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Floral Visitors of Critically Endangered *Arisaema cucullatum* (Araceae) Endemic to Kinki Region of Japan

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Abstract *Arisaema* (Araceae) is one of the most species-rich plant groups in Japan, while 20 out of 64 *Arisaema* taxa are categorized as endangered species (CR or EN) in the Red List of Japan. Although we need to comprehend their reproductive biology to conserve these species, the knowledge regarding their pollination systems is still limited. In this study, we examined the potential pollinators of *A. cucullatum*, a critically endangered species endemic to Kinki Region of Japan. To this end, we collected the insects trapped inside the spathes of *A. cucullatum* as well as the two sympatric *Arisaema* species in the two native populations, and identified by both morphology and DNA barcoding based on mitochondrial cytochrome oxidase subunit I (COI). Sciaridae were the most abundant family in the trapped insects of *A. cucullatum*, and one sciarid species accounted for 78.9% of the collected insects. Meanwhile, the insect assemblages trapped inside the spathes of *A. kishidae* and *A. yamatense* that grow sympatrically with *A. cucullatum* were distinct from those of *A. cucullatum*. Accordingly, the difference in the pollinator assemblages may be important for reproductive isolation among them, although further observation in different populations is needed.

Key words: *Arisaema*, conservation biology, fungus gnats, Nara Prefecture, pollination, reproductive isolation, Sciaridae.

Introduction

Japanese *Arisaema* (Araceae) comprising 64 taxa is one of the most species-rich plant groups in Japan (Iwatsuki *et al.*, 2016; Murata *et al.*, 2018). Moreover, 20 out of 64 taxa are categorized as endangered species (CR or EN) in the Red List of Japan (Ministry of the Environment, Japan, 2015). Even though in the increasing concern for the sustainability of these species, conservation measures for these plants have been hardly performed. For example, although we need to comprehend the reproductive biology of these endangered species to establish their conservation strategies, the knowledge regarding the pollination system of the species in Japan is still based on the pollinator records for only ＜10% of the diversity (5 spp.; Sasakawa, 1993, 1994a, 1994b; Nishizawa *et al.*, 2005; Tanaka *et al.*, 2013; Kakishima and Okuyama, 2018).

In this study, we examined floral visitors of an endangered species, *Arisaema cucullatum* M. Hotta, which belongs to sect. *Pistillata* (Fig. 1A, B). The sect. *Pistillata* is the largest group (54 taxa) in Japanese *Arisaema* (Murata *et al.*, 2018), but the pollinators in this group were reported

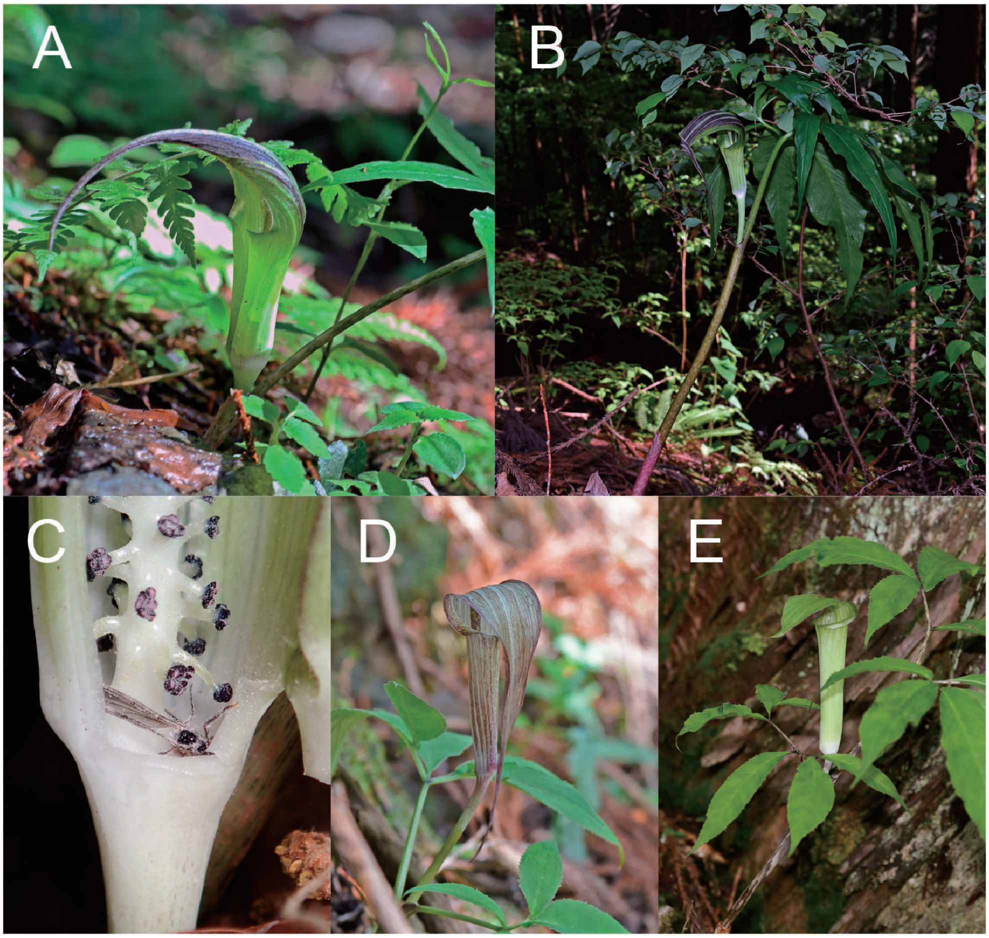

Fig. 1. The three *Arisaema* species studied in the present study in its native habitat. A. *A. cucullatum* with male inflorescence. B. *A. cucullatum* with female inflorescence. C. The trapped sciarid *Leptosciarella* sp. in the spathe of *A. cucullatum*. D. *A. kishidae* with male inflorescence. E. *A. yamatense* with male inflorescence.

from only two species, *A. serratum* (Thunb.) Scott (Sasakawa, 1993, 1994a, 1994b; Nishizawa *et al.*, 2005) and *A. yamatense* (Nakai) Nakai (Sasakawa, 1993). Most species of the sect. *Pistillata* in Japan are closely related to each other and there are many similar species (Murata, 1995). *Arisaema cucullatum* is similar to *A. seppikoense* Kitam. in several vegetative characters but distinguished by the incurved cucullate spathe (Hotta, 1963) (Fig. 1A, B). The distribution of *A. cucullatum* had been originally known in only Nara and Mie Prefectures in Kinki Region of Japan (Hotta, 1963). A population in Wakayama Prefecture next to Nara and Mie Prefectures has recently been found (Naito *et al.*, 2015). All the Red Lists of Japan and Nara, Wakayama and Mie Prefectures categorize this species as Critically Endangered (Wakayama Prefecture, 2012; Ministry of the Environment, Japan, 2015; Mie Prefecture, 2015; Nara Prefecture, 2016). In fact, the number of flowering individuals in the Wakayama population was less than 10 in these years (Naito *et al.*, 2015). In addition, *A. cucullatum* does not vegetatively reproduce through accessory buds so that pollinator-mediated reproduction is essential for the *in-situ* conservation of this species, although the pollinators have never been reported. Therefore, we surveyed the insects trapped inside the spathes of *A. cucullatum*, and compared their composition with those of two *Arisaema* species belonging to sect. *Pistillata*, *A. kishidae* and *A. yamatense*, growing sympatrically with *A. cucullatum* to examine if the potential pollinator assemblages of *A. cucullatum* is distinct from these species (Fig. 1D, E).

Materials and Methods

Insect collection

We surveyed the insects trapped inside the spathes of *Arisaema cucullatum*, *A. kishidae* and *A. yamatense* in the two populations (Site 1 and Site 2) in Tenkawa Village, Nara Prefecture (precise localities are not shown for conservation). The site 1 is located in a plantation forest of *Cryptomeria japonica* and a deciduous broad-leaved forest. The site 2 is located in a deciduous broad-leaved forest along a road and is 2 km away from the site 1.

Most species of the sect. *Pistillata* make male or female inflorescence every year depending on the plant size (Schaffner, 1922; Maekawa, 1924; Kinoshita, 1986; Takasu, 1987). The exit holes of the spathes found in only the male inflorescence. A cotton plug was installed for preventing insects going out from an exit hole (Kakishima and Okuyama, 2018). We collected insects inside 10 and four spathes and plugged the holes in the site 1 on 21 May 2019 and in the site 2 on 22 May 2019, respectively. Then, we collected the trapped insects from both male and female inflorescence on 23 May 2019. Thus, the length of the trap periods is two and one days in the site 1 and 2, respectively. The collected insects were stored in 99.5% ethanol for identification by morphology and DNA barcoding.

DNA extraction and sequencing

DNA barcoding was based on the sequences of mitochondrial cytochrome oxidase subunit I (COI). The legs of the collected insects were 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 55℃ for 3 h and heat inactivated at 95℃ 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) or ExTaq DNA polymerase (TaKaRa, Shiga, Japan) with Ampdirect (Shimadzu, Kyoto, Japan), with the forward primer LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3″) and the reverse primer HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.*, 1994). The PCR was performed as follows: the initial denaturation at 98℃ for 1 min, and 35 or 40 cycles of 98℃ for 10 s, annealing at 55℃ for 30 s, and extension at 72℃ for 1 min. The amplified DNA fragments were subjected to direct Sanger sequencing using the Applied Biosystems 3500xl Genetic Analyzer (Applied Biosystems, MA, USA). Nucleotide sequences newly obtained in this study are deposited in DDBJ under accession numbers LC500531–LC500560.

Phylogenetic analysis and identification

Species identification of the collected insects was based on both the morphology and the nucleotide sequences. Firstly we examined the collected insects by a binocular microscope (SZ, Olympus, Japan). Male genitalia of some fungus gnats were removed from the body with sharpened tweezers for species identification. They were treated with 10% KOH solution at room temperature overnight, and then observed under a stereoscopic microscope in 99.5% Ethanol. When it were difficult to identify some specimens by their morphology because of female or broken bodies, we used DNA barcoding with uncorrected distance of 4% was used as the threshold for differentiating species (Okuyama *et al.*, 2018). The results of the DNA barcording were also used for confirming identification based on the morphology.

A phylogenetic analysis was conducted by RAxML 8 (Stamatakis, 2014) using a maximum-

Table 1. The number of the collected insects from three *Arisaema* speices

			A. cucullatum			*A. kishidae*		*A. yamatense*	
			Site 1		Site 2	Site 1		Site 1	
Order	Family	Species	Male	Female	Male	Male	Female	Male	Total
Diptera	Calliphoridae	*Melanomya nana*	1	0	0	0	0	0	1
	Cecidomyidae	Cecidomyidae gen.	0	1	0	0	0	0	1
	Ceratopogonidae	Ceratopogonidae gen.	0	0	0	0	1	0	1
	Chironomidae	Chironomidae gen.	0	0	0	1	0	0	1
	Mycetophilidae	*Allodia pravdini*	0	0	0	0	1	0	1
		Allodia sp.	0	0	0	0	1	0	1
		Mycetophila ocellus	0	0	0	1	0	0	1
		Mycetophila ruficollis	0	0	0	0	1	0	1
		Mycomya fuscata	0	0	0	0	0	3	3
		Mycomya neodentata	0	0	0	0	1	0	1
	Sciaridae	*Corynoptera* sp.	0	0	0	1	0	0	1
		Ctenosciara insolita	0	2	0	0	0	0	2
		Leptosciarella sp.	5	2	8	0	0	0	15
Total			6	5	8	3	5	3	30

Table 2. Pollinator visits to *Arisaema cucullatum* during 21–23 May 2019

Sex	Site	Number of inflorescence	Number of trapped insects	Visits per inflorescence	Visits per inflorescence per day
Male	Site 1	9	6	0.67 ± 1.12	0.33
	Site 2	4	8	2.00 ± 2.83	2.00
	Subtotal	13	14	1.08 ± 1.80	0.85
Female	Site 1	1	3	3.00	1.50
Total		14	17	1.21 ± 1.81	0.89

likelihood (ML) method with a GTR + G likelihood model for nucleotide substitutions. The ML tree and bootstrap proportions (BPs) were obtained by simultaneously running rapid bootstrapping with 1,000 iterations followed by a search for the most likely tree.

Results

We found 12 and four flowering individuals of *A. cucullatum* in the site 1 and 2, respectively. Among them, one female and nine male flowering individuals in the site 1 and four male in the site 2 were surveyed. In total, we collected 19 insect individuals from the spathes of *A. cucullatum* (Table 1; Fig. 1C). Two and three insect individuals were collected from the only female plant individual in the site 1 on 21 and 23 May, respectively. Six and eight insect individuals from the male inflorescence were collected in the site 1 and 2 on 23 May, respectively. On average, 1.21 insect individuals were found in the individual spathe of *A. cucullatum* on 23 May, and therefore 0.89 insect individuals per day were supposed to be trapped (Table 2).

Overall, 13 dipteran species were found in this study (Table 1; Fig. 2). In *A. cucullatum*, Sciaridae was the most abundant flower visitor (17 out of 19 individuals), in which two species, *Leptosciarella* (*Leptosciarella*) sp. and *Ctenosciara insolita* (Sasakawa), were recorded. *Leptosciarella* sp. was found in both sites and from both male and female, accounting for 78.9% (15 individuals) of the collected insects. Other than Sciaridae, one individual of *Melanomya nana* (Meigen) (Calliphoridae) and one individual of Cecidomyiidae were collected in *A. cucullatum*. In contrast, Mycetophilidae was most abundant among the insects in *A. kishidae* (five out of eight individuals) and in *A. yamatense* (all three individuals). The species of the collected insects were not overlapped among the three *Arisaema*

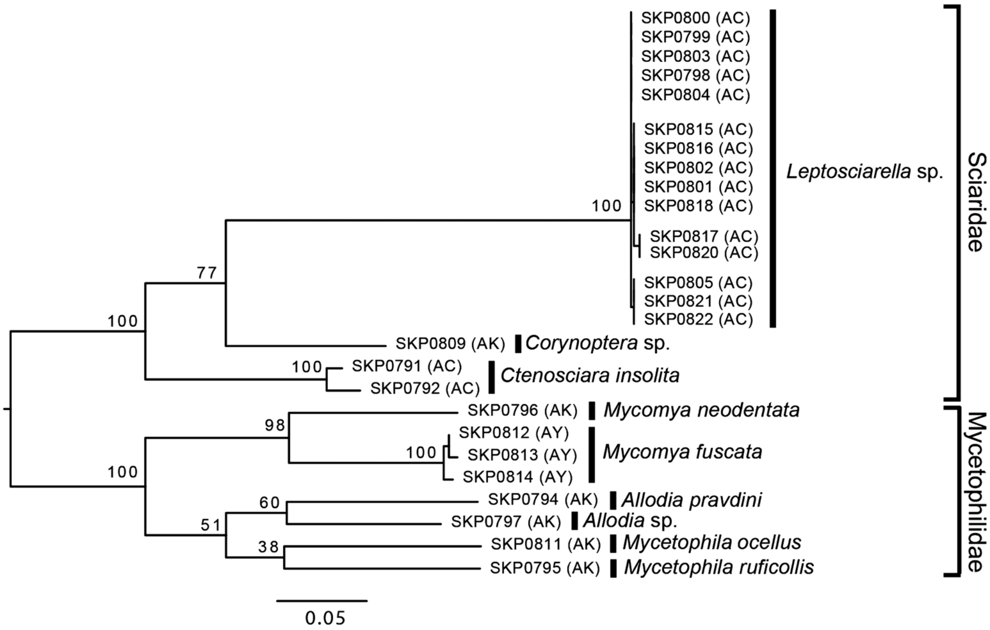

Fig. 2. A maximum likelihood tree of 25 individuals of fungus gnats (Sciaridae and Mycetophilidae) based on 617-bp of mitochondrial COI. Bootstrap supports are shown for nodes above the species-level. Each OTU label represents the sample name followed by the *Arisaema* species from which the sample was collected, i.e., *A. cucullatum* (AC), *A. kishidae* (AK) and *A. yamatense* (AY).

species.

Discussion

The principal flower visitors of three *Arisaema* species studied in the present study, namely *A. cucullatum*, *A. kishidae* and *A. yamatense*, were fungus gnats (Mycetophilidae or Sciaridae) as in the cases reported previously in other *Arisaema* species (Sasakawa, 1993, 1994a, 1994b; Vogel and Martens, 2000; Nishizawa *et al.*, 2005; Barriault *et al.*, 2009, 2010; Tanaka *et al.*, 2013; Kakishima and Okuyama, 2018). Other than the two sciarid species, only a few insect individuals were found to visit to the inflorescence in *A. cucullatum* (Table 1). This result suggests that *A. cucullatum* may rely on specific groups of sciarids for pollination. *Leptosciarella* sp. was the dominant species trapped in *A. cucullatum*, accounting for 78.9% of the collected insects (Table 1). In addition, although the morphology of *A. cucullatum* is different between male and female as in the case of other *Arisaema* species (Murata, 1986), i.e. female individuals have longer pseudostems (Fig. 1A, B), *Leptosciarella* sp. was found in the inflorescences of both sexes. Taken together, *Leptosciarella* sp. is likely to function as the most important pollinator of *A. cucullatum* in these study sites. Another sciarid species trapped in the inflorescences of *A. cucullatum* is *Ctenosciara insolita* (Table 1), which was also reported as the flower visitor of *A. serratum* in Toyama Prefecture [Sasakawa (1994b), as *Phytosciara* (*Dolichosciara*) *insolita*]. Although the species name *Arisaema serratum* was sometimes applied to several morphological types or taxa (*A. serratum sensu lato*) depending on the literatures (Murata, 1995), it is obvious that *A. cucullatum* is distinct from *A. serratum sensu lato* (Ohashi and Murata, 1980). Therefore, *C. insolita* was found to have relationships with multiple *Arisaema* species in two distant locations, although the mechanism underlying the relationships was unknown. A blow fly, *Melanomya nana*, is newly recorded from the inflorescence of *Arisaema*. The body size of *Melanomya* *nana* is larger than a hole of the spathe of *A. cucullatum*, which would not allow the fly to go out from the hole. Therefore, *M. nana* does not seem to be an effective pollinator.

Meanwhile, the principal flower visitors of *A. kishidae* and *A. yamatense* were mycetophilids (Table 1). Three individuals of *Mycomya fuscata* (Mycetophilidae) were collected from *A. yamatense* (Table 1). The result is congruent with the independent record of *Mycomia ornata* from *A. yamatense* in Kyoto Prefecture (Sasakawa, 1993), suggesting that the genus *Mycomia* might play important roles as the pollinators of *A. yamatense*. *Mycomia neodentata* collected from *A. kishidae* was different from *Mycomya* species collected from *A. yamatense*. Both *M. fuscata* and *M. neodentata* are newly recorded in Japan. Other mycetophilids collected from *A. kishidae* also includes rare or new records in Japan. *Allodia pravdini* is new to the Japanese fauna of fungus gnats and *Mycetophila ocellus* is the third record in Japan since Okada (1939, 1940).

Judging from the fact that most species of the sect. *Pistillata* are endemic and closely related to each other, it is most likely that the diversification of this group has centered in the Japanese Archipelago (Murata, 1995; Murata and Kawahara, 1995). In fact, a recent phylogenetic study using chloroplast DNA sequences revealed that there is only very small genetic variation in this group, including *A. cucullatum* (Ohi-Toma *et al.*, 2016). Therefore, it is of special interest to understand how these diverse species establish reproductive isolation among them in order to elucidate the mechanisms underlying the remarkable speciation. Although the number of the collected insects was not sufficient, the current study supported the view that the mechanisms of reproductive isolation among sympatric, closely-related *Arisaema* species involve pollinator difference, based on the observation that the three *Arisaema* species do not have common pollinator species in the two native populations. Further observation in different populations would be needed to confirm if this view on pollinator-isolation in Japanese *Arisaema* is appropriate. Apart from pollinator isolation, the difference in the flowering season may also play an important role in reproductive isolation (Murata *et al.*, 2018). In fact, *Arisaema cucullatum* was at the early flowering stage but *A. kishidae* was at the late stage in the study period.

In the present study, we could only find 16 flowering individuals of *A. cucullatum* in the two study sites, confirming that *A. cucullatum* is indeed critically endangered. So it is crucial to identify the environmental factors for their successful reproduction. To this end, the observation that the number of trapped pollinators per day was higher in the site 2 (2.00 individuals per day) than the site 1 (0.33 individuals per day) (Table 2) might provide some hints, although both study sites are common in that they were located on humid slopes along a river in a limestone area. The present observation also implies that *A. cucullatum* may rely on specific taxonomic groups of Sciaridae for their pollination (Table 1). If this view is correct, we need to elucidate the environmental factors suitable not only for the plants' growth but also pollinators' abundance for making conservation strategy.

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