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Pollination of Vietnamese *Aspidistra xuansonensis* (Asparagaceae) by female Cecidomyiidi flies: Larvae of pollinator feed on fertile pollen in anthers of anthetic bisexual flowers[1]

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- *Premise of the study: Aspidistra* is a species-rich, herbaceous monocot genus of tropical Southeast Asia. Most species are recently discovered and apparently endangered, though virtually nothing is known about their biology. Species of the genus are primarily distinguished using flower morphology, which is enormously diverse. However, the pollination process has not been directly observed in the center of diversity of the genus (N Vietnam and S China). Indirect and partly direct data on the only widely cultivated species of the genus (*A. elatior*) placed it among angiosperms with the most unusual pollination biology, though these data are highly controversial, suggesting pollen transfer by mollusks, crustaceans, flies, or possibly tiny soil invertebrates such as collembolans.
- *Methods:* Pollination of *Aspidistra xuansonensis* in the center of diversity of the genus was studied using visual observations and videos and light and scanning electron microscopy investigation of flowers and their pollinators. Pollinators and their larvae were molecularly barcoded.
- *Key results: Aspidistra xuansonensis* is pollinated by female cecidomyiid flies (gall midges). They oviposit on anthers, and larvae develop among the pollen mass. Molecular barcoding proved taxonomic identity of the larvae and the flies. The larvae neither damage floral parts nor cause gall formation, but feed on pollen grains by sucking out their content. The larvae move out of the flowers before decomposition starts. *Carebara* ants steal developing larvae from flowers but do not contribute to pollination.
- *Conclusions:* More than one kind of myiophily is present in *Aspidistra*. Brood site pollination was documented for the first time in *Aspidistra*. The pollination system of *A. xuansonensis* differs from other kinds of brood site pollination in the exit of the larvae prior to the decomposition of floral parts.

Key words: *Aspidistra*; Asparagaceae; Cecidomyiidae; molecular barcoding; myiophily; pollen-eating; pollination; oviposition; larvae; tritrophic interactions; Vietnam.

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Aspidistra is a genus of rhizomatous, herbaceous, evergreen plants with an enormous diversity of floral structure from Southeast Asian tropical forests (Li et al., 2000; Liang and Tamura, 2000; Bogner and Arnautov, 2004; Tillich, 2005, 2014; Kocyan and Renner, 2007; Averyanov and Tillich, in press; Vislobokov et al., 2014a). In terms of floral organization (groundplan: Endress, 1994), the diversity found in *Aspidistra* is remarkable in the species-rich order Asparagales (Kocyan, 2007; Remizowa et al., 2010; Endress, 2014; Vislobokov et al., 2014a). For example, tepal number varies from two (Vislobokov et al., 2014a) to 12 (Tillich et al., 2007), which are the lowest and the second-highest (after *Neoastelia*, Asteliaceae: Takhtajan, 2009) figures for Asparagales. *Aspidistra* is one of only three genera of Asparagales where true polyandry is found (i.e., multiplication of stamens resulting in occurrence of flowers with stamen number exceeding the tepal number: Kocyan, 2007). In terms of construction (gestalt: Endress, 1994), flowers of *Aspidistra* show a drastic variation in shape of the perianth tube, tube to lobe length ratio and stigma shape. For example, linear

tepal lobes are much longer than the tube and possess conspicuous basal appendages in *A. grandiflora*, whereas in *A. locii* free lobes are absent and the entire perianth is balloon-like with a narrow distal opening (Bogner and Arnautov, 2004; Tillich et al., 2007; Tillich, 2014).

Aspidistra is a species-rich genus with pronounced endemism. Most species have been recently described from South China and North and Central Vietnam, and the rapid species discovery has shown *Aspidistra* to be one of the taxonomically diverse genera of the flora of Southeast Asia. Floral characters are essential for distinguishing *Aspidistra* species. Given the diversity of floral construction, the differences in floral characters could indicate variable pollination biology (Tillich, 2014). The evolutionary history of *Aspidistra* (which is likely a recently evolved clade, Kocyan and Renner, 2007) should be analyzed in the framework of flower biology. However, direct field observations of pollination are not available for the vast majority of species. This lack is partly because the flowers of *Aspidistra* (along with some Orchidaceae, Triuridaceae, Burmanniaceae, and Aristolochiaceae: e.g., Hall and Brown, 1993; Endress, 1995; Dixon, 2003) are held at ground level, sometimes under the forest litter, and are not always visible to the researchers. Also, flowering of many species is confined to the start of the wet season: field researchers working in other seasons observe only vegetative morphology. *Aspidistra* is easy to cultivate, and for many species, flowering was observed only in botanical gardens, where the plants mostly do not set fruits (Bogner and Arnautov, 2004; Bogner, 2005; Tillich, 2005). Observations of cultivated plants cannot therefore substitute for field studies. Field observations are urgently needed because of rapid deforestation in Southeast Asia and loss of native habitats.

The majority of information currently available on pollination in *Aspidistra* is based on the studies of *A. elatior*, which is reportedly native to Japan (Liang and Tamura, 2000, but see Averyanov and Tillich, 2012). Stamens of *A. elatior* are located within a perianth tube under a table-shaped stigma with a receptive upper surface, so that self-pollination is impossible (Buchenau, 1867). Delpino (1868) presented strong evidence of cross-pollination in cultivated *A. elatior*. He found pollen grains of *Aspidistra* along the exit trajectories of the pollinators moving from the narrow gaps between the perianth tube and the stigma. When exiting a flower, the pollinators leave no pollen on its stigma. Data on the pollinators of *A. elatior* are highly controversial. Delpino (1868, see also Hildebrand, 1870; Loew, 1895; Ivanina, 1982) hypothesized that small nematocerans (gnats) could act as pollinators. Wilson (1889) suggested pollination of *A. elatior* by slugs. Kato (1995) made the first observations on *A. elatior* in natural habitats and found no evidence of pollination by slugs. Instead, a terrestrial pollen-eating crustacean *Platorchestia japonica* (Amphipoda) was found to be a potential pollinator (Kato, 1995). Conran and Bradbury (2007) documented fruit set in *A. elatior* cultivated in Australia where the amphipod species mentioned by Kato (1995) was absent. They concluded that pollinator substitution is possible in *Aspidistra* and speculated about soil invertebrates including collembolans as possible pollinators. Bräuchler and Ngoc (2005) observed the flowering of *A. renatae* in central Vietnam and suggested pollination by small ground insects but did not document any actual visits. Bogner and Arnautov (2004) suggested pollination of *A. locii* by small insects, perhaps flies. Features of floral morphology suggest pollination of *Aspidistra* species by fungus gnats (Diptera: Mycetophilidae, Sciaridae; Vogel, 1978; Tillich, 2005). Vislobokov et al. (2013) made the first direct field observations of floral visitors in a mainland species of *Aspidistra* (*A. phanluongii* in southern Vietnam) and documented pollination by *Megaselia* flies (Phoridae). Like the Japanese plants of *A. elatior*, *A. phanluongii* occurs outside the diversity center of the genus, and the results may not be reliably extrapolated to other species. Also, the study of *A. phanluongii* left an open question on the rewards provided by the flowers for the pollinators.

In the present study, we provide the first field observations of the pollination of a species of *Aspidistra* in the center of diversity of the genus. The species investigated here (*A. xuansonensis*) has been recently described from Phu Tho province in northern Vietnam (Vislobokov et al., 2014b). Using the data on *A. xuansonensis*, we confirm earlier ideas on the occurrence of myiophily in *Aspidistra* and answer the question regarding rewards for pollinators. We document for the first time the development of larvae of pollinating insects (female Cecidomyiidae flies) in the anthers of *Aspidistra* flowers, where the larvae consume fertile pollen grains. In addition to field observations, molecular barcoding was used to demonstrate taxonomic identity of pollinating insects and larvae. Together with our earlier work (Vislobokov et al., 2013), our study suggests the presence of different types of myiophily in *Aspidistra* (see also Tillich, 2014) and possible taxon-specific interactions between pollinators and at least some species in the genus. Our data increase general knowledge on pollination systems of tropical flowers because specialized feeding of pollinators' larvae on fertile pollen in anthers of anthetic bisexual flowers is a condition that differs from common examples of oviposition during pollination. We document regular capture of larvae of pollinating flies by ants, thus revealing tritrophic interactions in the flowers of *A. xuansonensis*.

MATERIALS AND METHODS

***Field observations*—**Plants of *Aspidistra xuansonensis* N. Vislobokov var. *xuansonensis* were observed by the first author (N. Vislobokov) in the type locality in northern Vietnam (Phu Tho province, Thanh Son district, Xuan Son National Park) between 1–12 November 2013. Detailed observations on floral visitors were performed between 08:30 and 16:30 hours. In addition, sporadic observations were made at night. For observations of successive stages of anthesis, flowers and flower buds were labeled individually. Twenty flowers were monitored in detail using visual observations, videos and photography. Insects were captured by air stream (exhauster: Uys and Urban, 2006; Golub et al., 2012), killed by ethyl acetate fumes and fixed in 70% ethanol in groups of 1–9 flies (collected from the same flower) per 1 mL test tube. Time of each insect visit was noted in the field journal. Flowers of *A. xuansonensis* var. *xuansonensis* at various stages of anthesis were fixed in 70% ethanol. Pollinated flowers were fixed together with insect larvae developing in their anthers.

***Scanning electron microscopy (SEM)*—**For SEM of insects and flowers, the material was dissected in 70% ethanol and subsequently transferred to 100% acetone via 80 and 96% ethanol and a mixture of ethanol (96%) and acetone (100%) in a 1 : 1 proportion. The material was then critical-point dried in a Hitachi HCP-2 critical point drier (Hitachi, Japan) using liquid carbon dioxide. Dried samples were mounted onto stubs using double-sided sticky tape, coated with gold using an Eiko IB-3 ion-coater (Eiko Engineering, Japan) and observed using a CamScan 4DV (CamScan, UK) scanning electron microscope at Moscow State University.

***Pollen grains*—**For the analysis of pollen grains attached to the flies, fixed flies were washed in 70% alcohol, which was then centrifuged for 5 min at 3000 rpm. Then 0.1 mL of sediment was taken from each sample, stained using safranin, and investigated by light microscope Micromed-2 (Ningbo Sheng Heng Optics & Electronics, Zhejiang, China). Pollen grains were counted in a 612-mm^2 view area of the slide for each sample. For measuring the diameter of pollen grains, 165 grains from a flower that just opened were investigated with a light microscope Nikon Eclipse Ci (Nikon, Japan), and images were obtained using a Nikon DWS-Vi1 camera (Nikon, Japan). Pollen diameter was measured

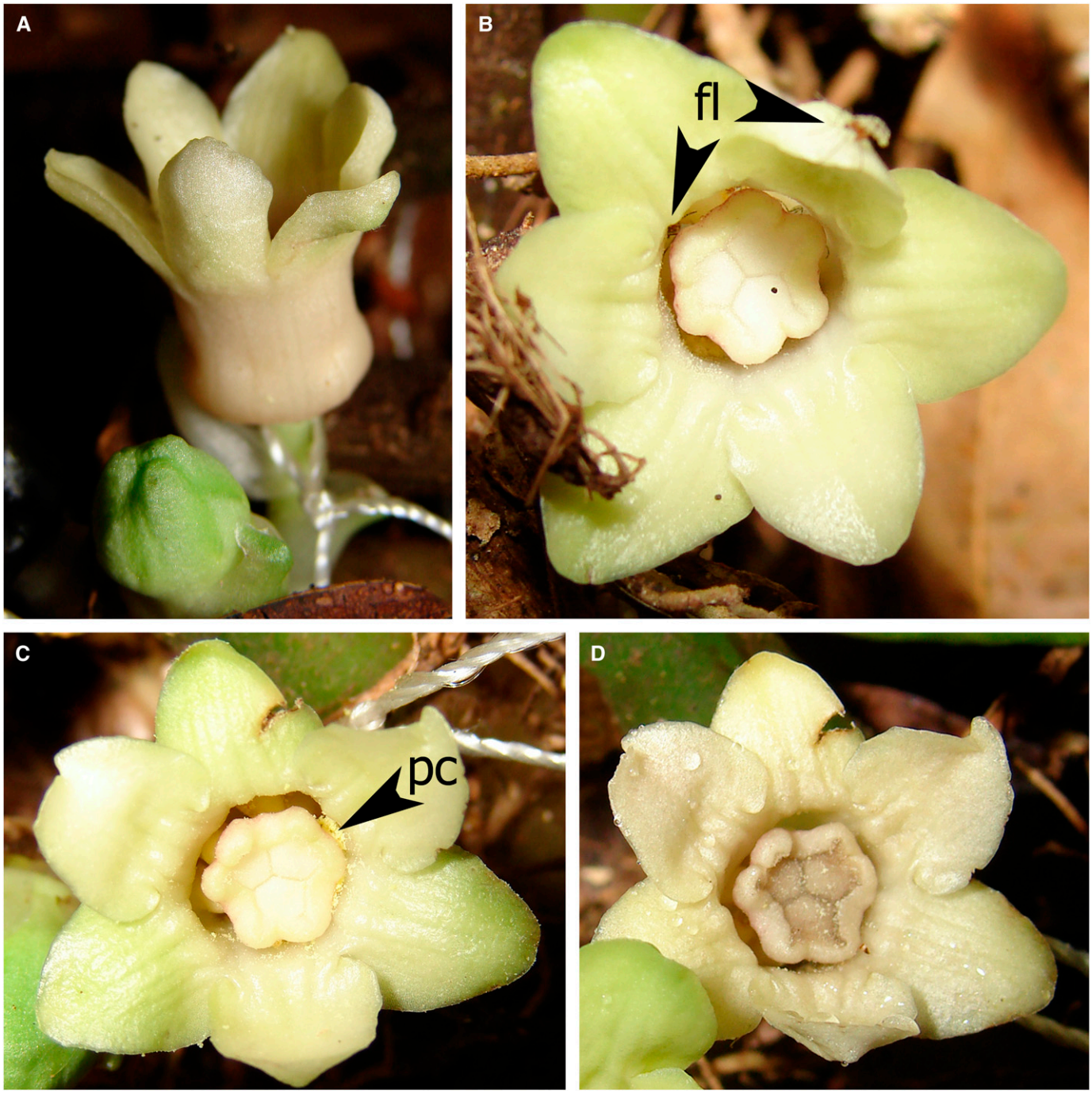


Fig. 1. Flowers of *Aspidsitra xuansonensis* var. *xuansonensis*. (A) A flower bud developing in forest litter and a flower at the beginning of anthesis. (B) Cecidomyiidi flies visiting a flower. (C) Clumps of pollen grains inside the flower after visits of Cecidomyiidi flies. (D) A flower after 6 d of anthesis with characteristics of senescence. pc = clump of collapsed pollen grains; fl = Cecidomyiidi flies.

from digital images using Image Scope software (Electron and x-ray analysis, Russia). Distribution fitting was performed in STATISTCA 7 software (StatSoft, Tulsa, Oklahoma, USA).

***Dissection of flies and larvae*—**Contents of the digestive tract of the flies and larvae as well as the presence and quantity of eggs in the flies were examined. Prepared material was studied using a light microscope Micromed-2 (Ningbo Sheng Heng Optics & Electronics) as well as SEM.

***DNA barcoding of flies and larvae*—**The mitochondrial gene cytochrome *c* oxidase I (COI) was chosen for DNA barcoding because COI is the main established standard reference for animal bioidentification (Hebert et al., 2003), including the insect family Cecidomyiidae (Kolesik and Butterill, in press). Total genomic DNA was isolated from animals (see Appendix 1), fixed by 96% ethanol, and air dried. The isolation was performed using a Diatom DNA Prep 200 kit (Laboratory IsoGen, Moscow, Russia), following the manufacturer's instructions with the reduction of all reagent volumes by

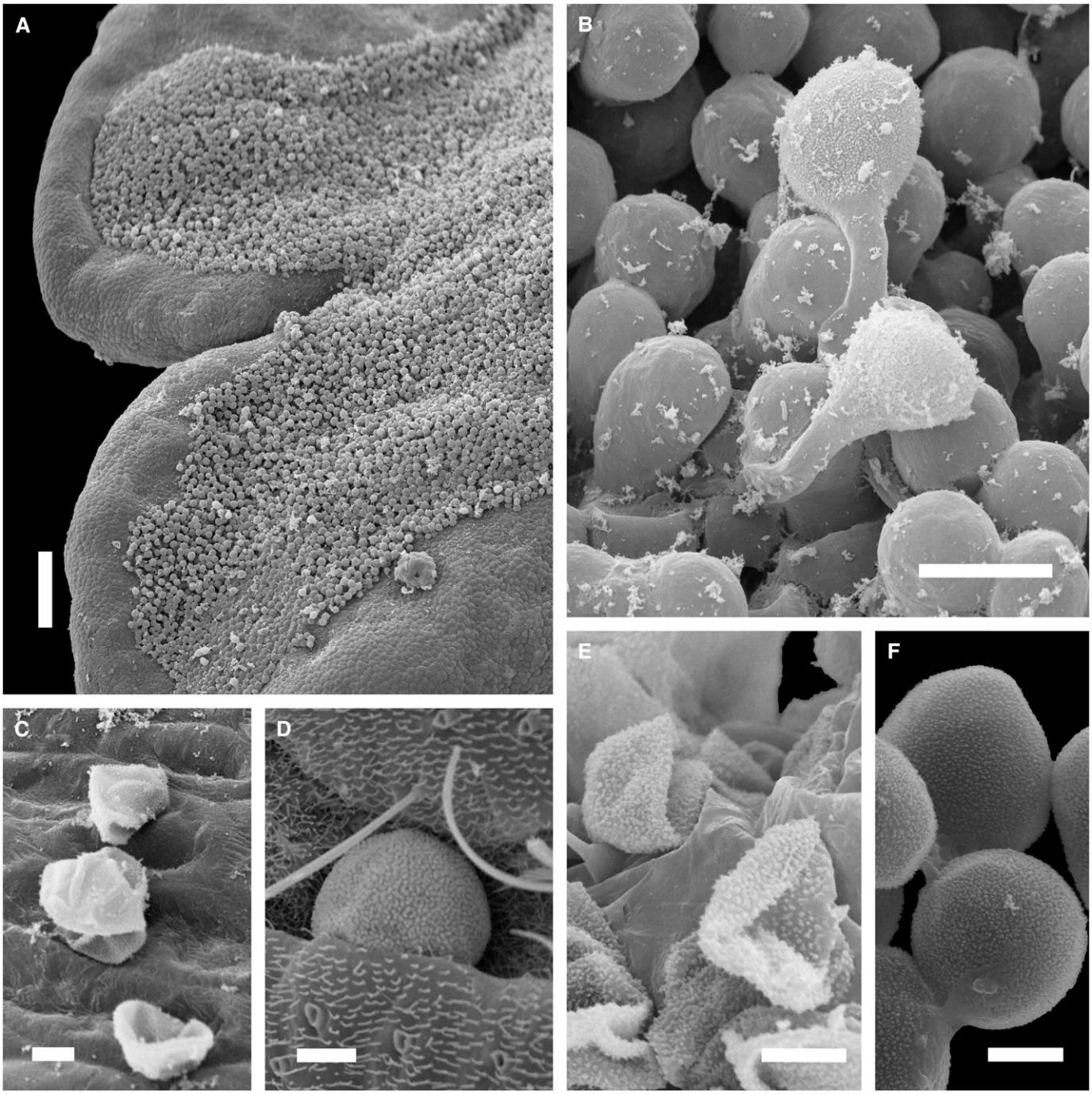


Fig. 2. Pollen and stigma of *Aspidstra xuansonensis* var. *xuansonensis* (SEM). (A) Upper surface of stigma with papillose receptive area. (B) Detail of receptive surface of stigma with germinated pollen grains (note pollen tubes). (C) Nonreceptive smooth lower surface of stigma with collapsed pollen grains whose content was consumed by larvae. (D) Uncollapsed pollen grain on abdomen of a cecidomyiid fly. (E) Collapsed pollen grains on body of a larva. (F) Pollen from recently opened anthers. Scale bars: A = 100 μm; B = 30 μm; C–F = 10 μm.

a factor of four. Polymerase chain reaction (PCR) was performed on a thermocycler T3000 (Biometra, Goettingen, Germany) using Encyclo PCR kit (Evrogen JSC, Moscow, Russia), according to recommendations of the manufacturer. Polymerase chain reaction (PCR) was conducted in a 10 μL volume under the following conditions: 96°C for 3 min (preliminary denaturation); 96°C for 20 s, annealing temperature 45°C for 30 s, and 72°C for 1 min (for 15 cycles); 96°C for 20 s, annealing temperature 48°C for 30 s, and 72°C for 1 min (for 20 cycles); and a final extension at 72°C for 3 min. The primers LCO1490 and HCO2198 (Folmer et al., 1994) were used. The PCR products were purified using a gel extraction and PCR cleanup kit (Cytokine Ltd, St. Petersburg, Russia). Automated sequencing was performed on an ABI 3100 sequencer using the Big Dye Terminator v.3.1 sequencing kit (Applied Biosystems, Foster City, California, USA). The COI region was sequenced in its entirety on both strands. An initial set of taxa for comparison with newly obtained sequences (Appendix 1) was identified using the BLAST option of the GenBank database. This search suggested that taxa belonging to subfamily Cecidomyiinae, were the most similar to our samples. The published COI data set for Cecidomyiidae from the study of

Beckenbach and Joy (2010) was modified and used for more precise identification of the phylogenetic position of our material. As in the study of Beckenbach and Joy (2010), the trees were rooted with *Bradysia amoena* (Sciaridae). The two new sequences (Appendix 1) were analyzed together with 54 GenBank accessions. The DNA sequences were aligned using the program MUSCLE (Edgar, 2004) and then manually adjusted in BioEdit (Hall, 1999). The COI data matrix was analyzed using both maximum parsimony (MP) and Bayesian inference (BI). Maximum parsimony (MP) analyses were performed using PAUP* (version 4.0b08; Swofford, 2003) with equal weighting of characters and tree-bisection-reconnection (TBR) branch swapping. For each heuristic search, 1000 random sequence addition replicates were run, all shortest trees were saved. Bootstrap values for branches were calculated using 1000 replicates with TBR branch swapping and random addition of taxa. One thousand most parsimonious trees from each replicate were saved. Bayesian analysis of molecular data were performed using the program MrBayes (version 3.2.2; Ronquist et al., 2012). The GTR model of substitutions with gamma rate categories was selected by the Akaike information criterion (Akaike, 1974) in the program Modeltest (Posada and Crandall, 1998). Bayesian inference was performed with two simultaneous runs with four chains in each; 25 million generations were performed; trees were sampled every 1000 generations. The number of generations to be discarded was determined by cold chain log likelihood examination using the program Tracer v. 1.5 (Rambaut and Drummond, 2007).

RESULTS

Flower morphology and phenology—*Aspidistra xuansonensis* var. *xuansonensis* is a terrestrial herbaceous rhizomatous plant growing on the slopes of limestone hills under the canopy of polydominant tropical forest at elevations of 350 to 450 m a.s.l. Flowers are up to 19 mm long and up to 23 mm wide. They possess 6 (rarely 4, 5, or 7) tepals in two whorls forming a pronounced basally widened perianth tube (Fig. 1A–D). Free lobes of the tepals are greenish white inside and greenish white or somewhat purple spotted outside. Stamens are arranged in two whorls. The number of the stamens is equal to the number of the tepals. The stamens are inserted at the lower part of the perianth tube, at the radii of the tepals. Anthers are sessile and located within the perianth tube below the level of the stigma. The gynoecium consists of (2)3(4) united carpels with a small superior ovary, a style, and an obconic stigma, which is white on the upper surface and white or purple on the lateral sides. The flowers develop on a short rhizome at ground level. Each peduncle gives rise to a single flower. Peduncle length varies, but mature flowers always emerge just above the forest litter. Young green flower buds grow within the forest litter for a minimum of 7 d. Later, the peduncle length increases, and when the perianth tube diameter reaches 6–8 mm, a flower starts opening gradually over 1 d (Fig. 1A). Anthers dehisce simultaneously with the opening of the perianth. The upper surface of the stigma is receptive and somewhat concave (Figs. 1B, 1C, 2A, 2B). Flowers do not secrete nectar or produce any scent detectable to humans. Anthesis of a flower takes 5–6 d, followed by senescence (Fig. 1D).

Flower visitors and pollination—*Flowers were visited by flies and ants only. Flies landing on flowers were recorded between 08:50 and 16:00 hours. Ant visits were recorded between 09:50 and 15:50 hours (Table 1). Two of the captured flies were identified as a species of *Drosophila*. All other captured flies (Figs. 3A–C) were identified as belonging to supertribe Cecidomyiidi, subfamily Cecidomyiinae, family Cecidomyiidae. Morphologically, these flies resemble the genera *Cecidomyia* or *Contarinia* (precise species-level identification is problematic in the absence of male individuals in our collection). Flies were documented as remaining inside the flower from 9:00 to 16:20 hours (Table 2). All flies found inside flowers were those of Cecidomyiidi. Only female flies of Cecidomyiidi were captured visiting *Aspidistra*. Although species and genus level taxonomy of Cecidomyiidi is largely based on male morphology (Yukawa, 1971; Gagné and Jaschhof, 2014), female Cecidomyiidi visitors appeared identical (e.g., in venation) and may have represented the same species. Ants visiting the flowers belong to the genus *Carebara* (Formicidae). Sporadic observations at night failed to reveal any active flower visitors.

Cecidomyiidi flies land on perianth lobes. A female fly remains motionless on the perianth lobes for as long as 3 min. Later, it moves along the perianth lobes and upper surface of stigma across its receptive zone, allowing cross-pollination if the fly visited another flower earlier. The flies are able to penetrate under the stigma only through the gaps between the perianth tube and the stigma lobes. In most cases, the gaps are so narrow that squeezing through them requires the flies to make a considerable effort. Often they make several attempts until they succeed in penetrating the flower. Stamens are located directly under the gaps between the perianth tube, and the stigma lobes and the flies therefore become covered with pollen (Fig. 3B, D). Once the flies have entered the flower (Fig. 1B), they remain there for a minimum of 6 h. Videos of the visits and their penetration inside the flowers are presented in Appendix S1 (see Supplemental Data with the online version of this article). Our observations show the absence of any other insects or their larvae in the flowers before the first visit of a Cecidomyiidi fly. Shortly after the long visits of the flies, their larvae become conspicuous inside the anther thecae among the pollen mass. The larvae are moderately long, flattened, and without large bristles (Fig. 4A, D). They can develop in the

TABLE 1. Floral visitors to *Aspidistra xuansonensis* var. *xuansonensis*.

Observation period (hours)	Observation duration (min)	No. observed visits of *Carebara* ants	No. *Carebara* ants/min	No. observed arrival events of Cecidomyiidi flies	No. arrivals of Cecidomyiidi flies/min	No. Cecidomyiidi larvae leaving flower	No. Cecidomyiidi larvae leaving/min	No. observed visits of *Drosophila* flies	No. *Drosophila* flies/min
08:00–09:00	33	—	—	1	0.03	—	—	—	—
09:00–10:00	100	—	—	—	—	—	—	—	—
10:00–11:00	99	1	0.01	—	—	—	—	—	—
11:00–12:00	112	8	0.07	5	0.04	—	—	2	0.02
12:00–13:00	150	6	0.04	—	—	7	0.05	—	—
13:00–14:00	139	4	0.03	—	—	—	—	—	—
14:00–15:00	146	6	0.04	1	0.01	—	—	—	—
15:00–16:00	167	5	0.03	—	—	—	—	—	—
16:00–17:00	36	—	—	—	—	—	—	—	—

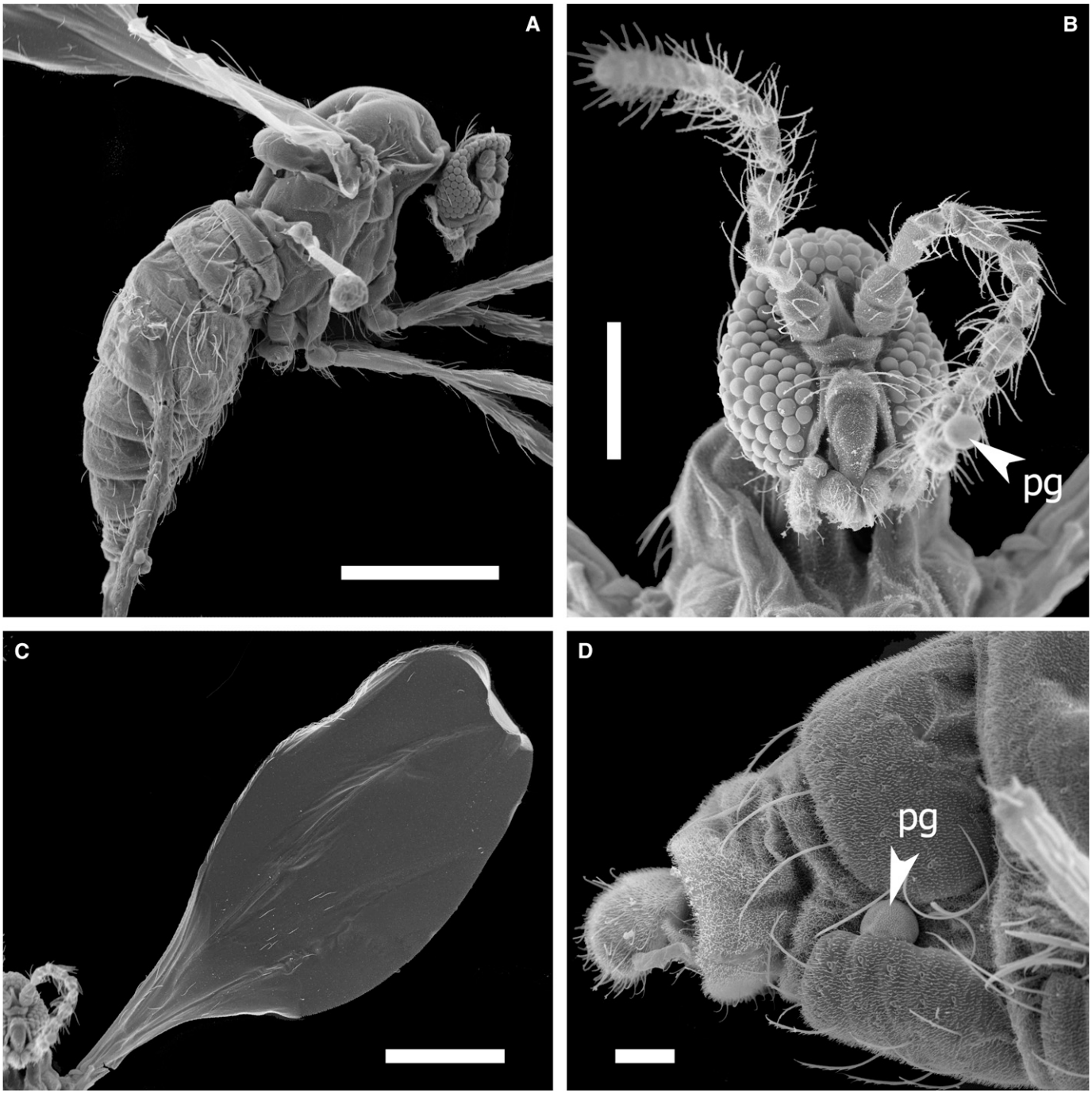


Fig. 3. Cecidomyiidi flies (SEM). Flies (A) captured from flowers of *Aspidstra xuansonensis* with a few *Aspidistra* pollen grains on the head (B) and abdomen (D) remaining after liquid fixation. Wings (C) morphology is an important character in the taxonomy of Cecidomyiidi. pg = pollen grain. Scale bars: A, C = 300 μm; B = 100 μm; D = 30 μm.

inner and outer anther whorls. Four to 20 larvae per flower were recorded (flowers with evidence of larval escape were excluded from this count). Larvae in different stages of development were observed in the same flowers (e.g., eggs and young to old larvae). No pupae were found in the flowers examined. Approximately 2 d after the visits of the adult female flies larvae covered by collapsed pollen grains (Fig. 4A, B) leave the flowers through the same gaps between stigmatic and perianth lobes that initially allowed the entrance of the adult flies (see videos: Appendix S2 in online Supplemental Data). The larvae move toward the edges of the perianth lobes and fall down into the forest litter. After the exit of the larvae, clumps of pollen grains are visible inside the flower (Fig. 1C). Pollen grains from these clumps are collapsed in the same way as the pollen on larval bodies (Figs. 2C, 2E, 4B).

TABLE 2. Presence of Cecidomyiidi flies inside a flower of *Aspidistra xuansonensis* var. *xuansonensis*.

Observation period (hours)	Observation duration (min)	Total duration of flies inside a flower (min)[a]	Mean No. of flies per flower (ratio of parameters from columns 3 and 2)
08:00–09:00	33	—	—
09:00–10:00	100	51	0.51
10:00–11:00	99	60	0.61
11:00–12:00	112	132	1.18
12:00–13:00	150	145	0.97
13:00–14:00	139	—	—
14:00–15:00	146	21	0.14
15:00–16:00	167	154	0.92
16:00–17:00	36	27	0.75

[a] Sum of the total presence durations of all observed individual flies inside all observed flowers; this sum can exceed the observation period when more than one fly is observed simultaneously.

The larvae were the only animals developing in the flowers examined. No soil invertebrates were documented.

Ants frequently visited flowers to catch larvae of the pollinator and remove them, apparently for food. In a video (Appendix S2), an ant penetrates the flower chamber, and a few minutes later, leaves the flower holding a larva with its mandibles. No pollen of *Aspidistra* was found on the bodies of the ants.

***Analyses of pollen grains and dissections of insects*—**Pollen grains washed from the bodies of adult flies were counted and analyzed. Pollen grains from flies (Fig. 2D) were compared with pollen from anthers of *A. xuansonensis* (Fig. 2F). Pollen grains from anthers show a normal distribution of diameter measurements (χ^2 test = 6.16, df = 3 (adjusted), $p = 0.10$). Pollen grains from flowers not yet visited by pollinators are all spherical, uncollapsed, all stain identically, and appear fertile. Pollen grains from the bodies of Cecidomyiidi flies are identified as those of *Aspidistra,* with rare exceptions (Table 3). Only a few pollen grains were found on the bodies of *Drosophila* flies, and none were identified as belonging to *Aspidistra* (Table 3). Pollen grains attached to Cecidomyiidi flies are spherical without any damage (Fig. 3B, D). Only eggs were found in the abdomen of flies (11 or 12 eggs or more), and no solid matter was observed in the digestive tract. Large quantities of noncellular debris with drops of an oleaginous substance were found in larval digestive tracts (Fig. 4C).

***Phylogenetic relationships of the pollinator*—**DNA-barcoding by COI applied to Cecidomyiidi flies and larvae shows their near identity, which supports the notion that the larvae and the flies belong to the same species. Our sequences of larvae and flies (Appendix 1) form a well-supported and isolated clade in phylogenetic analyses performed together with the available GenBank accessions of Cecidomyiidae (Fig. 5). The aligned matrix of COI data had 439 characters; 188 positions were parsimony informative, 37 were parsimony uninformative, and 214 characters were constant. Maximum parsimony analyses recovered 74 shortest trees of 1220 steps (CI = 0.29, RI = 0.62). Bayesian inference yielded a congruent tree (Fig. 5). Our taxon appears to be related to *Sitodiplosis*, *Macrodiplosis*, *Aphidoletes*, and *Contarinia*, but precise taxonomic assessment using our molecular data are impossible due to the incompleteness of GenBank data on the Cecidomyiidae.

DISCUSSION

***Flowers of* Aspidistra xuansonensis *are pollinated by cecidomyiid flies*—**Our data demonstrate the function of cecidomyiid flies as pollinators of *A. xuansonensis*. Identification of the pollinator at the species level (which is likely a yet undescribed taxon) is problematic. Our DNA-barcoding for COI shows affinities of the pollinators with the genera *Sitodiplosis*, *Macrodiplosis*, *Aphidoletes*, and *Contarinia*. Our material also shows morphological similarities to *Contarinia*. Larvae of *Contarinia* and *Clinodiplosis* species are reported as free-living in the flowers of temperate Asteraceae and Fabaceae (Mamaev and Krivosheina, 1993). We propose that captured Cecidomyiidi flies likely belong to the genus *Contarinia* in its traditional circumscription (Gagné and Jaschhof, 2014). However, as can be seen from the molecular phylogenetic tree (Fig. 5), *Contarinia* may not be monophyletic, and generic limits in this group likely need a revision using additional molecular and morphological data.

Cecidomyiidae are a large and mostly phytophagous family of Diptera (Gagné and Jaschhof, 2014). Imago stages are commonly nonfeeding, have early mating, males die immediately after mating, whereas females live longer than males, migrate, and oviposit (Mamaev and Krivosheina, 1993). As a result, the sex ratio in a population of adult flies is shifted toward the predominance of females. In the present study, all captured flies were females. The larvae of Cecidomyiidae are often parasitic or herbivorous, develop in leaves (Stokes, 1957; Kolesik and Butterill, in press), angiosperm flowers (Traveset, 1994; Graham, 1995; Ollerton, 1996; Kolesik and Butterill, in press), or gymnosperm cones (Hedlin, 1973), and either lead to proliferation of plant tissue with the formation of galls in the case of parasitism or develop without significant deformation of plant tissues in the case of herbivory (Mamaev and Krivosheina, 1993; Gagné and Jaschhof, 2014). Adult gall midges can travel long distances only passively within an air stream. Actively, they can cover distances of up to 5 m (Kolomoets et al., 1989).

The activity of gall midges as pollinators has been reported in the literature (e.g., Thien et al., 1983, 2003; Young, 1984; Larson et al., 2001; Sakai, 2002b; Du et al., 2012). However, there are relatively few cases when gall midges are stable pollinators (Young, 1985; Feil and Renner, 1991; Feil, 1992; Ollerton, 1996; Gagné et al., 1997; Renner et al., 1997; Sakai et al., 2000; Yuan et al., 2007, 2008; Luo et al., 2010; Yukawa et al., 2011). According to our data, flies remain inside *Aspidistra* flower for a long time, similar to the behavior of gall midges that were constant pollinators (Yuan et al., 2007, 2008). The fertility of female cecidomyiids is 150–200 eggs per individual (i.e., much more than the numbers of larvae per flower found in the present study), and the majority of species visit several places for oviposition (Mamaev, 1975), which leads to cross-pollination (or possibly geitonogamy). The fact that we found some flies with as few as 10 eggs strongly suggests that the female cecidomyiid flies visiting flowers of *A. xuansonensis* use more than one flower for oviposition. Our data do not rule out the possibility of geitonogamy in *A. xuansonensis*, but we believe that it does not play a major role, because when a given plant has several flowers they open sequentially. Also, other Vietnamese species of *Aspidistra* were reported to be apparently self-incompatible (e.g., Bogner, 2005).

Larvae need food for development inside the flower. Larvae consume developing seeds of the host plant *Geum reptans* (Skuhrava et al., 2006). In the case of *Artocarpus* (Moraceae),

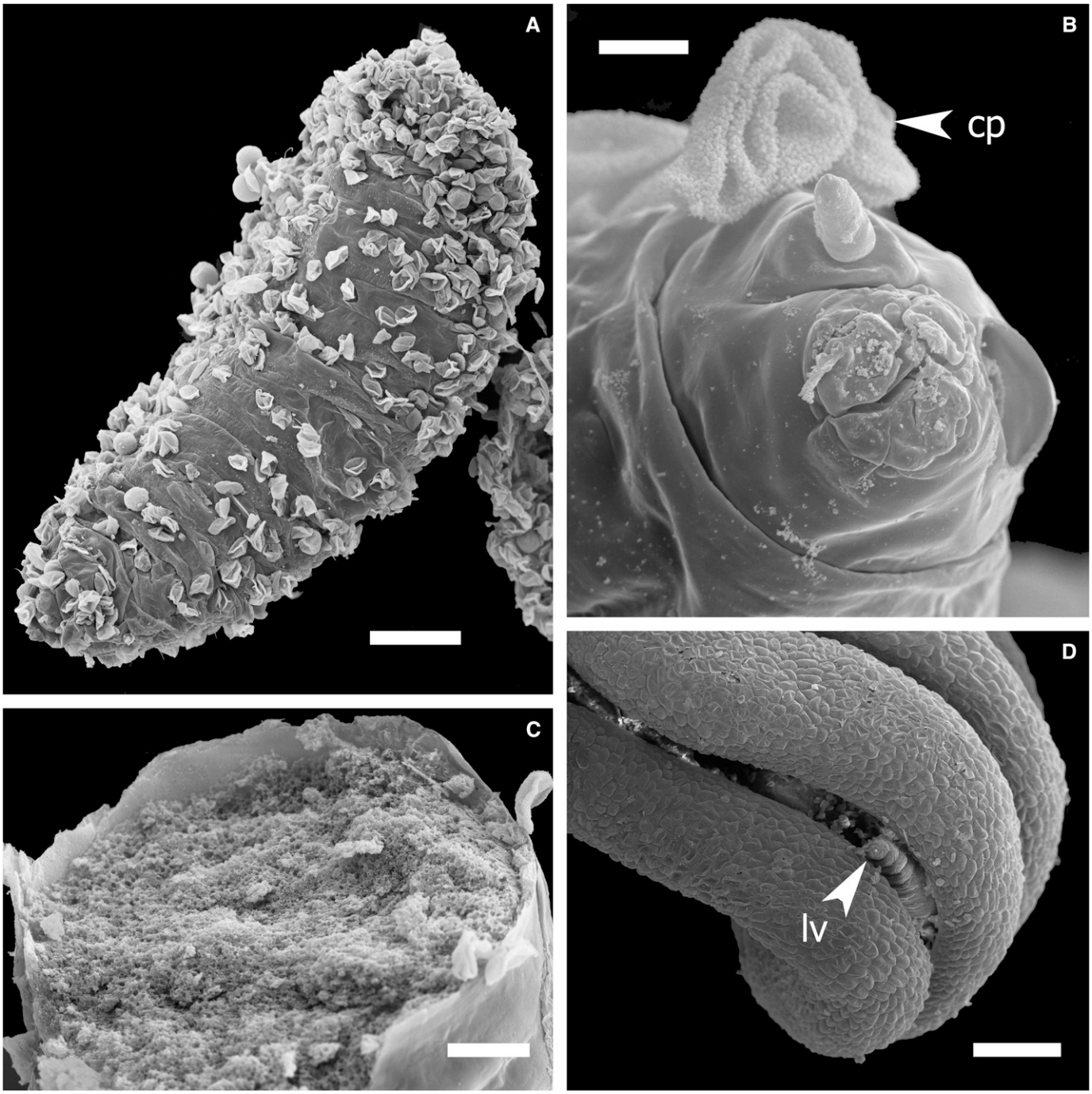


Fig. 4. Larvae of Cecidomyiidi flies from *Aspidistra xuansonensis* flowers (SEM). (A) Side view of larva covered by collapsed pollen grains. (B) Mouthparts of a larva with collapsed pollen grain. The mouthpart structure does not allow the consumption of whole pollen grains, which are too large to be ingested. (C) Cross section of a larva. Large amounts of noncellular debris with drops of oleaginous substance were found in digestive tract of the larvae. Fragments of sporoderm were not found in the digestive tract. (D) Larvae developing inside an anther with an undamaged anther wall. When all pollen grains are consumed larvae leave the anthers and ultimately the flowers. cp = collapsed pollen grain; lv = larva. Scale bars: A = 100 μm; B = 10 μm; C = 30 μm; D = 300 μm.

flies are attracted by a microscopic parasitic fungus that damages flowers, and larvae of the pollinator feed on the fungus (Sakai et al., 2000). No parasitic fungus was found in the flowers of *A. xuansonensis*, and no damage to developing seeds was recorded. *Aspidistra* flowers do not produce nectar (Buchenau, 1867; Vislobokov et al., 2013; present study), and a larval diet of nectar is not recorded in the literature (Woodcock et al., 2014). No damage to perianth, stamen filaments, anther walls, or gynoecium was found in *A. xuansonensis*, but collapsed pollen grains appear in flowers during larvae development. The presence of different types of pollen (feeding and fertilizing), as in some bee-pollinated angiosperms (Buchmann, 1983; Pacini

TABLE 3. Pollen from samples of flies.

Sample collection no.	Taxon of flies	No. flies in sample	*Aspidistra* pollen		Pollen from plants other than *Aspidistra*	
			No. pollen grains	No. grains/fly	No. grains	No. grains/fly
13101	Cecidomyiidi (Cecidomyiidae)	8	32	4	0	—
13109	Cecidomyiidi (Cecidomyiidae)	9	125	13.9	1	0.1
13112	Cecidomyiidi (Cecidomyiidae)	4	10	2.5	0	—
13113	Cecidomyiidi (Cecidomyiidae)	4	15	3.8	2	0.5
13119	Cecidomyiidi (Cecidomyiidae)	5	6	1.2	1	0.2
13120	Cecidomyiidi (Cecidomyiidae)	1	5	5	0	—
13107	*Drosophila* (Drosophilidae)	2	0	—	2	1

and Bellani, 1986; Nepi et al., 2003; Paulino et al., 2013), is unlikely in *Aspidistra* because of the absence of different stamen types and the uniform nature of pollen grains within anthers. The larvae consume the contents of the pollen grains by piercing individual grains and sucking out the contents, with a method similar to thrips and some nonpollinating fruit flies (Kirk, 1984; Gao, 2011). Consumption of whole pollen grains (as documented for Syrphidae flies: Haslett, 1983) is impossible for the larvae of Cecidomyiidae because of their extraintestinal digestion and reduction of mouthparts (Mamaev and Krivosheina, 1993) (see also Fig. 4B). Pollen-eating cecidomyiid flies pollinate some Schisandraceae (Yuan et al., 2007, 2008), but the diet of their larvae is unknown and consumption of pollen grains by adult flies has not been recorded for other cecidomyiids (Gagné and Jaschhof, 2014).

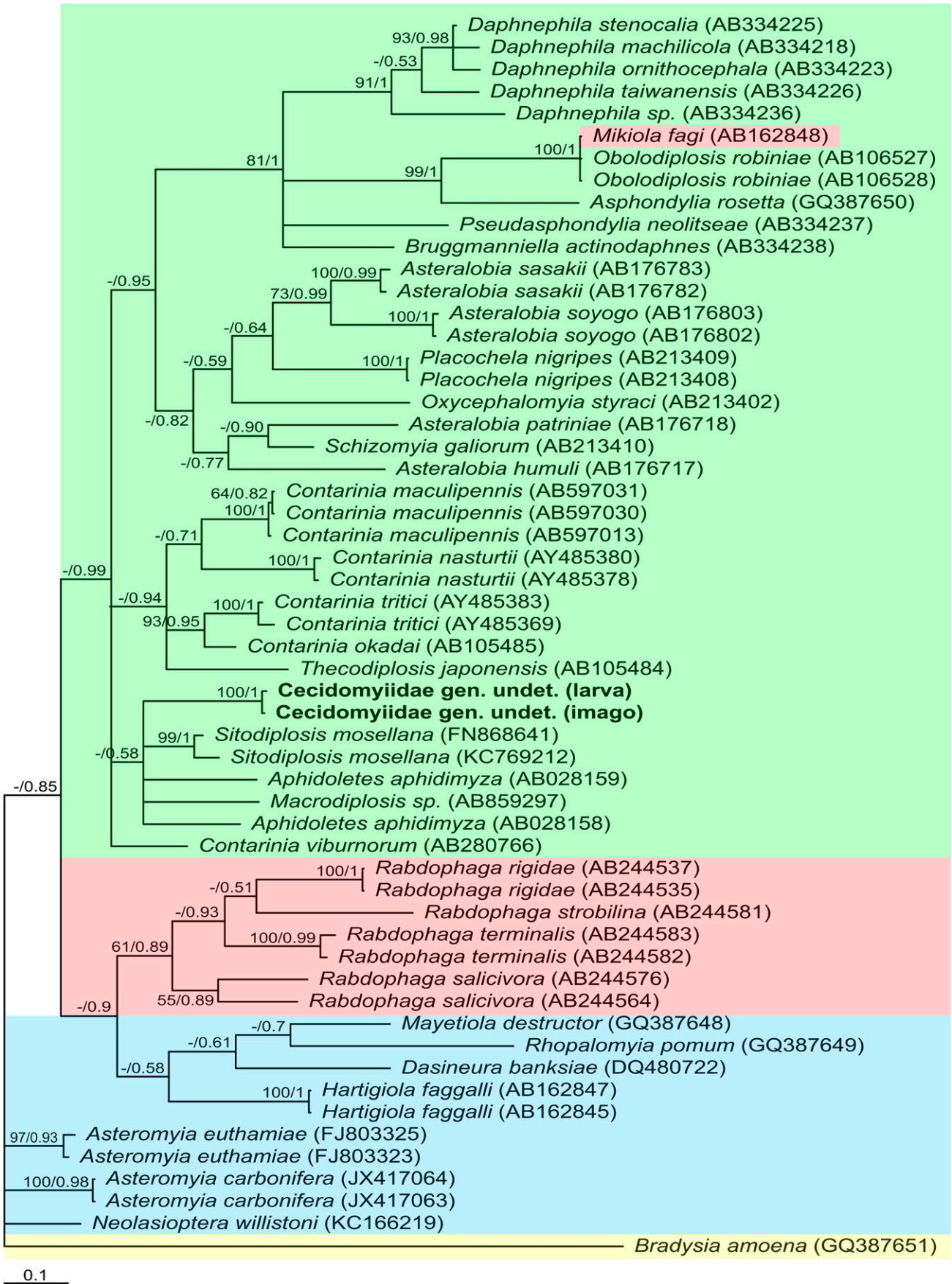
The larvae investigated in this study are phytophilic heterobionts because they migrate to soil and forest litter for pupation (Mamaev, 1975; Olfert et al., 1985; Mamaev and Krivosheina, 1993). When larvae leave the flowers of *Aspidistra* and fall onto the forest litter, the significance of this migration is likely a search for a place suitable for pupation. The food source (pollen) is fully consumed by the larvae prior to exit.

The pollination system in* A. xuansonensis *is unusual among brood site pollination in angiosperms—Pollinators breeding on flowers may be divided into three groups based upon ovipositing sites and food used by insect larvae (Sakai, 2002a). (1) Ovule parasites are recorded in only a few plant lineages, for example, fig wasps (Janzen, 1979; Wiebes, 1979) and yucca moths (Baker, 1986; Pellmyr and Thompson, 1992). Specificity of pollinators to the host plant is very high. (2) So-called pollen parasites (Sakai, 2002a) have larvae that feed on pollen grains of intact flowers attached to the plant. Consumption of pollen is common for different pollination systems (Gilbert, 1972; Faegri and Van der Pijl, 1979; O'Brien et al., 2003; Du et al., 2012), but in the majority of cases described, the pollen is consumed by adult pollinators. Larvae that develop in flowers by consuming pollen grains seem to be a rare phenomenon, though its apparent rarity could be due to insufficient knowledge of the diets of insect larvae. Sakai (2002a) mentioned larvae that develop in flowers by consuming pollen grains only for thrips. However, larvae of some flower-brooding dipteran pollinators also feed on pollen during development. For example, the chloropid fly *Elachiptera formosa* oviposits on *Peltandra virginica* (Araceae) during the female phase of the protogynous inflorescences with unisexual flowers, and larvae feed on pollen during the male phase through the ingestion of entire pollen grains (Patt et al., 1995). In contrast, thrips suck out the contents of individual pollen grains (Kirk, 1984). (3) Postpollination larval development in decomposing flowers and inflorescences takes place in some Diptera (Cecidomyiidae, Drosophilidae and Phoridae) and Coleoptera (Curculionidae and Nitidulidae) (Sakai, 2002b). The pollinator of *Aspidistra xuansonensis* falls into the group of "pollen parasites" in the classification by Sakai (2002a). Adult pollinator flies do not consume pollen grains, but pollen-eating larvae develop in the open flowers and leave the flowers for pupation before decomposition begins. This study provides the first evidence of pollen consumption by sucking the contents from individual pollen grains for Cecidomyiidae larvae.

In other angiosperms, the development of insect larvae in female (or hermaphroditic) flowers usually leads to ovule damage (Feil, 1992). When larvae of flower-brooding pollinators damage male flowers only (Feil, 1992; Sakai, 2002a; Yukawa et al., 2011), they leave female flowers undamaged to set fruit (Feller et al., 2002). In the case of mycetophagous larvae of pollinators, female flowers are not infected by the fungus, and the larvae do not develop there (Sakai, 2002a). We documented a pollination mechanism for hermaphroditic flowers in which the larvae exit before senescence of the flowers. This pollinator behavior is demonstrated here for the first time for a member of Cecidomyiidae and for *Aspidistra*. We are not aware of similar pollination systems recorded in the angiosperms. In contrast to the pollinator of *A. xuansonensis*, when larvae of other pollinators develop in anthetic angiosperm flowers or inflorescences, they pupate in the flower (Patt et al., 1995; Miyake and Yafuso, 2005).

***Myiophily is apparently diverse in* Aspidistra—**This work is the second observation of reproductive biology of *Aspidistra* in natural populations. Our previous study (Vislobokov et al., 2013) showed that *A. phanluongii* is pollinated by flies. The present results confirm the occurrence of myiophily in *Aspidistra*. Of course, these studies do not reject the possible occurrence of other types of pollinators in *A. elatior*, such as amphipods (Kato, 1995) and other invertebrates (Conran and Bradbury, 2007). Several authors have provided indirect but strong arguments in favor of myiophily in *Aspidistra* (Vogel, 1978; Endress, 1995; Bogner and Arnautov, 2004; Tillich, 2005). As *A. xuansonensis* occurs in the center of diversity of the genus and has the basic flower morphology of *Aspidistra*, it is quite likely that myiophily is present in many other species of this genus.

More than one type of fly pollination can be found in *Aspidistra* (see also Tillich, 2014). In the two systems studied in detail so far, pollinators belong to different families of Diptera. *Aspidistra phanluongii* is pollinated by flies of the genus *Megaselia* (Phoridae), while *A. xuansonensis* is pollinated by gall midges


Daphnephila stenocalia (AB334225)
Daphnephila machilicola (AB334218)
Daphnephila ornithocephala (AB334223)
Daphnephila taiwanensis (AB334226)
Daphnephila sp. (AB334236)
Mikiola fagi (AB162848)
Obolodiplosis robiniae (AB106527)
Obolodiplosis robiniae (AB106528)
Asphondylia rosetta (GQ387650)
Pseudasphondylia neolitseae (AB334237)
Bruggmanniella actinodaphnes (AB334238)
Asteralobia sasakii (AB176783)
Asteralobia sasakii (AB176782)
Asteralobia soyogo (AB176803)
Asteralobia soyogo (AB176802)
Placochela nigripes (AB213409)
Placochela nigripes (AB213408)
Oxycephalomyia styraci (AB213402)
Asteralobia patriniae (AB176718)
Schizomyia galiorum (AB213410)
Asteralobia humuli (AB176717)
Contarinia maculipennis (AB597031)
Contarinia maculipennis (AB597030)
Contarinia maculipennis (AB597013)
Contarinia nasturtii (AY485380)
Contarinia nasturtii (AY485378)
Contarinia tritici (AY485383)
Contarinia tritici (AY485369)
Contarinia okadai (AB105485)
Thecodiplosis japonensis (AB105484)
Cecidomyiidae gen. undet. (larva)
Cecidomyiidae gen. undet. (imago)
Sitodiplosis mosellana (FN868641)
Sitodiplosis mosellana (KC769212)
Aphidoletes aphidimyza (AB028159)
Macrodiplosis sp. (AB859297)
Aphidoletes aphidimyza (AB028158)
Contarinia viburnorum (AB280766)
Rabdophaga rigidae (AB244537)
Rabdophaga rigidae (AB244535)
Rabdophaga strobilina (AB244581)
Rabdophaga terminalis (AB244583)
Rabdophaga terminalis (AB244582)
Rabdophaga salicivora (AB244576)
Rabdophaga salicivora (AB244564)
Mayetiola destructor (GQ387648)
Rhopalomyia pomum (GQ387649)
Dasineura banksiae (DQ480722)
Hartigiola faggalli (AB162847)
Hartigiola faggalli (AB162845)
Asteromyia euthamiae (FJ803325)
Asteromyia euthamiae (FJ803323)
Asteromyia carbonifera (JX417064)
Asteromyia carbonifera (JX417063)
Neolasioptera willistoni (KC166219)
Bradysia amoena (GQ387651)
93/0.98
-/0.53
91/1
81/1
100/1
99/1
-/0.95
100/0.99
73/0.99
100/1
-/0.64
-/0.59
100/1
-/0.82
-/0.90
-/0.77
64/0.82
100/1
-/0.71
100/1
-/0.99
-/0.94
100/1
93/0.95
100/1
99/1
-/0.58
-/0.85
100/1
-/0.51
-/0.93
100/0.99
61/0.89
55/0.89
-/0.9
-/0.7
-/0.61
-/0.58
100/1
97/0.93
100/0.98
0.1

(Cecidomyiidae). Current knowledge suggests that pollination systems differ in the degree of pollinator specificity. For example, the orchid *Rhizanthella gardneri* with subterranean flowers is pollinated by termites, wasps, gall midges (Cecidomyiidae), and *Megaselia* flies (Dixon, 2003). On the other hand, flowers of *Aristolochia littoralis* occurring at ground level are specifically pollinated by males of *Megaselia* flies (Hall and Brown, 1993). It is possible that pollinator specificity is high in *A. phanluongii* and *A. xuansonensis* because all captured pollinators belong to a single species in both cases. The two pollination systems described in *Aspidistra* differ considerably because *Megaselia* flies do not oviposit in flowers of *A. phanluongii*. With further research, we will test hypotheses on the occurrence of taxon-specific interactions with pollinators and their possible roles in the evolution of *Aspidistra*.

***Evidence of tritrophic interactions in flowers of* Aspidistra—**This study documented regular flower visits by ants capturing pollinator larvae. The ants collected were taxonomically homogenous, and videos demonstrate similar behavior for all ants visiting *A. xuansonensis* flowers. These data indicate the occurrence of three-sided ecological interactions in which pollinator larvae feed on pollen and ants act as predators. We do not have any evidence of ant contribution to pollination. The floral construction and pollination biology of *Aspidistra* appears to expose pollinators to predators. Pollinators spend considerable time within the floral chamber, and they cannot leave it rapidly because the gaps between the perianth tube and the stigma are too narrow. Because the flowers are located at ground level, pollinators and their larvae can be easily found by nonflying predators such as ants. Vislobokov et al. (2013) noted that ants make regular visits to anthetic flowers of *Aspidistra phanluongii* and do not act as pollinators. The flies pollinating this species do not oviposit in the flowers, but one can hypothesize that the ants capture tiny soil invertebrates, which sometimes occur in flowers of this species. Ants were also recorded on flowers of the related genus *Tupistra* (e.g., *Tupistra khangii*), where their role could be similar (Vislobokov et al., 2014c). Hildebrand (1870) suggested that some spiders can catch flies as they leave *Aspidistra* flowers. His observations were made on cultivated material, but some of our preliminary observations on *A. phanluongii* confirm the occurrence of similar interactions in the field (N. A. Vislobokov, unpublished data). Various small soil arthropods can enter *Aspidistra* flowers for various reasons because the flowers are so close to the ground (Kato, 1995; Conran and Bradbury, 2007). Soil arthropods were, however, completely absent from the flowers of *A. xuansonensis* investigated here, probably because they are slightly elevated above the soil.

Conclusions—Flowers of *A. xuansonensis* are pollinated by adult female cecidomyiid flies. The flies use flowers as brood sites. They oviposit on anthers, so that larvae develop among the pollen mass. Pollen-eating larvae develop in open hermaphroditic flowers and feed on fertile pollen grains by sucking out their contents. Dimorphism of the flowers, stamens, or pollen grains related to the feeding of larvae is not recorded in *A. xuansonensis.* Larvae leave the flowers for pupation before decomposition starts. To our knowledge, this combination of characters has not been reported from other pollination systems described in the angiosperms. The types of fly pollination in *Aspidistra* are diverse. Further studies are needed to determine the degree of coadaptation between various species of *Aspidistra* and their pollinators. The construction and biology of *Aspidistra* flowers make them attractive as sites of food accumulation for predators. In the case of *A. xuansonensis*, ants were observed regularly capturing developing larvae of the pollinator.

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Fig. 5. Molecular barcoding of the flies pollinating *Aspidistra xuansonensis* and larvae developing in the flowers of this species. Congruent tree of the maximum parsimony analyses (CI = 0.2943, RI = 0.6215) and the Bayesian inference for available accessions of Cecidomyiidae. A member of Sciaridae (yellow) is used as an outgroup. Figures above the branches are bootstrap support values / posterior probabilities. The tree is based on the aligned matrix of COI data containing 439 characters. GenBank accession numbers are provided after taxon names. Branch lengths are proportional to the numbers of the expected nucleotide substitutions. Accessions generated for the present study are labeled Cecidomyiidae gen. indet. The tree contains members of subfamily Cecidomyiinae–supertribe Cecidomyiidi (green) and supertribe Lasiopteridi (blue), and subfamily Lestremiinae–supertribe Lestremiidi (red). The supertribe Cecidomyiidi is monophyletic (except the accession of *Mikiola fagi*, that may be based on a misidentified specimen). The tree supports taxonomic uniformity of the larvae and adult flies collected from flowers of *A. xuansonensis.* Our taxon belongs to supertribe Cecidomyiidi and appears to be related to *Sitodiplosis*, *Macrodiplosis, Aphidoletes*, and *Contarinia*.

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APPENDIX 1. Accessions of Cecidomyiidi flies used in molecular study and GenBank accession numbers for CO1 sequences.

Taxon	Locality	Collection date	Voucher	GenBank accession
Cecidomyiidi (imago)	Vietnam, Phu Tho Province, Thanh Son District, Xuan Son National Park, 355 m a.s.l., N 21°07.988′, E 104°56.579′	7 Nov. 2013	*N.A.Vislobokov 13109F*	KM023673
Cecidomyiidi (larva)	As above	10 Nov. 2013	*N.A.Vislobokov 13121L*	KM023672