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Herbarium Specimens Reveal the History and Distribution of Seed-feeding Fly Infestation in Native Japanese Orchids

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Abstract Fly infestation in the capsules of various wild orchids has become a serious issue for conservation in Japan in recent years. Herbivory caused by flies is easily recognized through the observation of coarctate pupa just beneath the pericarp or remnant puparium following adult emergence. We examined the capsules of herbarium specimens of five orchid species [*Cephalanthera erecta* (Thunb.) Blume, *C. falcata* (Thunb.) Blume, *C. longibracteata* Blume, *C. longifolia* (L.) Fritsch, and *Cypripedium japonicum* Thunb.] in eight herbaria. We found that 63 of 194 fruiting specimens (32.5%) were damaged by seed-eating flies. The morphology of the coarctate pupa, puparium, and emergence holes were indicative of *Japanagromyza tokunagai* (Sasakawa). The oldest infested capsules were collected in 1891. Since then, fly-infested specimens have been collected throughout Japan, from Hokkaido Prefecture in the north to Kagoshima Prefecture in the south. We suggest that current-day severe infestation damage on orchids is not due to the population increase or geographic expansion of recently introduced flies but rather to the population increase in a native species that is widely distributed across Japan.

Keywords: Agromyzidae, herbarium, Orchidaceae, seed-feeding fly, spatio-temporal analysis.

Introduction

Fly infestation of capsules or ovaries of wild orchid species presents a conservation issue in Japan (reviewed in Ogura-Tsujita *et al.*, 2019). The populations of many Japanese orchid species have sharply declined in recent years; 67% of these species are considered endangered by the Japanese Ministry of Environment (Ministry of the Environment, 2020). No seeds are produced when seed-feeding fly larvae predate the immature capsules of orchids. The failure to reproduce sexually not only leads to population decline; it can accelerate declines in genetic diversity, which can lead to a more serious conservation crisis. Our previous research and that of others has determined that 50 taxa of 24 native Japanese orchid genera can be infested by *Japanagromyza tokunagai* (Sasakawa) (Diptera: Agromyzidae), as well as other dipteran species, and that damage from fly infestation occurs across all of Japan, i.e., from Hokkaido Prefecture to Okinawa Prefecture (Ogura-Tsujita *et al.*, 2019). Infestation by *J. tokunagai* on Japanese orchids became obvious in the 1970s (Ogura-Tsujita *et al.*, 2019), but it remains unknown how these infestations began or how they came to be so widespread.

Evidence of fly infestation in orchid capsules is easily recognized by observing coarctate pupae, which are typically found just below the pericarp, or puparia that are left after the emergence of adult flies. Because these larval skins persist, capsules on herbarium species may be a source of historical data on the timeline and distribution of *Japanagromyza tokunagai* infestations. It was recently suggested that the current level of infestation on Japanese orchids is the result of recently naturalized seed-feeding flies that originated in other geographic areas (Onuki, 2007; Suetsugu *et al.*, 2018). Spatio-temporal analysis using herbarium specimens may help to test this hypothesis. Here, we examined evidence of fly infestation in the capsules of herbarium specimens and used collection information including date, geographic area, and species, to shed light on the expansion of fly infestation in Japanese orchids.

Materials and Methods

We examined herbarium specimens of five orchid species: *Cephalanthera erecta* (Thunb.) Blume, *C. falcata* (Thunb.) Blume, *C. longibracteata* Blume, *C. longifolia* (L.) Fritsch, and *Cypripedium japonicum* Thunb. These species have been previously investigated for seed-feeding fly infestations (Ogura-Tsujita *et al.*, 2019), and are widely distributed in Japan with relatively large historical population sizes. Therefore, they are suitable for use in a spatio-temporal analysis of fly infestation.

We examined specimens preserved in eight Japanese herbaria: Herbarium, Hokkaido University Museum (SAPS); Herbarium of Sapporo Museum Activity Center; Herbarium, Tohoku University Botanical Garden (TUS); Herbarium, Institute of University Materials, Fukushima University (FKSE); Herbarium, National Museum of Nature and Science (TNS); Makino Herbarium, Tokyo Metropolitan University (MAK); Herbarium, Kanagawa Prefectural Museum of Natural History (KPM); and Herbarium, Osaka Museum of Natural History (OSA). We examined capsule-bearing specimens of all five species in these herbaria. Coarctate pupae and/or puparia were investigated using a stereomicroscope and the number of fly-infested specimens, the number of fly-infested fruits per specimen and the number of pupae and/or puparia per specimen were recorded.

Results and Discussion

1. Fly infestation detected from herbarium specimens

We examined a total of 194 fruiting specimens of all five orchid species across the eight herbaria. Of these, 63 (32.5%) showed evidence of fly infestation (Table 1). Detailed information for fly-infested specimen is provided in Appendix. Fly-infested specimens had remnant coarctate pupae and/or puparia, as shown in Fig. 1. Coarctate pupae were typically approximately 2 mm in length and 1 mm in width, with characteristic anterior spiracles (Fig. 1A, B). Emergence holes in capsules were typically 1–2 mm in diameter and typically bore puparia fragments (Fig. 1C, D). These characters are consistent with observations of *Japanagromyza tokunagai* (Suga *et al.*, 2018b; Ogura-Tsujita *et al.*, 2019). *Japanagromyza tokunagai* is the only known species in the genus to damage the seeds of *Cephalanthera* and *Cypripedium* (Ogura-Tsujita *et al.*, 2019). Furthermore, puparium morphology was examined (Fig. 1E, F) and found to be highly similar to that of *J. tokunagai.* Specifically, the structure of the rows of cuticular processes on the abdominal segments was consistent with *J. tokunagai*, as were the shape of the anterior and posterior spiracles. As a result of these observations, we identified the residual puparia found on herbarium specimens as belonging to *J. tokunagai*.

Previous field studies have determined that two species are primarily responsible for the inhibition of seed production in wild orchids in Japan: *Japanagromyza tokunagai* and *Chyliza vittata* Meigen (Diptera: Psilidae) (Ogura-Tsujita *et al.*, 2019). *Japanagromyza tokunagai* has been reported to feed on 47 taxa of 22 orchid genera, including the five species examined in this study.

Table 1. The number of fly-infested (+) and un-infested (−) fruiting specimens of *Cephalanthera erecta*, *C. falcata*, *C. longibracteata*, *C. longifolia* and *Cypripedium japonicum* in each decade from 1881 to 2020

Collection year	*Cephalauthera erecta*		*C. falcata*		*C. longibracteata*		*C. longifolia*		*Cypripedium japonicum*		Total	
	+	−	+	−	+	−	+	−	+	−	+	−
1881–1890				1		3					0	4
1891–1900	1	1		1	2	2	1			1	4	5
1901–1910				2	4	2			1	1	5	5
1911–1920		1		2		2					0	5
1921–1930			1	1		1		1		1	1	4
1931–1940	1	2	1	2	1	7		1	1	1	4	13
1941–1950			3	2	1	1			1		5	3
1951–1960	1	3	2	4	4	9	1		3	1	11	17
1961–1970		3	2	3	4				1	1	7	7
1971–1980		2	1	3		7		2	1	5	2	19
1981–1990	2	4	2	2	2	11		1			6	18
1991–2000	1	3	4		3	9		1			8	13
2001–2010	2	1	3	4		5		2		1	5	13
2011–2020		1	1	1	1	2	1		2	1	5	5
Total	8	21	20	28	22	61	3	8	10	13	63	131
Infestation rate for each species	27.6%		41.7%		26.5%		27.3%		43.5%		32.5%	

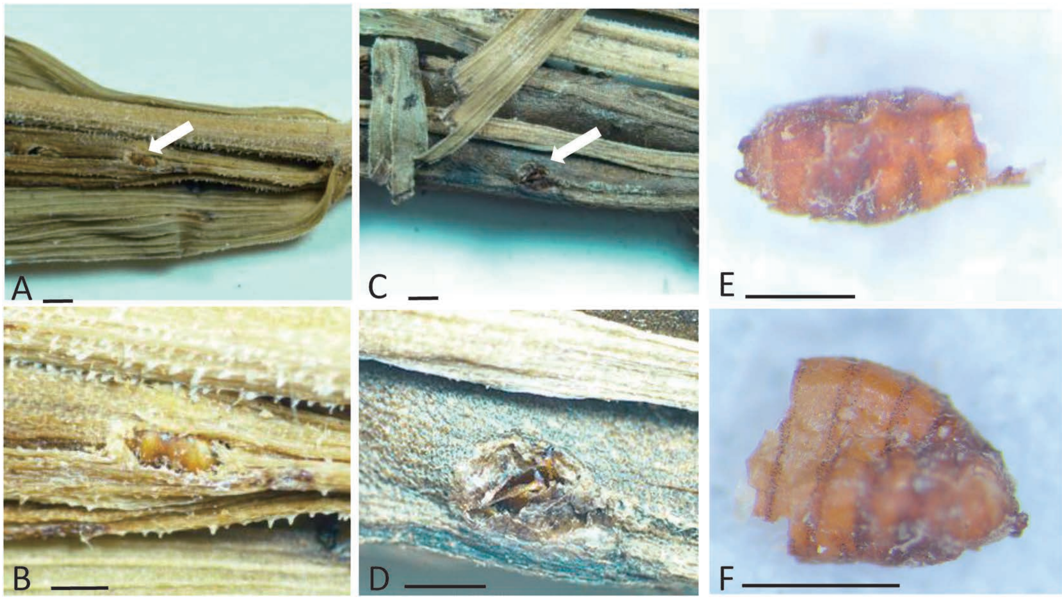

Fig. 1. Fly infestation in capsules of herbarium specimens of *Cephalanthera longibracteata*. A, B. Pupa. C, D. Puparium. E, F. Puparium dissected from a specimen. Arrowheads indicate a pupa (A) or a puparium (C). A, B, E, and F were taken from a specimen collected by M. Majima *s. n.* in Hokkaido Prefecture, Chitose-shi, Mt. Eniwadake in July 1906 (SAPS). C and D were taken from a specimen collected by S. Tanaka *s. n.* in Hokkaido Prefecture, Chitose-shi, Lake Shikotsuko on 1 October 1967 (SAPS). Scale bars = 1 mm.

Table 2. The number of fly-infested (+) and un-infested (−) fruiting specimens collected in each district of Japan in each decade from 1881 to 2020

Collection year	Kyusyu		Shikoku		Kinki		Hokuriku		Chubu		Kanto		Tohoku		Hokkaido		Total	
	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
1881–1890				1								3					0	4
1891–1900				1						1	1	1			3	2	4	5
1901–1910		1	1								1	1	1	2	2	1	5	5
1911–1920										1		3				1	0	5
1921–1930			1	1		1										2	1	4
1931–1940		1	1			1			1		1	3	1	3		5	4	13
1941–1950	1		1		1	1					1	2	1				5	3
1951–1960	1	1	1	2		2		4	1	2	5	3	2	1	1	2	11	17
1961–1970		1					2	1				1	3	3	2	1	7	7
1971–1980		1								4	1	4	1	6		4	2	19
1981–1990					2	2	1			1	1	3	2	8		4	6	18
1991–2000					1				1	1	2		3	4	1	8	8	13
2001–2010					1	3			1	3	1	2	2	3		2	5	13
2011–2020									1		4	1		1		3	5	5
Total	2	5	5	5	5	10	3	5	5	13	18	27	16	31	9	35	63	131
Infestation rate for each district	28.6%		50.0%		33.3%		37.5%		27.8%		40.0%		34.0%		20.5%		32.5%	

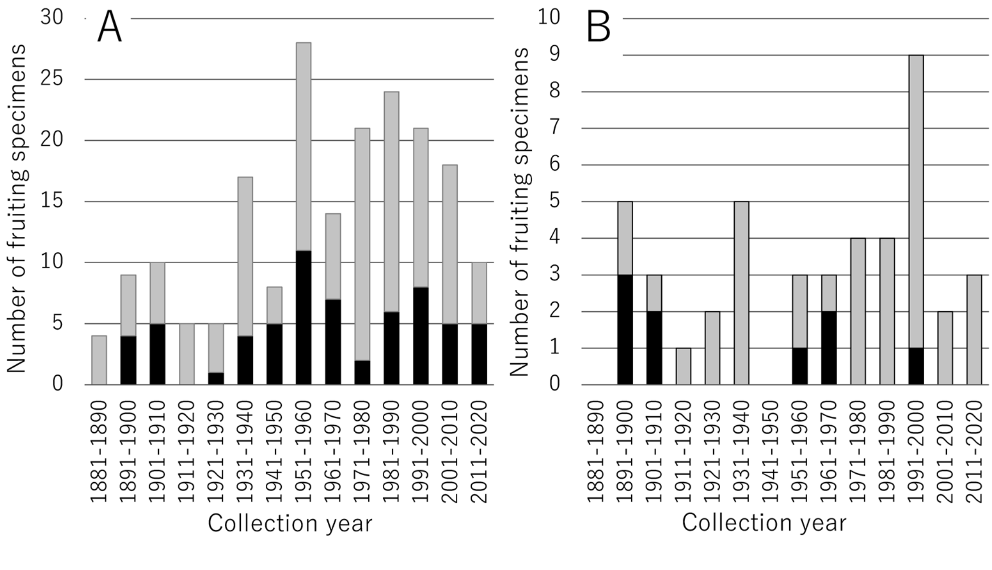

Fig. 2. Number of fly-infested (black) and un-infested (gray) fruiting specimens in Japan (A) and in Hokkaido Prefecture (B) in each decade from 1881 to 2020.

Damage from this species has been recorded from Fukushima Prefecture to Okinawa Prefecture (Ogura-Tsujita *et al.*, 2019). *Chyliza vittata* has been reported to damage the seeds of *Calanthe nipponica* Makino, *Epipactis helleborine* (L.) Crantz, *Gastrodia elata* Blume, and *Neottia furusei* T.Yukawa & Yagame. Observations of damage by this species have been reported from cool-temperate to subarctic regions in Hokkaido and Nagano Prefectures (Kato *et al.*, 2006; Suetsugu, 2013, 2016a, 2016b; Sueyoshi, 2013; Sugiura, 2016; Ogura-Tsujita *et al.*, 2019; Suetsugu *et al.*, 2019). Pupal length is approximately 2 mm and 5 mm in *J. tokunagai* and

Fig. 3. Geographical distribution of fly infestation of fruiting specimens in each prefecture of Japan. Prefectures in which fly infestation was detected are shown in dark hatch, and prefectures without detection are shown in pale hatch. The left- and right-hand figures in each prefecture denote the number of fly-infested specimens and the total number of fruiting specimens, respectively.

C. vittata, respectively; thus, the pupae and/or puparia observed in this study are unlikely to belong to *C. vittata*.

2. Geographical and temporal distribution of fly-infested specimens

Infested capsules were found on specimens collected across a wide range of time periods (Table 1, Fig. 2A). The earliest fly-infested specimens, individuals of *Cephalanthera erecta* and *C. longibracteata* from two sites in Hokkaido, were collected in 1891 (Appendix). Thereafter, between 1–11 damaged specimens were found in each decade, excluding the 1910s (Table 1, Fig. 2A). For the remaining three species, the first records of fly infestation were in 1894 for *C. longifolia*, 1904 for *Cypripedium japonicum*, and 1923 for *C. falcata* (Table 1, Appendix). The earliest evidence of fly infestation throughout Japan was that observed in Hokkaido in 1891, followed by records in the Kanto region in 1899, Shikoku in 1904, Tohoku in 1906, and Kinki in 1950 (Table 2, Appendix).

Specimen data therefore suggest that seed-eating flies spread throughout Japan from Hokkaido Prefecture to Kagoshima Prefecture (Fig. 3, Appendix). Fruiting specimens of the five species were available for 31 of the 47 Japanese prefectures covering most of the distribution range of these species (Fig. 3); infestations were observed in specimens collected from 25 prefectures. However, we note that *J. tokunagai* has been found feeding on the capsules and inflorescences of *Cephalanthera erecta*, *C. falcata*, *Cymbidium goeringii* (Rchb.f.) Rchb.f., *C. kanran* Makino, *Cypripedium macranthos* Sw., and *Oreorchis coreana* Finet on Jeju Island, South Korea (Kim and Lim, 2019). Therefore, examination of specimens preserved in other Japanese herbaria, as well as those in other countries, may help to clarify the spread and extent of fly infestation in orchids more accurately.

Although previous reports have suggested that *C. vittata* was the only seed-feeding fly in Hokkaido (Nakamura *et al.*, 2014; Ogura-Tsujita *et al.*, 2019), our results provide the first evidence of the occurrence of *J. tokunagai* in Hokkaido (Table 2, Fig. 3). Nine of the 44 fruiting specimens collected in Hokkaido were infested by *J. tokunagai* (Fig. 3). However, our results suggest that fly infestation was less severe in Hokkaido relative to other areas. Compared to the infestation rate for Japan as a whole (32.5%), infestation in Hokkaido was relatively low, at 20.5% (Table 2). Furthermore, infestations were only observed on specimens collected in 1891, 1894, 1904, 1906, 1953, 1967, 1968, and 1991, although specimens of the five orchid species were collected continuously over this period. This indicates that *J. tokunagai* is relatively rare in Hokkaido Prefecture (Fig. 2B, Appendix).

3. Infestation rates

Our examination of herbarium specimens revealed significant damage resulting from fly infestation in all target species (Table 1). In *Cypripedium japonicum*, 43.5% (10 out of 23) of fruiting specimens showed evidence of damage, whereas 90–92% of capsules in Ibaraki Prefecture and 83% of capsules in Yamanashi Prefecture were found to be damaged in a recent field survey (Suga *et al.*, 2018c). In *Cephalanthera falcata*, 41.7% (20 out of 48) of fruiting herbarium specimens showed evidence of infestation, but recent field surveys reported capsule damage rates of 91% and 89% in Ibaraki Prefecture and the Tokyo Metropolitan area, respectively (Suga *et al.*, 2018c). In *C. erecta*, 27.6% (8 out of 29) of fruiting specimens were infested, whereas 42% of capsules examined in Ibaraki Prefecture were found to be damaged (Suga *et al.*, 2018c). In *C. longifolia*, 27.3% (3 out of 11) of fruiting specimens were infested, whereas 64% of surveyed capsules in the field in the Tokyo Metropolitan area were found to be infested (Suga *et al.*, 2018c). In *C. longibracteata*, 26.5% (22 out of 83) of fruiting specimens were infested, whereas 68% of surveyed capsules in Ibaraki Prefecture were found to be infested (Suga *et al.*, 2018c). These data highlight the fact that infestation rates in the field may be much higher than levels reflected in herbarium specimens.

These differences in damage rates may be due to under-estimation of the infestation rate in the herbarium specimens. In immature capsules, *J. tokunagai* remains in the larval stage, and thus may not be easily recognizable. Infestation is therefore only observed following coarctate pupation. In a study from Chiba Prefecture, Suga *et al.* (2018a) reported that most individuals of *J. tokunagai* pupate in June or later in *Cephalanthera falcata* and *Cypripedium japonicum* capsules. However, several specimens in this study were collected prior to June. It is therefore possible that capsules appeared visually intact even though larvae were present; alternatively, coarctate pupae or emergence holes may have been overlooked if they were located on the underside of the herbarium specimens, as our examination was non-destructive. Furthermore, ovaries or immature capsules that fell off the plant shortly after infestation would have been overlooked in this study. Therefore, infestation rates from field and herbarium surveys cannot be directly compared.

An alternative explanation for the discrepancies in these rates is the possibility that the population of *J. tokunagai* in Japan has increased in recent years due to a decrease in its parasites. Several parasitic wasps are known to prey on *J. tokunagai* and thus could influence its population size (Kim and Lim, 2019; Matsuo *et al.*, 2019; Suetsugu *et al.*, 2019). Habitat change and other factors may have reduced parasitic wasp populations, resulting in an increase in the *J. tokunagai* population. A third possible explanation is that specimen collection sites and field observation locations differed. Collection details recorded on herbarium specimen labels indicate that specimens were largely collected in natural ecosystems, whereas the field observation data were mostly collected in peri-urban ecosystems, such as secondary woodland, urban parks and gardens (Ogura-Tsujita *et al.*, 2019). Therefore, observed differences in infestation rates between herbarium specimens and field surveys could be due to different biological interactions present in the different habitats.

4. Conclusions

We assessed the temporal and geographic expansion of seed-feeding fly damage in native Japanese orchids using herbarium specimens and determined that i) infestation by *J. tokunagai* is evident in all regions and on all species over an extended period of time; ii) the record of infestation by *J. tokunagai* is more-or-less uninterrupted from 1891 to the present day (i.e. over a 129-year timespan); iii) all five orchid species examined in this study have been infested by *J. tokunagai* since at least 1923; and iv) nationwide damage has occurred since the time of earliest collections. Therefore, it is likely that *J. tokunagai* is not a recent foreign or domestic inter-regional introduced invasive species, but rather a component of the native Japanese fauna. This study highlights the utility of herbarium specimens in documenting and explaining patterns of insect herbivory. We note that the specimens assessed in this study do not reflect the entirety of Japan and that the number of specimens available for examination was limited. We suggest that future surveys target geographic gaps in herbarium records to provide greater understanding of the ecology of seed-feeding flies in Japan. Finally, assessing the genetic diversity of flies collected from herbarium specimens may allow for an understanding of population and migration dynamics.

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Appendix. List of fly-infested fruiting specimens of five Japanese native orchid species preserved in herbaria

Species	Locality			Collection date	Collector and collection number	Voucher
	District	Prefecture	Detailed locality	Year-Month-Day		
Cephalanthera erecta	Hokkaido	Hokkaido	Sapporo-shi	1891-7-7	Tokubuchi *s. n.*	SAPS
	Kanto	Saitama	Hannou-shi, Mt. Tenranzan	1939-6-5	T. Makino *s. n.*	MAK193684
		Kanagawa	Ashigarakami-gun, Yamakita-machi	1999-7-5	Mashiko *et al. s. n.*	KPM-NA0115518
	Chubu	Nagano	Shimoina-gun, Oshika-mura	2006-9-25	M. Maeshima *s. n.*	MAK411161
		Aichi	Tahara-shi, Cape Irago	1954-12-27	N. Satomi *s. n.*	TUS177757
	Kinki	Osaka	Kaizuka-shi, Baba	2003-7-6	C. Shimizu 2003-074	OSA280824
			Izumi-shi, Nabetani Pass	1981-10-25	C. Shimizu *s. n.*	OSA204582
		Wakayama	Iwade-shi, Sakaidani	1985-8-5	S. Yamashita 6452	OSA104880
Cephalanthera falcata	Tohoku	Miyagi	Ishinomaki-shi, Kitazakai	1993-10-25	Y. Sasaki 93-0689	TUS51304
			Higashimatsushima-shi, JASDF Matsushima Airbase	1995-9-4	Y. Ueno *et al.* 38193	TUS263153
			Sendai-shi, Taihaku-ku, Akiu-machi	1961-6-20	H. Ohashi 3271	TUS26214
			Iwanuma-shi, Mt. Inokurayama	1990-5-30	T. Koga & T. Kurosawa *s. n.*	TUS151723
			Iwanuma-shi, Mt. Inokurayama	1990-10-16	T. Koga & T. Kurosawa *s. n.*	TUS152354
		Fukushima	Fukushima-shi, Mizubayasi Park	2009-7-19	T. Ando *et al.* 319	FKSE64528
			Fukushima-shi, Mizubayasi Park	2010-6-21	T. Ando *et al.* 64	FKSE64337
			Iwaki-shi, Yamada-machi	1933-6-24	J. Nozaki *s. n.*	TUS342078
	Kanto	Ibaraki	Tsukuba-shi, Tsukuba University Campus	1980-7-10	Collector unknown	TNS01083089
		Saitama	Niiza-shi, Nobitome, Heirinji Temple	1951-9-20	H. Ohashi *s. n.*	TUS50660
		Chiba	Funabashi-shi, Tsuboi	1991-10-17	H. Nakamura 1510	KPM428808
		Kanagawa	Sagamihara-shi, Shiroyama-machi	2009-7-4	F. Sakai & K. Sakai 43558	KPM-NA0163598
			Minamiashigara-shi, Mt. Maruyama	2015-7-19	E. Nomura NMR-2236	KPM-NA0297255
	Hokuriku	Ishikawa	Kanazawa-shi, Mt. Utatsuyama	1969-6-22	T. Fukui *s. n.*	TUS60503
	Kinki	Mie	Nabari-shi, Akame Valley	1950-8-19	T. Kodama *s. n.*	OSA
		Hyogo	Ako-gun, Kamigori-cho	1992-9-12	S. Miyake 1261	TNS665112
	Shikoku	Ehime	Shikokuchuou-shi, Doi-cho	1950-8-8	T. Yamanaka 5408	TUS409791
		Tokushima	Kaifu-gun, Mugi-cho	1923-9-12	A. Kimura *s. n.*	TUS232703

Appendix. List of fly-infested fruiting specimens of five Japanese native orchid species preserved in herbaria (continued)

Species	Locality			Collection date	Collector and collection number	Voucher
	District	Prefecture	Detailed locality	Year-Month-Day		
Cephalanthera falcata	Kyusyu	Nagasaki	Tsushima-shi, Mine-machi	1944-5-5	H. Marui *s. n.*	TNS708860
		Kagoshima	Satsuma-cho	1960-5-19	S. Sugaya & K. Sohma *s. n.*	TUS26207
Cephalanthera longibracteata	Hokkaido	Hokkaido	Tokoro-gun, Saroma-cho, Mt. Horoiwayama	1953-10-25	T. Misumi *s. n.*	SAPS
			Kamikawa-gun	1891-8-11	K. Miyabe *s. n.*	SAPS
			Kitahiroshima-shi, Mt. Sanbetsusan	1991-7-28	Y. Tateishi *et al.* 15131	TUS75925
			Chitose-shi, Mt. Eniwadake	1906-7-?	M. Majima *s. n.*	SAPS
			Chitose-shi, Lake Shikotsuko	1967-10-1	S. Tanaka *s. n.*	SAPS
			Tomakomai-shi	1904-10-17	K. Miyabe & J. Hanzawa *s. n.*	SAPS
	Tohoku	Iwate	Kamaishi-shi, Kasshi-cho	1994-8-23	H. Nakamura *s. n.*	MAK451585
		Yamagata	Nishimurayama-gun, Mt. Asahidake	1960-8-15	M. Mizushima *s. n.*	MAK336564
	Tohoku	Yamagata	Mt. Iidesan	1906-8-10	T. Makino *s. n.*	MAK237948
		Fukushima	Onuma-gun, Aizumisato-machi	1967-10-1	T. Saito *s. n.*	FKSE40230
			Aizuwakamatsu-shi, Mt. Otoyama	1953-7-13	T. Suzuki *s. n.*	TUS346926
			Koriyama-shi, Tamura-machi	1950-8-17	H. Saze *s. n.*	FKSE416
			Nishishirakawa-gun, Kashi-onsen	1967-8-9	K. Sohma 4446	TUS
	Kanto	Tochigi	Ashikaga-shi, Oiwa-cho	1903-8-31	S. Aiba *s. n.*	TNS54274
		Tokyo Met.	Nerima-ku, Shakujii Pond	1951-11-7	Y. Yambe *s. n.*	KPM45176
			Suginami-ku, Omiya-Hachiman Shrine	1899-6-25	T. Makino *s. n.*	MAK193757
			Hachioji-shi, Nakayama	2011-12-15	F. Sakai & K. Sakai *s. n.*	MAK404736
		Kanagawa	Sagamihara-shi, Mt. Shotosan	1985-10-10	M. Akiyama *et al. s. n.*	KPM72653
	Chubu	Nagano	Kitasaku-gun, Karuizawa-machi	1936-7-22	K. Shirai *s. n.*	TNS66016
			Chiisagata-gun, Wada Pass	1995-9-28	H. Nakamura *s. n.*	MAK452812
	Hokuriku	Niigata	Nagaoka-shi, Oguni-machi	1982-10-2	S. Iwano 18317	TUS146204
			Minamiuonuma-gun, Yuzawa-machi	1963-10-20	Collector unknown	TNS791967
Cephalanthera longifolia	Hokkaido	Hokkaido	Sapporo-shi, Minami-ku, Ishiyama	1894-7-2	K. Miyabe *s. n.*	SAPS023726
	Kanto	Kanagawa	Odawara-shi, Oniyanagi	2014-4-27	K. Ito OD30366	KPM-NA0297913
	Shikoku	Kochi	Kami-shi, Kahoku-cho, Arase	1954-5-12	T. Yamanaka *s. n.*	TUS402587
Cypripedium japonicum	Hokkaido	Hokkaido	Tomakomai-shi, Hokkaido Univ. Tomakomai Experimental Forest	1968-9-25	Nishikawa *s. n.*	SAPS025932
	Tohoku	Miyagi	Katta-gun, Shichikasyuku-machi	1977-8-21	R. Suzuki *s. n.*	TNS374938
	Kanto	Saitama	Hanno-shi, Agano	1953-6-7	T. Kawasaki *s. n.*	TNS01063947
		Tokyo Met.	Hachioji-shi, Asakawa	1953-6-14	T. Yano *s. n.*	MAK341275
		Chiba	Inbanuma Pond	1947-6-22	B. Makino *s. n.*	MAK299987
		Kanagawa	Yotsukaido-shi	2015-7-28	Y. Yamashita 735	FKSE87328
	Chubu	Shizuoka	Yokohama-shi, Asahi-ku, Kamikawai-cho	1952-8-9	N. Deguchi *s. n.*	KPM80272
	Shikoku	Tokushima	Fujinomiya-shi	2016-10-23	Y. Yamashita *et al.* 1225	FKSE94275
		Kochi	Miyoshi-shi, Mt. Tsurugisan	1904-8-12	J. Nikai *s. n.*	TNS049109
			Takaoka-gun, Ochi-cho, Mt. Yokokurayama	1934-8-22	G. Koidzumi *s. n.*	OSA17392