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Bryophytes facilitate outcrossing of *Mitella* by functioning as larval food for pollinating fungus gnats

Fig. 1 documents a previously unrecognized tripartite interaction between the plant genus *Mitella* and allies (Saxifragaceae), their specialized pollinating fungus gnats (Diptera, Mycetophilidae), and living mosses and liverworts (Bryophytes) that host the specialized pollinator larvae. Here we hypothesize a novel facilitation mechanism by bryophytes for outcrossing of flowering plants, where the pollination success of these plants is supported by co-occurring bryophytes mediated through their pollinators.

Diptera (true flies) is one of the largest groups of insects acting as pollinators (Proctor et al. 1996, Ollerton 2017). They are ubiquitous, diverse (e.g., Hebert et al. 2016), and, in some cases, specialized to flower-feeding habitat, thereby being indispensable as pollinators for a wide range of plant communities, especially where other pollinators are less abundant (Orford et al. 2015). Fungus gnats (family Mycetophilidae and allies) act as principal pollinators for some plant species in temperate forests (Proctor et al. 1996, Okuyama et al. 2008, Ollerton et al. 2017, Mochizuki and Kawakita 2018). With few exceptions, they are regarded as obligate fungal feeders at the larval stage, yet the larval habitats of a large proportion of their high diversity are still unknown (Jakovlev 2011, 2012). There are several observations, almost 100 years old, of larvae of the genus *Boletina* emerging from liverworts (Cheetham 1920) and larvae of the genus *Gnoriste* from mosses (Lenz 1927), which have been anecdotally repeated in reviews and textbooks without ever being further confirmed (e.g., Jakovlev 2011, 2012).

The genus *Mitella* and allies (Saxifragaceae) constitutes an interesting example of plant lineages that have coevolved with a specific group of pollinating fungus gnats. The pollinators are almost exclusively confined to the genera *Boletina*, *Coelosia*, and *Gnoriste* (Okuyama et al. 2008), genera that are closely related to each other (Rindal et al. 2009, Martinsson et al. 2011) and likely to have similar biology. These specialized plant–pollinator interactions have evolved multiple times in both Pacific Northwestern America and East Asia (Okuyama et al. 2008). In extreme cases, species such as *Tolmiea menziesii*, *Mitella furusei*, and *M. stylosa* rely on a single species of a genus with elongated mouthparts, *Gnoriste*, for pollination (Goldblatt et al. 2004, Okuyama et al. 2004, 2008, Okamoto et al. 2015). These facts indicate a long and tight evolutionary relationship between the pollinators and the plants, but little is known about the habitats of the larval stage of these pollinators. Accordingly, the circumstances that enable these specific interactions to be maintained stably for a long geological time remain an enigma.

Over our 14 years of field observations of *Mitella* plants and their pollinators, we repeatedly observed oviposition of females of the species *Gnoriste mikado* on mosses near the *Mitella* plants (Fig. 1A). Following this observation, we searched for the larvae of *G. mikado* in mosses at the riparian forest of Kibune, Kyoto, Japan where *Mitella* plants grow, and found several last instar larvae of fungus gnats during wintertime. The larvae clearly fed on the moss of the family Mniaceae (*Rhizomnium*, *Mnium*, and *Plagiomnium* spp.), their diet being visible from outside through the translucent abdomen (Fig. 1B). The larva made a mucinous cocoon at pupal stage (Fig. 1C), and eventually we successfully reared adults of *G. mikado*. We also found that it is easy to induce oviposition of wild-collected females of *G. mikado* by placing them in a case together with mosses of family Mniaceae, although the laid eggs never hatched. Taken together, *G. mikado*, an exclusive pollinator of some *Mitella* species (Fig. 1E; Okuyama et al. 2004, 2008, Okamoto et al. 2015), is likely to rely strictly on mosses of the family Mniaceae as their brood site.

During parallel studies of bryophyte-feeding insects in Japan, one of the authors (M. Kato) noticed that fungus gnats of the genus *Boletina* frequently emerge from liverworts of the genera *Conocephallum*, *Apopellia*, and *Pallavicinia*. Again, we observed that the larvae of the *Boletina* spp. fed on the liverworts by spinning a mucous silk network of nests on, beneath, or among thalli (Fig. 1D). We applied the integrative approach of morphology and DNA barcoding, using the mitochondrial cytochrome oxidase subunit I (COI) gene (see Appendix S1: *Materials and Methods*), to examine if the bryophyte-living larvae belong to the same species that are known pollinators of *Mitella* (Okuyama et al. 2008, Katsuhara et al. 2017). Among seven distinct genetic groups, corresponding to seven morphological species of *Boletina* spp. feeding on the liverworts, we confirmed that five of them, *Boletina nasuta*, *B. conjuncta*, *B. curta*, *Boletina* sp. A, and *Boletina* sp. B are the same species we found visiting *Mitella* flowers (Fig. 2, Data S1).

Other than for host–parasite and tri-trophic interactions, tripartite interactions in which one of the species benignly uses each of the others as hosts during different life stages are not well documented. Our study is unusual in that it demonstrates a possible tripartite interaction between a flowering plant and a bryophyte that are the hosts of different life stages of a third species, a fungus gnat, that pollinates the flower but whose larvae live on the bryophyte. The interaction seems benign from the bryophyte perspective, as we did not find any bryophytes killed by the insect feeding, although the herbivory may reduce fitness of the bryophytes to some extent. Accordingly, it is possible, perhaps even likely, that the bryophytes indirectly limit the distribution ranges of *Mitella* and *Tolmiea* spp. by being the host for the larval stage of the fungus gnats that pollinate these flowering plants. Indeed,

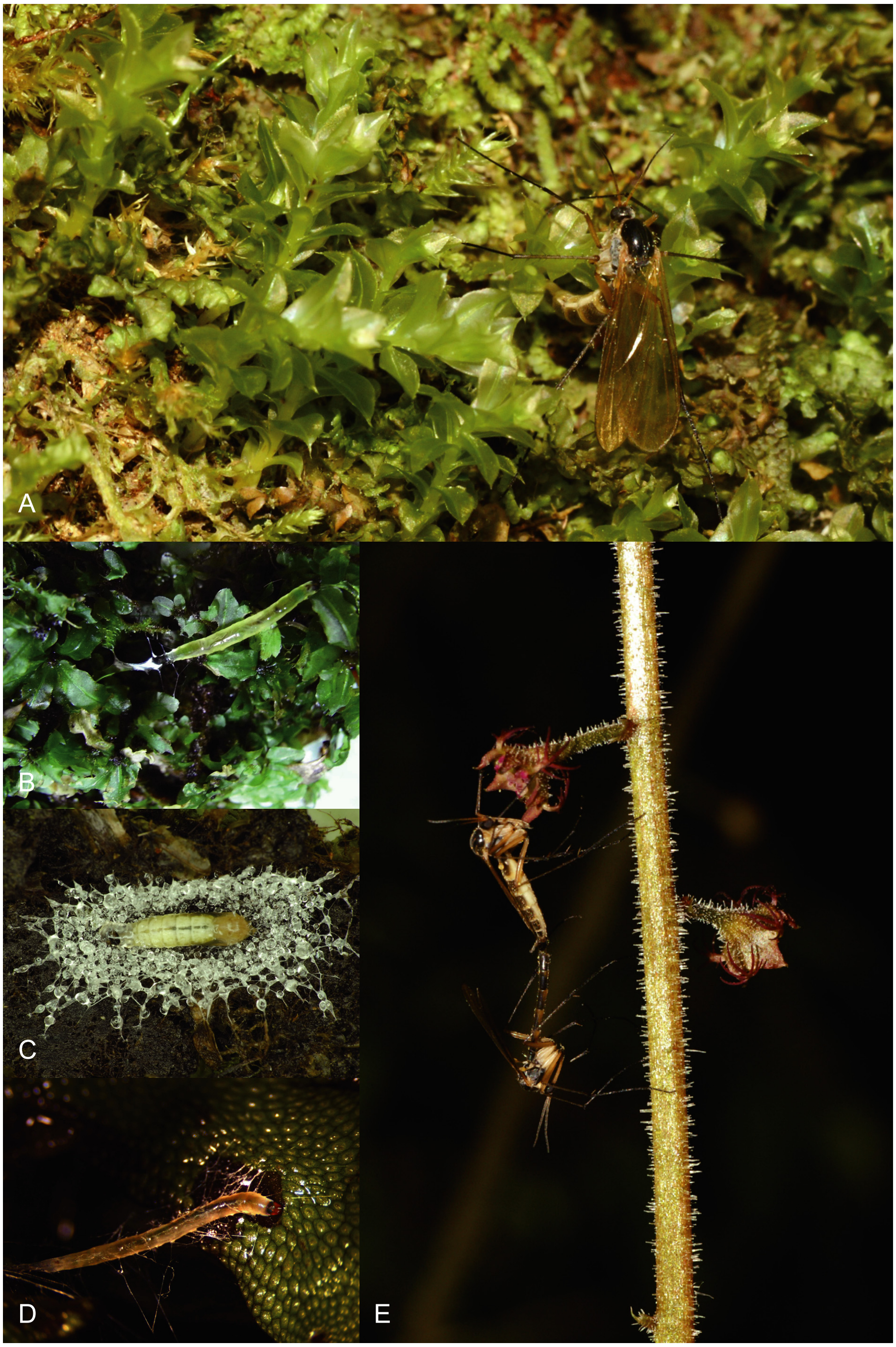

Fig. 1. The tripartite interactions of *Mitella*, pollinating fungus gnats, and their host bryophytes. (A) A female *Gnoriste mikado* ovipositing on moss *Plagiomnium acutum*. (B) A last instar larva feeding on *Plagiomnium* sp. (C) A pupa of *G. mikado* surrounded by mucous cocoon. (D) A larva of *Boletina* sp. feeding on liverwort *Conocephallum* sp. showing a typical feeding trench covered with web. (E) A pair of *G. mikado* copulating on an inflorescense of *M. furusei*.

Mitella and *Tolmiea* spp. always co-occur with the bryophytes of the genera *Conocephallum*, *Apopellia*, and *Pallavicinia*, or the family Mniaceae that host the pollinator fungus gnats as long as we observed them in a number of natural habitats. Further evidence for or against this hypothesis could be provided by a study of the spatial coexistence between *Mitella* and bryophytes that are the hosts for the pollinators. If *Mitella* and the bryophytes each have patchy but strongly coincident distributions, that would lend strong support to our hypothesis that the bryophytes must provide the necessary habitat for the larval stage of the fungus gnats in order for *Mitella* to be successfully reproduced.

A tripartite interaction linked by different habitat preferences of the life stage of one of the species would be an

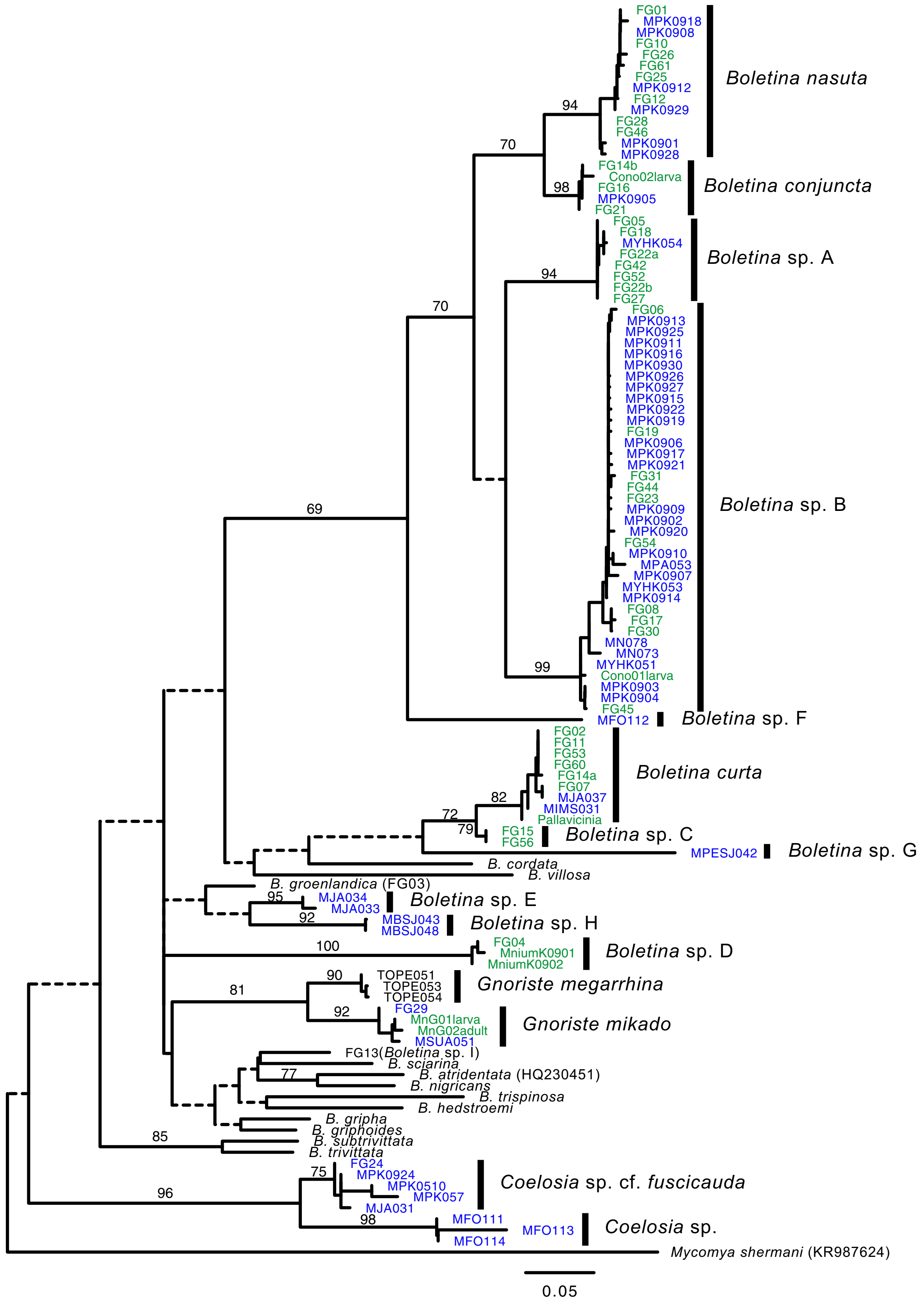

Fig. 2. A neighbor-joining tree of 101 fungus gnat individuals collected in the present study and some related taxa based on 644 base pairs

(Fig. 2. *Continued*)

(bp) of mitochondrial cytochrome oxidase subunit I gene. Bootstrap supports for nodes above the species level are shown (only when >50%). Colored OTUs were the individuals either reared from bryophytes (green) or collected from *Mitella* flowers (blue). Note that *Boletina nasuta*, *B. conjuncta*, *B. curta*, *Boletina* sp. A, *Boletina* sp. B, and *Gnoriste mikado* were collected both from bryophytes and *Mitella* flowers.

intriguing and a not well-studied topic in both empirical and theoretical ways. For example, *Mitella pauciflora* and *M. furusei*, each of which are pollinated by *Boletina* and *Gnoriste*, respectively, are the pair of sister species that has been ideal as the model for studying pollinator shifts and pollinator-mediated reproductive isolation (Okamoto et al. 2015). Although the ultimate cause of the pollinator shift between these species remains unclear, it is worth asking if it is associated with the habitat differences between the bryophyte species that host each of the pollinators.

Species of *Boletina* and *Gnoriste* are diverse and abundant fungus gnats in the Holarctic Region, especially in subarctic forest floors and along banks of Arctic and mountain streams with riparian vegetation (Edwards 1925, Martinsson et al. 2011). Although their taxonomy, life-history requirements, and potential role as pollinators are considerably understudied in proportion to their diversity, our finding raises the possibility that such tripartite interactions of plants, pollinators, and their host bryophytes are widespread across temperate to arctic riparian environments.

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