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Pollinator Assemblages of *Arisaema heterocephalum* subsp. *majus* (Araceae), a Critically Endangered Species Endemic to Tokunoshima Island, Central Ryukyus

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Abstract The pollinator assemblages of a critically endangered aroid species endemic to Tokunoshima Island (Central Ryukyus, Japan), *Arisaema heterocephalum* subsp. *majus*, were studied. The insects trapped inside the spathes were collected in the native populations. Tentative identification of the collected insects was made using DNA barcoding based on mitochondrial cytochrome oxidase subunit I (COI). Sciaridae was the most abundant family collected, followed by Mycetophilidae, suggesting that *A. heterocephalum* subsp. *majus* depends its pollination largely on fungus gnats as reported in the other species of the genus *Arisaema.*

Key words: *Arisaema*, fungus gnat, pollination, Sciaridae, Tokunoshima.

Introduction

The genus *Arisaema* is one of the most species rich plant groups native to Japan archipelago (Iwatsuki *et al.*, 2016). Murata *et al.* (2018) recognized 64 taxa (53 species, 9 subspecies, and 2 varieties) native to Japan, of which 58 taxa are endemic. Although infrageneric taxonomy of the genus remains controversial, Ohi-Toma *et al.* (2016) proposed subdivision of the genus into 15 sections based on the molecular phylogeny. According to their system, the species in Japan are classified in three sections out of the 15 sections, i.e., *Pistillata* (56 taxa), *Flagellarisaema* (4 taxa) and *Clavata* (4 taxa). Among them, sect. *Clavata* in Japan is characteristic in that the distribution is restricted to isolated islands and it is not native to the mainlands (Hokkaido, Honshu, Shikoku, and Kyushu islands). *Arisaema heterocephalum* Koidz. in sect. *Clavata* is distributed in the Ryukyu Islands, Japan and comprises three subspecies (Murata and Ohashi, 1980; Serizawa, 1982; Murata, 1985;). *A. heterocephalum* subsp. *majus* (Seriz.) J.Murata is endemic to the limestone area in the small western part of Tokunoshima Island of Central Ryukyus. The Red List of Japan and the IUCN Red List categorize this taxon as Critically Endangered and Endangered, respectively, although the latter regards it as the synonym of *A. heterocephalum* subsp. *heterocephalum* (Ministry of the Environment, Japan, 2015, 2016). In fact, the number of the flowering individuals is very small (probably <100 based on authors' personal observation) in the native populations of this species (henceforce we regard this subspecies as "species"). Thus, the chance of sexual reproduction is limited. Because accessory buds of tubers usually do not geminate unless the tubers are split (Murata, 1984; Murata *et al.*, 2018), sexual reproduction by seeds is important for *A. heterocephalum* subsp. *majus* to sustain its population. Nevertheless, the basic knowledge on its sexual reproduction is almost lacking, although it is essential for designing the conservation strategy. Therefore, to understand its pollination system in the native habitat, here

we surveyed the insects trapped inside the spathes of *A. heterocephalum* subsp. *majus*.

Materials and Methods

Insect collection and DNA extraction

We surveyed the insects trapped inside the spathes of *Arisaema heterocephalum* subsp. *majus* in the two native populations (Isen 1 and Isen 2), which are located close (ca. 0.3 km) to each other in Isen Town, Tokunoshima Island (precise localities are not shown for conservation). The population Isen 1 is located in a habitation area nearby a small building and farmland, while the population Isen 2 is located in a secondary forest. We visited these populations on February 8, 2018, and plugged cotton into the exit hole of the spathes of the male individuals not to allow insects to escape. The spathes of the female individuals do not have the exit hole so that the plugging procedure is not necessary. Because the male inflorescences were allowed to trap insects for several days, we revisited these populations on February 11, 2018, to collect the trapped insects from both male and female individuals. This procedure enables us to calculate average insect visits per day on an individual spathe of the male. Insects were stored in 100% ethanol for the subsequent analysis. To check natural fruit set of the species, we made a follow-up inspection for the two female flowering individuals in Isen 1 on May 16 and June 27, 2018.

Tentative identification of the collected insects was made using DNA barcoding based on mitochondrial cytochrome oxidase subunit I (COI). A leg of the adult gnats was used for DNA extraction. Tissue was smashed in 20 µl of quick extraction buffer (16 mg/ml Chelex-100 [Bio-Rad Laboratories, Hercules, CA] and 1.25 mg/ml proteinase K [Wako, Osaka, Japan] in distilled water), and then incubated at 55ºC for 3 h and heat inactivated at 95ºC for 10 min. A 0.5 µl supernatant of the solution was then used directly for polymerase chain reaction (PCR) using EmeraldAmp PCR Master Mix (TaKaRa, Shiga, Japan) with forward primer LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3″) and reverse primer HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). The temperature settings for the PCR was as following; the initial denaturation at 98ºC for 1 min, and 35 cycles of 98ºC for 10 sec, annealing at 55ºC for 30 sec, and extension at 72ºC for 1 min. The amplified DNA fragments were subjected to direct Sanger sequencing using the Applied Biosystems 3130xl or 3500xl Genetic Analyzer (Applied Biosystems, MA, USA).

The remaining extraction buffer was kept at −30ºC for future use, and the materials used in the present study were stored in National Museum of Nature and Science, Japan. Nucleotide sequences newly obtained in this study are deposited in DDBJ under accession numbers LC422844–LC422945.

Phylogenetic Analysis and Taxonomy

Phylogenetic analysis was conducted by PAUP* 4.0a162 (Swofford, 2018) using a Neighbor-Joining (NJ; Saitou and Nei, 1987) method with uncorrected p-distance. To obtain branch support, 1000 bootstrap replicates were generated.

Temporal species delimitation of the collected insects was made based solely on the nucleotide sequences, with uncorrected distance of 4% was used as the threshold for differentiating species (Okuyama *et al.*, 2018). The insect identification to family or generic level, where possible, was made based on morphology.

Results

We surveyed four female and 14 male flowering individuals of *A. heterocephalum* subsp. *majus* in Isen 1 population, while we found only three male flowering individuals and surveyed them in Isen 2 population. On average, 5.2 insect individuals were found in the individual spathe of the male and female plant, and 1.8 insect individuals per day were trapped in the male plant (Fig. 1A, B). In total, we collected 109 insect individuals from the spathes and we succeeded to

Fig. 1. Pollination biology of *Arisaema heterocephalum* subsp. *majus*. A: A fungus gnat (Mycetophilidae) trapped inside a spathe of the male inflorescence. Note that the insect is heavily coated with pollen grains. B: Fungus gnats trapped inside a spathe of the female inflorescence. C: Two fruiting female individuals on May 16, 2018. D: The same fruiting individuals on June 27, 2018.

Table 1. Insects collected from the spathes of *Arisaema heterocephalum* subsp. *majus*

Order	Family	Species	Number of individuals			Total
			Isen 1 Male	Isen 1 Female	Isen 2 Male	
Diptera	Cecidomyiidae	Cecidomyiidae sp. 1	1	1	0	2
		Cecidomyiidae sp. 2	0	1	0	1
		Cecidomyiidae sp. 3	1	0	2	3
	Ceratopogonidae	Ceratopogonidae sp. 1	1	0	0	1
	Drosophilidae	Drosophilidae sp. 1	0	0	1	1
	Mycetophilidae	*Rondaniella* sp.	9	2	0	11
		Phronia sp. 1	1	0	0	1
		Phronia sp. 2	0	0	1	1
		Mycetophilidae sp. 1 (c.f. *Brevicornu*)	1	0	0	1
		Mycetophilidae sp. 2 (c.f. *Exechia*)	1	0	0	1
	Phoridae	Phoridae sp. 1	1	0	0	1
	Psychodidae	*Psychoda alternata*	1	0	0	1
	Psychodidae	Psychodidae sp. 1	1	1	0	2
	Sciaridae	*Bradysia impatiens*	1	0	0	1
		Sciaridae sp. 1	5	1	0	6
		Sciaridae sp. 2	2	0	0	2
		Sciaridae sp. 3	4	1	0	5
		Sciaridae sp. 4	19	1	0	20
		Sciaridae sp. 5	3	0	0	3
		Sciaridae sp. 6	3	7	0	10
		Sciaridae sp. 7	19	1	0	20
		Sciaridae sp. 8	0	0	1	1
		Sciaridae sp. 9	1	0	0	1
		Sciaridae sp. 10	0	1	0	1
		Sciaridae sp. 11	1	0	0	1
		Sciaridae sp. 12	1	0	0	1
	Tipulidae	Tipulidae sp. 1	1	0	0	1
Hymenoptera	Braconidae	Braconidae sp. 1	0	0	1	1
Entomobryomorpha	Paronellidae	Paronellidae sp. 1	1	0	0	1
Unidentified		Unidentified spp.	5	0	3	8

obtain DNA sequences of the mitochondrial COI gene from 102 individuals. Overall, 29 arthropod species were found therein (Table 1). Among these insects, the most abundant family was Sciaridae (72 individuals), followed by Mycetophilidae (15 individuals). Not only these fungus gnats were most abundant, but also their body size is generally larger (>3 mm) than the other flower visitors (<2 mm except for Tipulidae), This indicates that the fungus gnats are able to carry much more pollen loads than the others because they are more likely to contact with anthers and stigma in the spathe and simply because their body surface area is much larger. Multiple individuals were obtained only for two cecidomyid species, one psychodid species, seven sciarid species and one mycetophilid species (Table 1, Fig. 2). Eight out of 11 species with multiple individuals were collected in both male and female plant. We confirmed that both of the two female individuals which we made a follow-up inspection set fruits.

Discussion

Arisaema heterocephalum subsp. *majus* is one of the most threatened taxa of the genus in Japan, with the number of flowering individuals in the wild being extremely scarce. Nevertheless, the present study revealed that the plants still have the potential to reproduce sexually as the pollinating insects, fungus gnats (Sciaridae and Mycetophilidae), were abundant in its native habitat.

Although pollinators have been surveyed for many species in *Arisaema* from Japan (Sasakawa, 1993, 1994; Nishizawa *et al.*, 2005; Tanaka *et al.*, 2013), Nepal (Vogel and Martens,

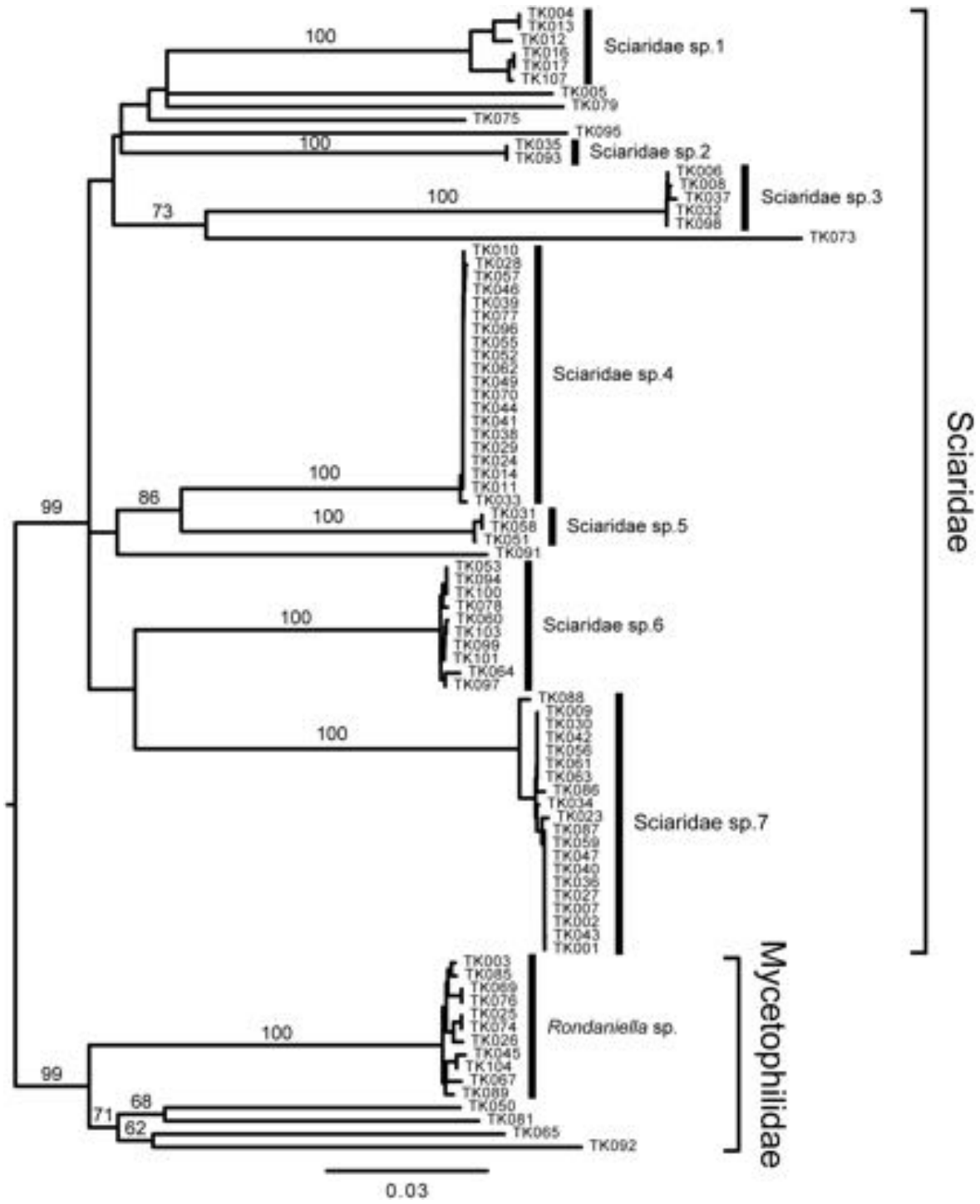

Fig. 2. A neighbor-joining tree of 87 fungus gnat (Sciaridae and Mycetophilidae) individuals collected in the present study based on 624-bp of mitochondrial cytochrome oxidase subunit I gene. Bootstrap supports for nodes above the species-level are shown (only when ＞50%).

2000) and North America (Barriault *et al.*, 2009, 2010), this study is probably the first report for the species of sect. *Clavata*. We confirmed that the major pollinators of *A. heterocephalum* subsp. *majus* were fungus gnats (Mycetophilidae and Sciaridae). The finding is similar to the pollinators reported in the other species. Most pollinator species were observed in both male and female plants (Table 1), even though the morphology of *A. heterocephalum* subsp. *majus* is different between male and female [i.e., female individuals have larger inflorescence, shorter peduncles, and many horn-like protuberances at the base of spadix-appendix inside spathe (Murata and Ohashi, 1980; Serizawa, 1982; Murata, 1985)]. We made a follow-up inspection for the two female flowering individuals, and confirmed that both of them set fruits successfully on June 27, 2018 (Fig. 1C, D). Therefore, although the species is rare, seed reproduction was likely to be still effective to sustain the natural populations. Because the limiting factor of seed reproduction seems to be the number of flowering individuals, especially that of females requiring better growth condition (Kinoshita, 1986), clarifying the environmental conditions for flowering of this species would be important for the conservation.

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