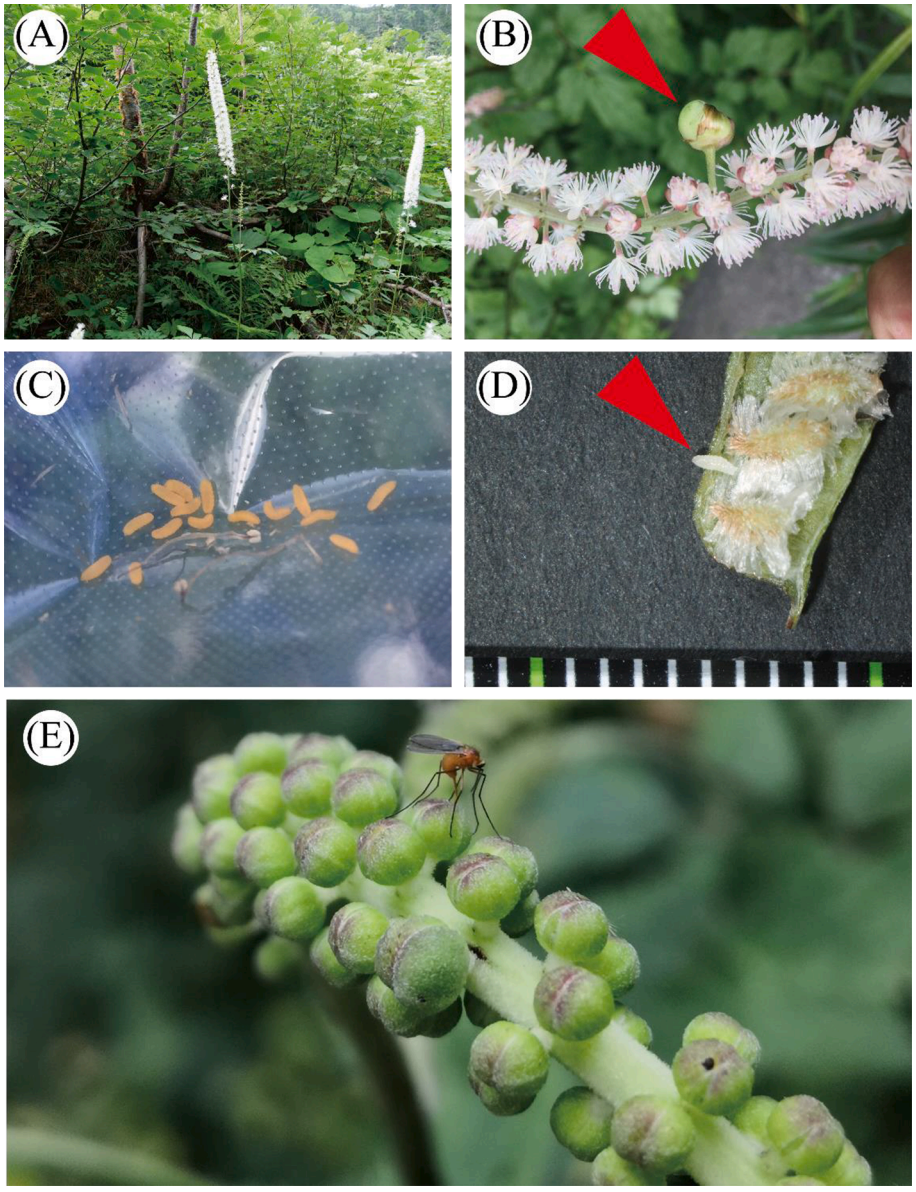

Fig. 1. (A) Inflorescences of morph I of *Cimicifuga simplex* (Norikura 2240 m population). (B) Enlarged view of a *C. simplex* morph I inflorescence. The red arrow indicates a gall induced by a gall midge of *Schizomyia* species. (C) Gall midge larvae (*Schizomyia* species) that had escaped from a gall were trapped in a nylon mesh bag covering the inflorescence. (D) A gall midge larva (*Contarinia* species, indicated by the red arrow) growing in a fruit of *C. simplex* morph III. Marks on the scale are 1 mm apart. (E) An adult gall midge (*Schizomyia* species) ovipositing on a flower bud of *C. simplex* morph II (Norikura 1470 m population). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

name and elevation). Gall midges of morph II were collected at Sanjiro 1250 m, Sakurashimizu 1300 m, Utsukushigahara 1350 m, and Norikura 1470 m. Gall midges of morph III were collected at Hora 700 m, Gakenoyu 920 m, Misuzuko 1000 m, and Utsukushigahara 1350 m (Fig. 2). To the best of our knowledge, each of the *C. simplex* morphs grows in relatively large numbers at the indicated sites.

Gall formation in the flower buds of *C. simplex* was intermittently observed at these study sites. This study was conducted over the flowering period of *C. simplex*, from early August to the end of October, in 2018. Then, from early September to the end of November 2019, *C. simplex* fruits were collected at each site to check for the presence of gall midge larvae inside them. One gall midge larva was collected from a gall per *C. simplex* plant and preserved in 70% ethanol.

Morphological examination of larvae

Larval specimens were mounted on slides in Canada balsam, following the technique outlined by Gagné (1994), and were observed under a bright-field, phase-contrast microscope (H550L, Nikon, Tokyo). Photomicrographs for stacking were taken with a digital camera (DP22, Olympus, Tokyo) attached to a semi-motorized fluorescence microscope (BX53, Olympus, Tokyo). The images were stacked by using image J software (NIH, Maryland, USA) with the Stack Focuser plugin. Terminology for larval morphology follows Gagné (1994). The slide-mounted specimens have been deposited in the collection of Laboratory of Systems Ecology, Faculty of Agriculture, Saga University, Japan.

Mitochondrial DNA-based phylogenetic analysis

DNA was extracted from the whole body of each specimen by using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocols. The mitochondrial *cytochrome oxidase I* (*COI*) gene was amplified by polymerase chain reaction (PCR) using Takara Ex Taq polymerase (Takara Bio, Shiga, Japan) and the primers COIS 5′-GGA TCA CCT GAT ATA GCA TTC CC-3′ and COIA 5′- CCC GGT AAA ATT AAA ATA TAA ACT TC-3′ (Funk et al., 1995b). This primer pair has been used previously for the effective detection of intraspecific variations in Cecidomyiidae (Tokuda et al., 2008; Yukawa et al., 2009). The PCR amplification was carried out for 30 cycles of 98 °C for 10 s, 50 °C for 30 s, and 72 °C for 60 s. After the amplification, the PCR product was purified by using an illustra ExoStar Clean-Up Kit (GE Healthcare, Chicago, IL, USA). Sequencing of both strands was performed with a BigDye Terminator v1.1 Cycle Sequencing Kit (ABI, Weiterstadt, Germany) on an ABI 3130 Genetic Analyzer.

We used the dataset of the 441 bp fragment of the mitochondrial *COI* gene to infer a phylogenetic tree by the maximum likelihood (ML) method with best fitted GTR + G model by MEGA7 software (Kumar et al., 2016). We conducted a BLAST search of the GenBank database to obtain sequences with high homology to our sequences for use as outgroups. We also added Japanese Cecidomyiidae species, *S. castanopsisae*, *S. achyranthesae*, *S. usubai* (Elsayed et al., 2018) and three *Dasineura* species (Yukawa et al., 2009; Tokuda et al., 2009) as ingroup or outgroup taxa. To determine clade support, bootstrap values were calculated 1000 times. In addition, the percentage of nucleotide substitutions between clades was calculated.

Results

Distribution and host-plant use by the gall midges

We were able to obtain gall midge larvae from all three morphs of *C. simplex*. Larval gall midge specimens were obtained from morph I at Norikura 2050–2340 m, from morph II at Norikura 1470 m and Utsukushigahara 1350 m, and from morph III at Hora 700 m. We were unable to confirm the presence of gall midges in the other studied populations. The larvae from morphs I and II had each induced a gall on a flower bud (Fig. 1b), whereas the larvae from morph III were each growing inside a fruit and no gall induction was observed (Fig. 1d). The seeds inside the fruit did not show any damage from the feeding by the larvae.

Larval morphological examination

The gall midge larvae obtained from the flower bud galls on morphs I and II were orange or white colored and morphologically similar to *Schizomyia* in the shape of the sternal spatula, the arrangement of lateral papillae, and the shape and composition of the terminal papillae

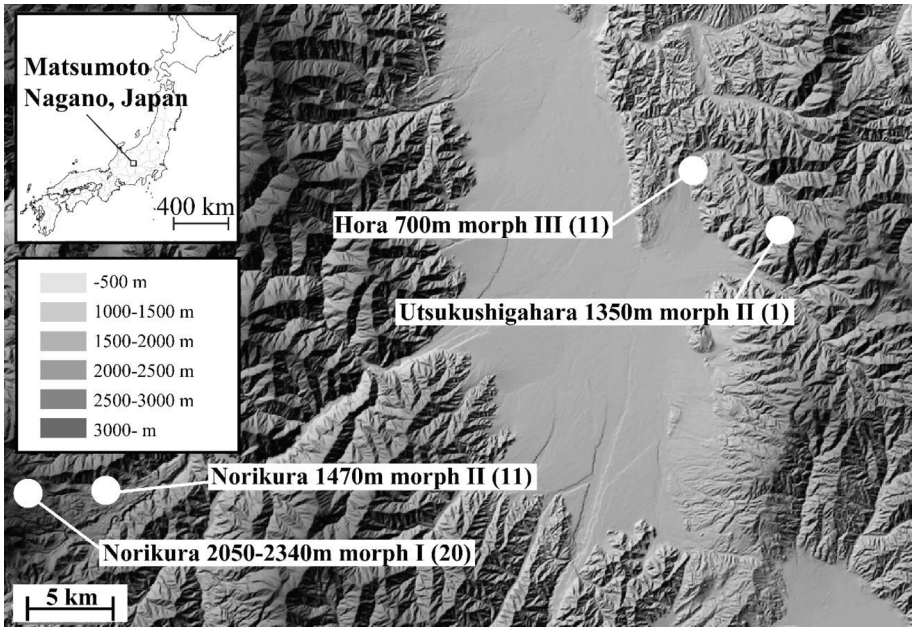

Fig. 2. Locations of *Cimicifuga simplex* populations (site and altitude and *C. simplex* morph) where gall midges were collected. The number of gall midge specimens collected at each site is shown in parentheses. Information on all study sites, including those from which no gall midge specimens were obtained, is given in Supplementary material Table S1.

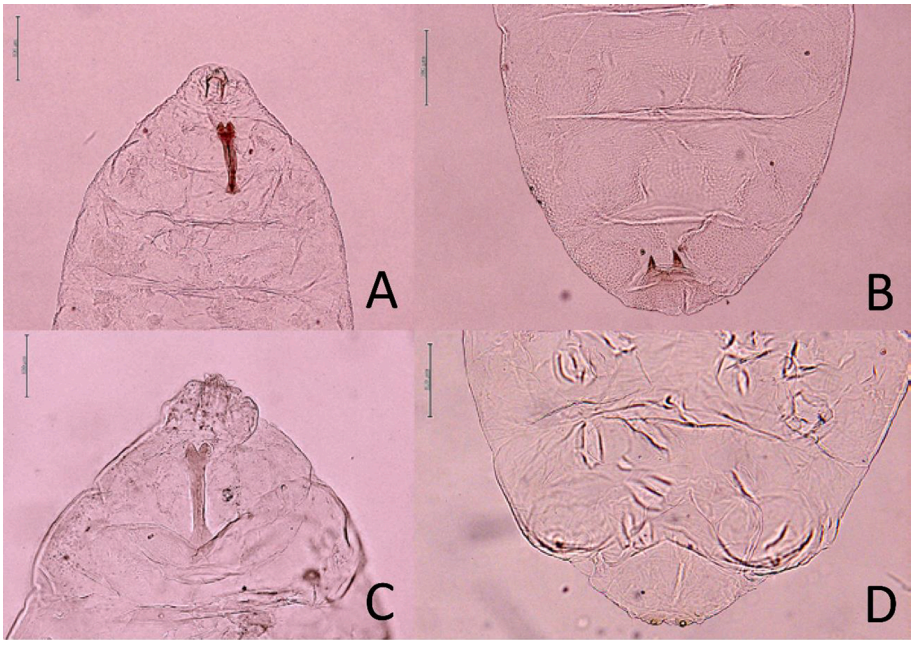

Fig. 3. Morphological features of larval gall midges associated with *Cimicifuga simplex*. A, B, *Schizomyia* species associated with *C. simplex* morph I (A, ventral view of head–mesothorax; B, ventral view of 8th and terminal abdominal segments); C, D, *Contarinia* species associated with *C. simplex* morph III (C, ventral view of head–mesothorax; D, ventral view of the 8th and terminal abdominal segments).

(Fig. 3A, B; for the detailed larval morphology of *Schizomyia*, see Elsayed et al., 2018). No distinct morphological differences were found between the larval groups collected from morphs I and II. Larvae obtained from fruit on morph III were white colored and possessed the typical morphological features of genus *Contarinia* with regard to the shape of the sternal spatula, the arrangement of lateral papillae, the shape and composition of terminal papillae, and the smooth integument (Fig. 3C, D; e.g. Gagné, 1995; Tokuda et al., 2006; Möhn, 1955). We treated this species temporally as belonging to *Contarinia* because it is the catch-all genus of Cecidomyiidae and these characters are common in known species of *Contarinia*.

On the basis of these morphological characteristics and the molecular phylogenetic results (see below), the gall midges that induced flower bud galls on morphs I and II were identified as *Schizomyia* spp., and the gall midges associated with fruit on morph III were identified as *Contarinia* sp.

Phylogenetic analysis

The phylogenetic analysis results showed that our gall midge specimens belonged to five major clades (Fig. 4), each of which was supported by a high bootstrap value. All gall midges collected from *C. simplex* morph II were grouped into clade 1, and all those collected from morph III were grouped into clade 5, whereas gall midges collected from *C. simplex* morph I were grouped into four clades (clades 1–4). Clade 1 comprised gall midges collected from both morphs I and II of *C. simplex*. The clades of the gall midges from morphs I and II were sister groups to *Schizomyia* species. Clade 5, composed of gall midges collected from morph III, was a sister group to *Contarinia* species. The genetic distance between clades belonging to *Schizomyia* spp. ranged between 3.5 and 5.0% (Table 1). All gall midges collected from morph II belonged to the same clade (clade 1) even though the specimens were collected from two geographically separated populations (Norikura 1470 m and Utsukushigahara 1350 m; Fig. 2).

Discussion

The *Schizomyia* individuals belonging to clades 1–4 and collected from morphs I and II, like the gall midges mentioned in Yukawa and Masuda (1996), were found in galls induced on *C. simplex* flower buds. The morphological features of the collected larvae are consistent with those of *Schizomyia* species (Fig. 3A, B), and the results of the molecular phylogenetic analysis supported that the specimens are closely related to *Schizomyia* (Fig. 4). Therefore, we regard the gall midges belonging to clades 1–4 as *Schizomyia* species. The morphological features of the *Contarinia* individuals belonging to clade 5 and collected from morph III are consistent with those of *Contarinia* species (Fig. 3C, D), and the molecular phylogenetic analysis showed that these specimens are closely related to *Contarinia* species (Fig. 4). Therefore, we regard the gall midges belonging to clade 5 as a *Contarinia* species.

Schizomyia individuals on morph I were collected in different altitude or different seasons (Fig. 4). This result suggests that isolation due to both distance and time contributed to the genetic differentiation of these clades. Some gall midges collected from morph I of *C. simplex* were included in clade 1, which consisted mainly of gall midges collected from morph II (Fig. 4). The monophyly of each of clades 1–5 was strongly supported. In general, the threshold for species diagnosis is usually based on a 3% base substitution in the mitochondrial *COI* gene (Hebert et al., 2003). Based on this criterion, the gall midges of each clade are different in nucleotide sequence at the different species levels. In particular, there are four mtDNA clades (putative species) of *Schizomyia* gall midges collected from morph I of *C. simplex*. However, because of low support at the nodes, the phylogenetic relationships among clades 1–4, including their outgroups, could not be robustly inferred. The general trend was that each clade consisted of gall midges from a single morph, with clade 1 being the exception.

The gall midges belonging to clade 5 were collected from within the fruits of *C. simplex* morph III. In addition, the gall midges did not induce a gall, and the fruit containing the gall midge larvae appeared at a glance to be fruiting normally. Thus, their plant utilization is completely different from that of the gall midges of clades 1–4. This plant utilization suggests that the larvae belonging to clade 5 are probably seed-eaters, but we observed no evidence of seed-eating during this study.

The results of our phylogenetic analysis indicate that the cryptic diversity of the gall inducer is corresponded within the cryptic diversity of the plant species. A similar corresponded relationship has been found

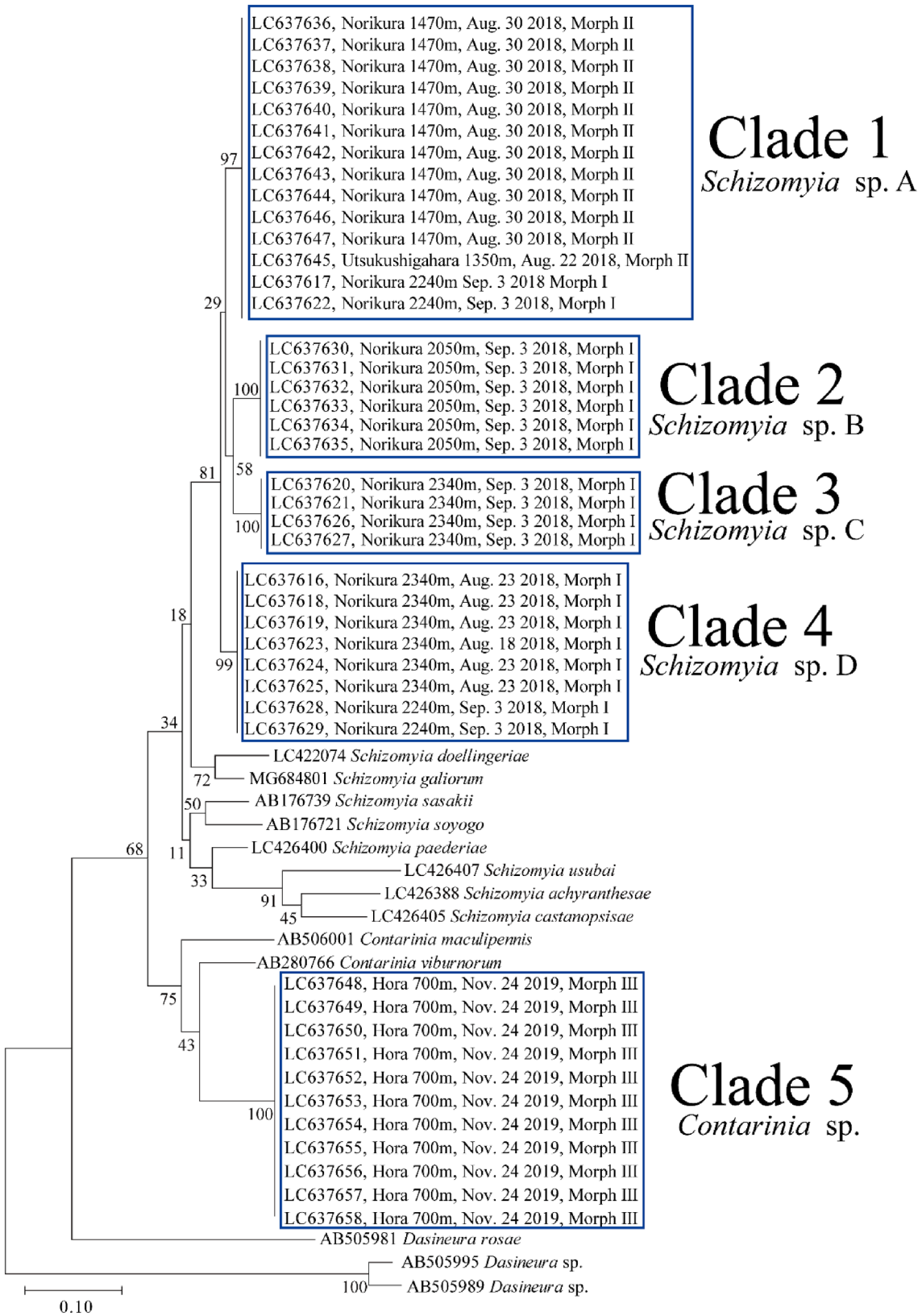

Fig. 4. Maximum likelihood phylogenetic tree of gall midges collected from *C. simplex* based on the mitochondrial *COI* region (441 bp). For specimens collected in this study, the GenBank accession number, collection site, collection date, and *C. simplex* morph are noted. For samples obtained from the GenBank database, the accession number and species name are noted. Bootstrap values are shown next to nodes.

Table 1
Genetic differences between clades based on the mitochondrial *COI* region of gall midges collected from *C. simplex*. See Fig. 3 for the phylogenetic tree.

	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5
Clade 1		3.8%	4.3%	3.5%	12.2%
Clade 2	3.8%		4.8%	5.0%	14.2%
Clade 3	4.3%	4.8%		4.0%	13.6%
Clade 4	3.5%	5.0%	4.0%		12.2%
Clade 5	12.2%	14.2%	13.6%	12.2%	

between symbiotic yeasts and their insect host species. Nearly 10 cryptic species have been discovered in *Platycerus* stag beetles in Japan (Kubota et al., 2020). Symbiotic yeasts, which contribute to the degradation of xylose, grow in the mycangium of these stag beetles, and the phylogeny of the symbiotic yeasts is known to correspond to that of the stag beetle (Kubota et al., 2020). Here, we found that five lineages of gall midges used three ecotypes of *C. simplex*. Moreover, four of the gall midge lineages used morph I of *C. simplex*. According to recent estimates, the diversity of Cecidomyiidae is the highest among insects (Hebert et al., 2016; Borkent et al., 2018; Brown et al., 2018). The association of multiple species of the same genus with a single host plant species, as in our results, is an important finding that may explain the extraordinary diversity of Cecidomyiidae. Further study is needed to determine why such diversification has occurred only in gall midges collected from morph I. In addition, for purposes of species description, more information is needed on the life histories of gall midges that use *C. simplex* and the morphological features of the adults need to be examined.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aspen.2021.09.006.

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