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VARIATION OF BREEDING SYSTEM, FLORAL REWARDS, AND REPRODUCTIVE SUCCESS IN CLONAL *CALYSTEGIA* SPECIES (CONVOLVULACEAE)[1]

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The reproductive biology of four Japanese *Calystegia* species (Convolvulaceae) was studied to examine the effects of clonality and population structure on reproductive success. *Calystegia soldanella, C. hederacea,* and *C. japonica* are self-incompatible, while *C. sepium* is self-compatible but needs pollinator services for self-pollination. The showy, bisexual flowers of *Calystegia* offer pollen and nectar that attract many kinds of insects such as bees and syrphid flies. Clones of *C. soldanella* often formed mats just above the high tide line on beaches and produced a great number of seeds. *Calystegia hederacea* and *C. japonica* were distributed as patches of separate clones and often failed to transfer pollen grains among clones. Fruit and seed sets in *C. hederacea* and *C. japonica* were not limited by pollinators but by the number of compatible pollen grains. Although *C. sepium* clones were also distributed in patches, high and stable fecundity was achieved by self-compatibility and pollinator attraction. We suggest that self-compatibility in *C. sepium* has evolved under fertilization limitation caused by geitonogamy or facilitated selfing.

Key words: *Calystegia*; clonal plant; Convolvulaceae; self-compatibility; fertilization limitation.

Breeding systems in flowering plants are often associated with their life forms (Silander, 1985; Barrett, Harder, and Worley, 1996). Many annual plants are self-compatible (SC) and often exhibit autogamy or cleistogamy since they may thus maintain stable fecundity when pollinator availability is unpredictable (Baker, 1965; Lloyd, 1980; Cruzan and Barrett, 1993; but see Barrett, Harder and Worley, 1996). In many taxa, however, self-incompatible (SI) species are predominantly perennial (Stebbins, 1975). Most tree species exhibit outcrossing in tropical rain forests (Bawa, 1974; Chan, 1981; Bawa and Beach, 1983; Kress and Beach, 1994; Murawski, Dayanandan, and Bawa, 1994). Thus, outbreeding may be advantageous in perennial polycarpic plant species (Silander, 1985; Barrett and Eckert, 1990; Karoly, 1992; Barrett, Harder, and Worley, 1996) because they have extended opportunities to reproduce.

Most clonal plants are polycarpic perennials, with breeding systems believed to be the results of their clonal growth (Handel, 1985; Silander, 1985; Back, Kron, and Stewart, 1996). As clonal plants get larger, individual flowers become surrounded by other flowers of the same genet and geitonogamy results (Handel, 1985). Although in some clonal species, dichogamy (separation of male and female function chronologically; see Lloyd and Webb, 1986) and sequential flowering prevent geitonogamous pollination within a single inflorescence (Wyatt, 1982; Back, Kron, and Stewart, 1996), geitonogamy may be unavoidable in a large clone (Back, Kron, and Stewart, 1996). Geitonogamy occurs more frequently in clumped (‘‘phalanx’’) species whose neighboring culms are often members of the same clone than in spreading [‘‘guerilla’’: guerilla and phalanx are defined by Lovett Doust (1981)] species whose nearest neighbors are often members of another clone (Silander, 1985). When the plant species are SI, geitonogamy decreases fitness (Handel, 1985; Richards, 1986; Klinkhamer and de Jong, 1993). Silander (1985) found that many ‘‘phalanx’’ species are SC or predominantly selfing, while most ‘‘guerilla’’ species are SI according to Stebbins’ (1950) data. This suggests that clonal architecture is associated with mating system strategies in clonal plant species.

Even in ‘‘guerilla’’ species, however, the number of clones in a population or distance between nearest neighbors may affect fecundity. *Rubus saxatilis*, which is an insect-pollinated, SI, clonal shrub, tends to have low fruit set in low-density populations (Eriksson and Bremer, 1993). Genet size has little effect on fruit set in *Rubus saxatilis*. Thus, not only clonal architecture but also population structure may have a great effect on reproductive success in clonal plants.

Here we investigate the reproductive ecologies of four *Calystegia* species to examine the effects of clonality and population structure on mating success. In four Japanese *Calystegia* species, all of which are insect-pollinated, clonal, herbaceous vines, fruit set in natural populations varies (Kitamura, Murata, and Hori, 1972). *Calystegia hederacea* and *C. japonica* rarely produce fruits in natural populations, while *C. soldanella* and *C. sepium* set fruits. Clones of *C. soldanella* dominate just above the high tide line on coastal beaches and dunes, where other plant species cannot exist because of frequent disturbance and high salinity (Ishikawa, Furukawa, and Oikawa, 1995). The other three *Calystegia* species are often found as patches of separate clones spreading vegetatively by slender rhizomes and climbing on other plants. This dif-

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TABLE 1. Summary of study sites and investigations (except breeding system tests and germination test for the four *Calystegia* species. The symbols a, b, c, and d indicate that the investigation was carried out in 1993, 1994, 1996, and 1997, respectively. Plot, PV, Nec., and pollen refer to number of plots in each site, pollinator visit observation, nectar measurement, and pollen counting, respectively. Seedlings were carefully searched for at each site.

Species	Site	Symbol	Location	Plot	Phenology	Fruit set	Seed set	PV	Nec.	Pollen	Seedling
C. soldanella	Makino	MA	(35°28′N, 136°04′E)	2	a	a		b		b	a, b
	Imazu	IM	(35°25′N, 136°03′E)	3	b	a, b		b		b	a, b
	Yoshikawa	YO	(35°08′N, 135°59′E)	6	a, b	a, b	b		d		a, b
	Hazaki	HA	(35°48′N, 140°55′E)	5		b	b				b
C. hederacea	Kyoto University (KU)	EG	(35°02′N, 135°47′E)	1	a	a	a			b	a, b
		PH		1	a	a, b	a, b	b	d	b	a, b
		BG		2	a	a, b	a, b		d	b	a, b
		FP		1	a	a	a		d	b	a, b
	Kibune	KB	(35°06′N, 135°46′E)	1		b	b				a, b
	Tsukuba city	TU	(36°05′N, 140°08′E)	1		b	b				
	Misato	MI	(36°23′N, 138°58′E)	1		b	b				
C. japonica	Yodo	YD	(34°53′N, 135°43′E)	3	a	a	a				a
	Katsura	KA	(35°00′N, 135°41′E)	4	b	b	b	b	c	b	b
	Tajimagahara nature reserve	TA	(35°52′N, 139°40′E)	7		b	b	b		b	b
C. sepium	Kibune	KB	(35°06′N, 135°46′E)	8	b	b	b	b	c	b	a, b

ference in population structure may lead to variation in reproductive output among species.

MATERIALS AND METHODS

Study species—We studied four perennial, clonal vine species of *Calystegia* (Convolvulaceae), *C. soldanella*, *C. hederacea*, *C. japonica*, and *C. sepium* (Ohwi, 1965). All members of the genus *Calystegia* in Japan have an extensive capacity for rhizomatous growth (>1 m per year; A. Ushimaru, personal observation). Their elongated rhizomes have many shoot buds and one clone often has many aerial shoots; thus the growth pattern is intermediate between the guerilla and phalanx strategies. All species produce rosy or white, bisexual, funnel-shaped, herkogamous (herkogamy: spatial separation of stigma and stamen in a flower; see Webb and Lloyd, 1986) flowers in leaf axils that are usually pollinated by insects. Flowers of all *Calystegia* open at sunrise and last for a day. In most cases, single flowers open sequentially from the bottom to the top of their shoots (Kitamura, Murata, and Hori, 1972) so that geitonogamy within a single shoot does not occur. Each flower has an incompletely two-locular ovary with two ovules in each locule, a filiform style, and five anthers. The seeds are dispersed by water in *C. soldanella* and by gravity in the other three species (Okamoto, 1992; A. Ushimaru, personal observation). In Japan, *Calystegia* seeds are rarely eaten by animals but are sometimes damaged by fungi (A. Ushimaru, personal observation). However, seeds of one species, *C. japonica*, were strongly preyed on by weevils at Tajimagahara Nature Reserve (Ishikawa, Toquenaga, and Matubayashi, 1994). Several flower characteristics, such as petal size and color, style length, filament color, and nectary color, often vary among clones. Leaf morphologies sometimes vary among clones. Flower type and leaf type were constant at each site in every year, and these characteristics were the basis for clone identification. A brief description of the four species follows.

Calystegia soldanella (L.) Roem. et Schult., a creeping herb, often colonizes coastal habitats throughout temperate and subtropical Eurasia and the Pacific. In Japan, it commonly occurs on the seashore of Hokkaido, Honshu, Sikoku, and Kyushu and the beaches of Lake Biwa.

Calystegia hederacea Wall., a herbaceous vine, is found throughout temperate southeast Asia and Japan. In Japan, it is a common weed in cultivated fields and in sunny disturbed sites.

Calystegia japonica Choisy., a climber, is distributed in temperate parts of Japan, Korea, and China. This species is sometimes classified as *C. sepium* var. *japonica* (Choisy) Makino (Makino, 1895; Iwatuki et al., 1993). In Japan, it occurs on sunny and disturbed riversides and roadsides. Seed production is infrequent in both Japan and Korea (Kitamura, Murata and Hori, 1972; Kim and Chung, 1995).

Calystegia sepium (L.) R. Br. (bindweed), a climber, occurs over temperate regions of the Northern Hemisphere. In Japan it occurs along roadsides and on banks of mountain rivers that are often disturbed by human activity or flooding.

Study sites—The locations of study sites for each species are shown in Table 1. We studied *C. soldanella* at three sites around Lake Biwa, Shiga, and at Hazaki Beach, Ibaragi. We established 2–6 1-m^2 plots in each site. Except for YO1, we observed several clones at each site.

We studied four patches of *C. hederacea* in the north campus of Kyoto University, Kyoto. Two 1-m^2 plots were placed in the Botanical Garden of the Faculty of Science (BG-A, B; near BG-B there are flowering clones of *Calystegia* planted in pots), a 1-m^2 quadrat in front of the Research Institute for Fundamental Physics (FP), a 2-m^2 plot around the Phytotron (PH), and a 4-m^2 plot in the Experimental Garden of the Faculty of Agriculture (EG). In addition to these sites, three other patches were observed at Kibune, Kyoto, at Tukuba, Ibaragi, and at Misato, Gunma. With the exception of sites EG and KB3, we observed only one clone per site. Two clones were observed at EG, and we named the clone that has larger flowers EG-L and the other EG-S, while at KB3 there was a patch of *C. sepium* near the patch of *C. hederacea*.

Patches of *C. japonica* were studied in riparian areas of the Uji River at Yodo (YD) and the Katura River at Matuo (KA), Kyoto. Tajimagahara nature reserve (TA), where *C. japonica* produces many fruits, is located in the floodplain of Arakawa River at Tajimagahara, Saitama. This species is often distributed as patches of separate clones, but the size of each clone often exceeds 9 m^2. There were three patches of *C. japonica* at YD, separated by ~100 m. We established one 2-m^2 plot in each patch. At both KA and TA, *C. japonica* dominated relatively large area (>20 m^2 in KA and >500 m^2 in TA). Therefore, we established four 2-m^2 plots at KA and seven 2-m^2 plots at TA within these patches. At TA several clones with distinct flowers were observed.

We studied four sites (1, 2, 3, and 4) where *C. sepium* had colonized the roadside at Kibune (KB), Kyoto. Sites 1, 2, 3, and 4 included one, more than three, one and two patches (1, 2-A, 2-B, 2-C, 2-D, 3, 4-A and 4-B), respectively. KB2-B was next to KB2-A. We placed one plot per patch.

Pollination experiments—At Lake Biwa and Hazaki, artificial self-, outcross-, and open-pollination experiments were conducted on *C. soldanella*. The breeding systems of the other three species were deter-

TABLE 2. Summary of experimental treatments performed to determine breeding system.

Treatment	Protected from insects	Anthers removed	Pollination type
Autogamy	Yes	No	None
Self	Yes	No	Hand, self
Geitonogamy	Yes	Yes	Hand, same clone, self
Apomixis	Yes	Yes	None
Outcross	Yes	Yes	Hand, outcrossed
Open	No	No	Open
Hybridization (species name)	Yes	Yes	Hand, outcrossed

mined in a cultivated field inside the Botanical Garden, Kyoto University. Their seeds were collected from EG, BG, KA, TA, KB1, and KB2 in 1993. In 1994, they were planted in pots and grown in order to examine breeding systems. For crossing experiments, flower buds were bagged on the afternoon before opening (Table 2). The pollination treatment was repeated the next morning and then flowers were bagged again. We used flowers from the same clone to test for geitonogamy. Bagged flowers without artificial pollination were used to assess autogamy. We removed stamens from bagged flowers to examine apomixis. Open-pollination assessed the effectiveness of pollinators. Cross-pollinations between congeneric species (*C. hederacea, C. japonica*, and *C. sepium*) were carried out in order to examine hybridization.

Germination of seeds—We compared germination rates of selfed and outcrossed seeds for *C. sepium* to assess inbreeding depression at germination. Twenty-one selfed and 20 outcrossed seeds were collected from several plants and were planted in pots filled with vermiculite and watered once a week. Germination was observed at 2 wk after planting.

Pollen tube growth—Selfed and crossed flowers of *C. hederacea, C. japonica*, and *C. sepium* were collected from the cultivated field 8 h after artificial pollination, pistils were stained with cotton blue, and pollen germination was assessed under a microscope.

Flowering and fruiting phenology—In several plots, we counted and marked all flowers at anthesis in each 5-d period and noted capsule production after 45 d for each species (Table 1).

Fruit and seed set under natural conditions—All flowers were marked within each plot and the numbers of flowers and capsules were counted for four species. At TA, a subsample of flowers was randomly chosen and monitored. We recorded fruit set for marked flowers, collected all capsules in each plot 45 d after anthesis, and counted the number of seeds per fruit. Seed set was calculated as total number of seeds per total number of ovules. Total number of ovules was estimated by multiplying the total number of flowers by 4.

Nectar—The standing volume of nectar was measured with microcapillary tubes, and sugar content (mass/mass) was estimated with a portable refractometer. This measurement was made for about ten flowers for 1 d at YO for *C. soldanella* and at KU for *C. hederacea in* 1997 and at KA for *C. japonica* and at KB for *C. sepium* in 1996. Flowers were marked and bagged before anthesis, and nectar was then collected at 1700. The average sugar production per flower was calculated.

Pollinator visitation—Pollinators were collected from all sites in 1993 and 1994. We observed flowers of the four *Calystegia* species for insect foragers at MA-A, IM-A, BG-A, KA-A, TA-C, and KB2-A. This observation was carried out for 1 d at each plot in 1994. We randomly chose and marked about ten flowers in each plot to count insect visitation to each flower for 15 min per hour throughout the day. Insects that carried pollen and made contact with anthers and stigmas were easily recognized by naked eye and recorded. The mean number of pollinator visits per flower each day was calculated as a multiple of the average number of pollinator visits per flower during observations.

Pollen—The mean number of pollen grains per ovule was calculated. About ten flowers were collected from IM, KU, KA, and KB. Five anthers in each flower were stored in 1.5-mL 70% ethanol. Pollen grains easily separated from anthers in ethanol, after which the anthers were removed and 3.5 mL water was added to each vial. We estimated the numbers of pollen grains per flower (in a solution) using a hematocytometer. In most cases *Calystegia* species have four ovules, therefore, the P : O (pollen : ovule) ratio was defined as the average number of pollen grains per flower divided by 4. In addition, we collected open-pollinated flowers at 1700 and the number of pollen grains attached on the stigma was counted under a microscope to examine pollen delivery.

Seedlings—Each site was carefully searched for seedlings in 1993 and 1994 to study seedling recruitment for every species.

RESULTS

Pollination experiments—Results of pollination experiments are summarized in Table 3. In *C. soldanella, C. hederacea*, and *C. japonica*, few fruits and seeds were set following self-pollination, suggesting *C. soldanella, C. hederacea* and *C. japonica* are largely self-incompatible. In *C. sepium* no significant differences in fruit and seed production were recorded between self-pollinated and outcrossed flowers. Also, germination rates of both outbred and inbred seeds were almost the same (0.95 and 1.00, respectively) in *C. sepium*. The pollen grains of *C. sepium* were rarely deposited on stigmas in bagged flowers (eight flowers were examined). Further, few fruits and seeds were set in autogamy treatment. Thus *C. sepium* is self-compatible and not autogamous.

Fruit and seed set in open-pollinated flowers of *C. soldanella* were significantly lower than in outcrossed ones, while the other three species had similar reproductive output in the two treatments.

Hybrids (F_1) were obtained from crossing among three of the *Calystegia* species. Most F_1 seeds germinated and grew, and subsequently they flowered and set seeds.

Pollen tube growth—No pollen tube growth was observed in stigmas of self-pollinated flowers of either *C. hederacea* or *C. japonica* ($N = 14$ and 13, respectively), whereas tube growth was observed in 100 and 81.8% of cross-pollinated stigmas of these species ($N = 9$ and 11, respectively). Both self- and outcross-pollen tubes grew in *C. sepium* ($N = 2$ and 2, respectively).

Flowering and fruiting phenology—*Calystegia soldanella* produced fruits throughout the entire period of anthesis except at YO1-C (Fig. 1). The flowering periods of *C. soldanella* were shorter than in other *Calystegia* species.

The *C. hederacea* clone set few fruits (Fig. 1). Two clones at the Kyoto University Experimental Garden (EG-S and EG-L) set fruits only when their flowering overlapped (Fig. 1).

Flowering phenology of *C. japonica* varied between years and/or sites (Fig. 1). Peaks of different plots were not synchronized with each other at KA. Small numbers

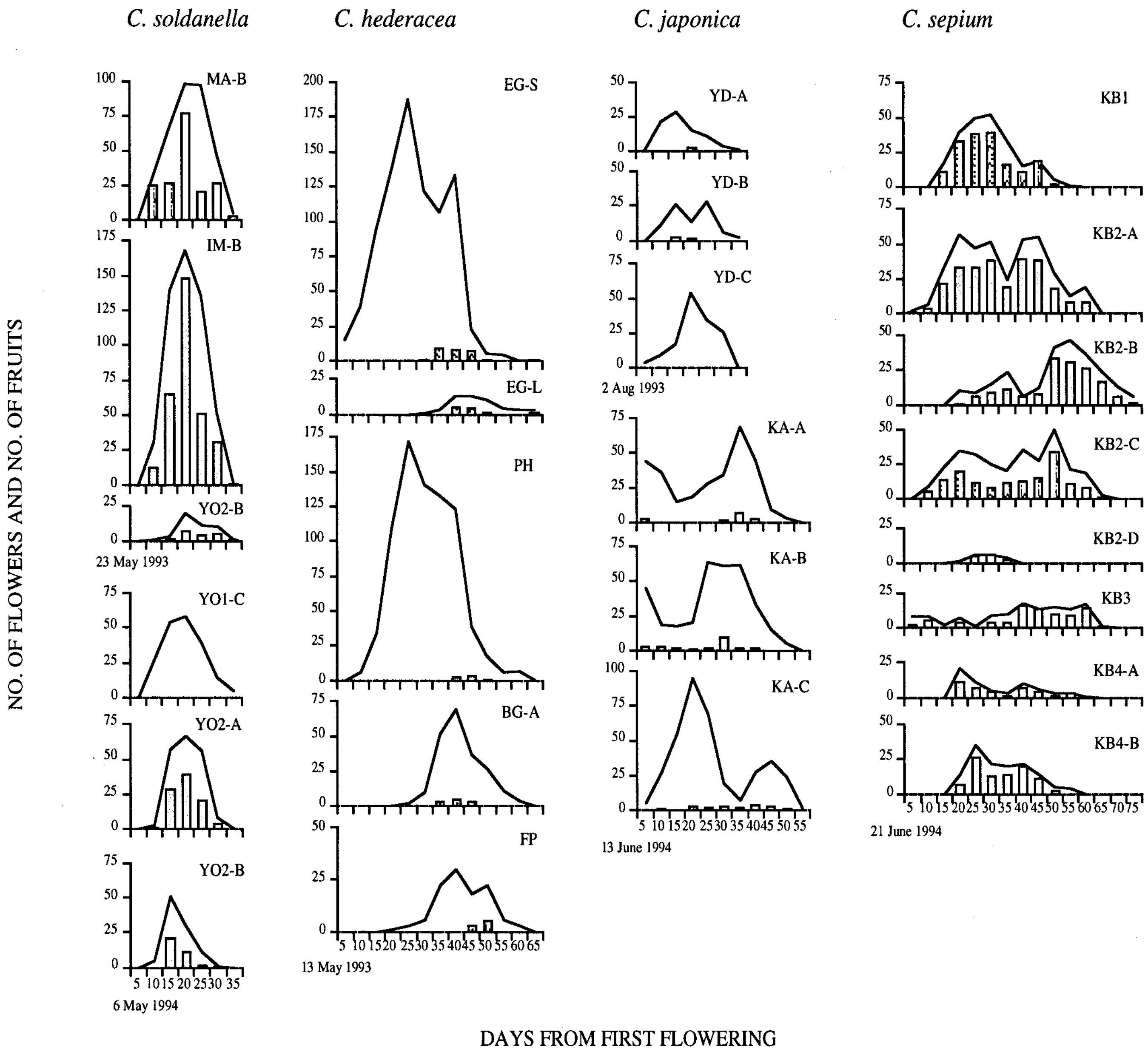

Fig. 1. Flowering and fruiting phenology of *Calystegia* species. Lines show the number of flowers and bars the number of fruits developed. Date of first flowering is given beneath the sites monitored in a particular year. Letters above each figure show the sites and clones where observations were carried out (cf. Table 1).

of fruits were produced during the flowering season (Fig. 1).

Although the blooming phenology of *C. sepium* was not synchronized among plots, fertilizations occurred throughout the flowering period in each plot (Fig. 1).

Fruit and seed set under natural conditions—Fruit set in *C. soldanella* varied from 0.0 to 72.1% under natural conditions (Table 4). Outcrossed flowers had significantly higher fruit and seed set than selfed flowers (Tables 3, 4). At YO1, fruit set in this species was extremely low (almost 0%), while at YO2 near to YO1, ~20–50% of flowers set fruits and seeds.

Fruit and seed set were low in *C. hederacea* in natural conditions (Table 4). The clones with highest fruit production were adjacent to a flowering conspecific or congeneric clone (Appendix 1). Further, in the cultivated field where many clones were planted, fruit and seed set for open pollinated flowers were much higher than fruit and seed set in natural conditions (Tables 3, 4).

Fruit and seed set of *C. japonica* were 0.0–56.7 and 0.0–13.0%, respectively. Open-pollinated flowers in the cultivated field produced 65% more fruit and 50% more seed than under natural conditions (Tables 3, 4). *Calystegia japonica* produced few fruits and seeds at YD and KA (Appendix 1). On the other hand, this species achieved relatively high fruit set (15.7–56.7%) and seed set (2.4–27.0%) at TA, and these values were signifi-

TABLE 3. Results of pollination treatments in four *Calystegia* species. Percentage fruit and seed set and average number of seed per fruit (±SE) were recorded after pollination treatments within and between species. The number of fruits is shown in parentheses for $N < 4$. — indicates that no data were recorded. A species name indicates an interspecific cross. Chi-square tests were performed between treatments. * indicates fruit and seed sets for its treatment are significantly ($P < 0.01$) lower than that for outcrossed treatment. ** indicates that fruit and seed sets for autogamy or apogamy treatments are significantly ($P < 0.01$) lower than that for self-pollination treatment.

Treatment	No. of flowers	Fruit set (%)	Seed set (%)	No. of seed per fruit
C. soldanella				
Autogamy	—	—	—	—
Self	84	3.6*	0.6*	2.0 (3)
Geitonogamy	—	—	—	—
Apomixis	—	—	—	—
Outcross	48	65	52	3.2 ± 0.18
Open	57	35	25*	2.8 ± 0.35
C. japonica				
Autogamy	13	0.0	0.0	0.0
Self	51	5.9*	0.49*	0.33 (3)
Geitonogamy	28	0.0*	0.0*	0.0
Apomixis	—	—	—	—
Outcross	47	68	43	2.5 ± 0.24
Open	34	76	52	2.7 ± 0.21
C. hederacea	18	39	26	2.7 ± 0.18
C. sepium	6	67	46	2.8 ± 0.48
C. hederacea				
Autogamy	31	0.0	0.0	0.0
Self	21	0.0*	0.0*	0.0
Geitonogamy	23	0.0*	0.0*	0.0
Apomixis	—	—	—	—
Outcross	47	83	46	2.2 ± 0.20
Open	31	68	35	2.0 ± 0.31
C. japonica	41	90	46	2.0 ± 0.18
C. sepium	8	25	9.4*	1.5
C. sepium				
Autogamy	38	5.3**	4.6**	3.5 (2)
Self	43	77	56.0	2.9 ± 0.23
Geitonogamy	—	—	—	—
Apomixis	14	0.0**	0.0**	0.0
Outcross	37	81	57	2.8 ± 0.22
Open	34	88	59	3.6 ± 0.20
C. hederacea	5	80	55	2.8 ± 0.63
C. japonica	18	89	65	2.9 ± 0.35

TABLE 5. Results of Mann-Whitney *U* tests for differences in fruit and seed sets of pairs of *Calystegia* species under natural pollination conditions. Results for fruit and seed sets are shown in the upper right cells and the lower left ones, respectively.

C. soldanella	38**	101.5	13**
30*	*C. hederacea*	41.5	0**
48.5	37*	*C. japonica*	3**
5**	8**	1***	*C. sepium*

Note: U value is shown for each test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

cantly higher than those at the other two sites (Mann-Whitney $U = 0, 4$, $P = 0.0017$ and 0.0087, respectively).

Flowers of *C. sepium* produced more fruits and seeds than those of the other three species (Table 4). The median fruit (67%) and seed set (46.3%) were close to those in the cultivated field (Tables 3, 4). The variation of fruit set among plots was relatively small. In isolated patches such as KB1 and KB3, *C. sepium* plants set as many fruits as those in patches that had a neighbor patch.

Pairwise species comparisons revealed that fruit and seed set in *C. sepium* were significantly higher than in all other species (Table 5), while *C. hederacea* set fewer fruits and seeds than the others. As for seed set, only between *C. soldanella* and *C. japonica* was there no significant difference (Table 5).

Flower density (NFL/m^2) is one of the indicators of clone size (because larger clones have more aerial shoots that produce more flowers per area) for the four species. Kendall rank correlation coefficient tests were performed for relationships between the number of flowers per plots and fruit and seed set for four *Calystegia* species. Fruit and seed set in *C. hederacea* were negatively correlated with flower density ($\tau = -0.473$, $P = 0.043$ and $\tau = -0.527$, $P = 0.024$, respectively). However, as mentioned above, small clones (EG-L, BG-B, and KB-C) had a neighbor conspecific or congeneric clone. Fruit and seed sets in the other three species were not correlated with the flower density.

Nectar—The total nectar per flower, sugar concentration, and mean sugar production per flower differed significantly among the four species (ANOVA and Fisher's PSLD procedure were applied; $F = 28.7$, df = 3, $P = 0.0001$ for nectar volume; $F = 4.4$, df = 3, $P = 0.0088$ for sugar concentration; $F = 29.7$, df = 3, $P = 0.0001$ for sugar production) (Table 6). *Calystegia japonica* and *C. soldanella* secrete $\sim$6 times as much nectar as *C. hederacea*, and about 2 times that of *C. sepium*. The sugar concentration of *C. sepium* is significantly higher than that of the other species. The rankings of sugar production (sugar mass per flower) were *C. japonica* $>$ *C. soldanella* $>$ *C. sepium* $>$ *C. hederacea*.

Pollinator visitation—The primary pollinators of *Calystegia* species are several kinds of bees, which comprised 56.7% of the total visitor species. Sixteen out of 33 bee species were caught on flowers of more than one *Calystegia* species. Thus the pollinator systems of the

TABLE 4. The range and median of fruit and seed sets under natural conditions for the four *Calystegia* species. The number of samples and the values of fruit and seed set refer to Appendix 1.

	C. soldanella	*C. hederacea*	*C. japonica*	*C. sepium*
Range of fruit set	0.0–72.1%	0.0–28.0%	0.0–56.7%	50.8–76.5%
Median (*N*)	36.3% (20)	3.1% (11)	14.7% (14)	67.0% (8)
Range of seed set	0.0–38.1%	0.0–10.0%	0.0–27.0%	19.2–66.2%
Median (*N*)	15.5% (11)	0.3% (11)	2.4% (14)	46.3% (8)

TABLE 6. Results of pollen count and nectar measurement. The number of pollen grains in anthers and on stigmas after open-pollination was counted. The minimum and maximum values and the number of samples are shown in parentheses. The P:O (pollen : ovule) ratio and the percentage of pollen grains deposited on stigmas wre calculated for each species. Sugar production per flower for the four species were measured. Size of corolla is described by Ohwi (1965) or Kitamura, Murata, and Hori (1972).

	C. soldanella	*C. hederacea*	*C. japonica*	*C. sepium*
No. of pollen grains per flower	15 032.5^{a} ± 1551.1 (11 200–27 250, 10)	10 815.4^{b} ± 513.6 (9219–141 250, 11)	15 037.5^{a} ± 882.5 (12 125–21 500, 10)	9055.0^{b} ± 568.7 (6300–12 750, 10)
P/O ratio	3758.1	2703.9	3759.4	2263.8
No. of pollen grains deposited on stigma	174.8^{a} ± 11.5 (121–246, 11)	136.0^{a} ± 26.6 (15–345, 16)	108.9^{a} ± 11.6 (22–239, 11)	118.6^{a} ± 8.2 (70–160, 11)
Pollen grains deposited on stigma (%)	1.2	1.3	0.7	1.3
Total nectar per flower (μL)	18.7^{a} ± 1.45 (8.8–30.0, 15)	6.8^{b} ± 0.42 (4.5–8.0, 10)	21.52^{a} ± 1.39 (15.0–30.5, 10)	11.1^{c} ± 0.79 (8.2–15.35, 10)
Sugar concentration of nectar (%)	25.1^{a} ± 1.53 (15–38, 15)	27.6^{ab} ± 0.57 (26–32, 10)	26.6^{a} ± 0.79 (22–29, 10)	30.7^{b} ± 0.47 (28–33, 10)
Sugar production per flower (μg)	5.01^{a} ± 0.36 (2.13–7.81, 15)	2.08^{b} ± 0.10 (1.63–2.56, 10)	6.30^{c} ± 0.34 (4.51–7.82, 10)	3.87^{d} ± 0.29 (2.61–5.58, 10)
Size of the corolla (cm)	4–5	3–3.5	5–6	5–6

Note: The average values (±SE) with the same letter are not significantly different at $p = 0.05$ for each species for each measurement.

four species are similar to each other. *Calystegia soldanella* flowers were most frequently visited by honey bees at MA and IM, whereas smaller halictid and megachilid bees frequently visited flowers of *C. japonica* and *C. hederacea*. Several types of pollinators were observed visiting *C. sepium* flowers: bumble bees, smaller bees, and syrphid flies (Table 6).

ANOVA and Fisher's PSLD procedure were performed on the number of pollinator visits during observation (NPO) in the different *Calystegia* species. Significant differences in NPO were only found between *C. hederacea* (BG) and *C. japonica* (TA) and between *C. sepium* (KB-B) and *C. japonica* (TA) ($F = 4.93$, df = 5, $P = 0.0007$). There was no relationship between NPO and fruit set ($\tau = -0.154$, $P = 0.303$), when the Kendall rank correlation coefficient test was applied to the fruit set and NPO data for four species at these sites where pollinator visits were observed.

Pollen—The mean P:O (pollen : ovule) ratios for each *Calystegia* species are 2264–3759 (Table 7). ANOVA and Fisher's PSLD procedure were applied for the comparison of P:O ratios among species. *Calystegia soldanella* and *C. japonica* had significantly larger P:O ratios than *C. hederacea* and *C. sepium*. The number of pollen grains per flower in *C. hederacea* was slightly larger than that in *C. sepium*.

The number of pollen grains on stigmas did not differ among the four species (Table 7). However, pollen grains of other species, such as *Pharbitis nil* (Convolvulaceae), were observed deposited on the stigmas of both *C. hederacea* and *C. japonica*. The ratio of average numbers of pollen grains received on stigmas to the average numbers of pollen grains produced per flower was ~1% for each species.

Seedlings—No seedlings of *C. soldanella* were found at any site around Lake Biwa. At Hazaki Beach, many seedlings of *C. soldanella* were observed on 27 May 1994. Seedlings often showed a clumped distribution. A few seedlings survived until 20 October (S. Ishikawa, personal communication, Tukuba University). Seedlings of the three climbing species were never encountered at any site.

DISCUSSION

Variation of breeding systems and floral rewards—Breeding systems varied within *Calystegia*. All four species were herkogamous and needed pollinator visits for pollination, though only *C. sepium* was self-compatible. Self-incompatibility has been reported in other genera (*Ipomoea* and *Merremia*) of Convolvulaceae (Kowyama, Shimano, and Kawase, 1980; Devall and Thien, 1989, 1993; Willmott and Burquez, 1996). The sporophytic SI system has been well studied in *Ipomoea* (Kowyama, Shimano, and Kawase, 1980; Devall and Thien, 1993). In *C. hederacea* and *C. japonica*, growth of self-pollen tubes was blocked on the surface of dry stigmas, suggesting that SI in *Calystegia* is sporophytic.

All four *Calystegia* species had relatively high P:O ratios, higher than the mean ratio for facultatively xenogamous plants (796.6) and lower than the mean ratio for xenogamous species (5859.2) as reported by Cruden (1977). In addition, these ratios for *Calystegia* species are 20 times as large as those for species living in highly disturbed areas (Cruden, 1977). In *Calystegia*, only ~1% of pollen grains was carried to stigmas, indicating that most pollen grains were either used as rewards for insects or deposited elsewhere. In most cases, pollen grains were almost entirely removed from anthers by noon (A. Ushimaru, personal observation). The P:O ratio of *C. sepium* was lower than those of the three SI species, suggesting that *C. sepium* reproduces through selfing as well as outcrossing. The fact that no inbreeding depression was expressed at seed production and germination also is consistent with selfing in *C. sepium*.

Interspecific differences in nectar production were also found in *Calystegia* species. The ranking of nectar production in the four species was almost the same as the

TABLE 7. Numbers of pollinator visits during observation of *Calystegia* species. Numbers of flowers observed are shown in parentheses under the site name. The mean number of pollinators per flower during observation (±SE) and the mean number of pollinators per flower per day were calculated.

Species	Site	Pollinators	No. of visits	No. visits/flower	No. visits/flower^{-1}d^{-1}
C. soldanella	MA	*Apis cenara japonica*	66	6.5 ± 0.74	25.8
	(11)	*Campsomeris* sp.	5		
		Syrphidae sp.	1		
	IM	*Apis mellifera*	48	4.5 ± 0.89	18.2
	(11)	Halictidae spp.	9		
C. hederacea	BG	Halictidae spp.	40	8.0 ± 0.97	32.0
	(9)	Megachilidae spp.	30		
		Syrphidae sp.	1		
C. japonica	KA	Halictidae spp.	33	4.6 ± 0.52	18.3
	(14)	Megachilidae spp.	9		
		Syrphidae sp.	2		
		Parnara guttata guttata	8		
	TA	Halictidae spp.	40	3.9 ± 0.68	15.7
	(15)	Syrphidae spp.	3		
		Parnara guttata guttata	15		
C. sepium	KB	*Bombus diversus*	3	7.9 ± 1.1	31.6
	(11)	*Cratina japonica*	10		
		Nomia sp.	22		
		Syrphidae spp.	52		
		Tachinidae sp.	1		

ranking of corolla size (*C. japonica* = *C. sepium* > *C. soldanella* > *C. hederacea*) (Table 7). Nectar production is likely to be affected by the size of the nectary, which in turn is related to corolla size. However, *C. sepium* with a large corolla secreted relatively small amounts of nectar. It is likely that *C. sepium* evolved reduced nectar production in association with selfing. Floral rewards in mixed-mating species are generally smaller than those in related outcrossing species (Banks, 1980; Spira, 1980; Haber and Frankie, 1982; Rathcke, 1988). However, SI *C. hederacea* produced even less nectar than *C. sepium*, which we cannot explain.

Variation of pollination and reproductive success under natural conditions—Fruit and seed sets varied enormously among species. High, stable fruit and seed sets throughout the entire flowering season were recorded only in *C. sepium*, while fruit and seed sets in SI species varied depending on site conditions.

Pollination experiments show that all *Calystegia* species were pollen limited under natural conditions. Here, two different types of pollen limitation should be recognized: one is pollinator limitation (lack of pollinator services) and the other is fertilization limitation (lack of compatible, intraspecific pollen on stigmas) (Garwood and Horvitz, 1985; Byers, 1995). Flowers of all four *Calystegia* species received many insect visits, and stigmas of each species were equally pollinated by them. The more limited reproduction of SI *Calystegia* species compared to *C. sepium* suggests that fertilization limitation occurs more frequently in SI plants than in SC plants and causes variation in reproductive success among the four *Calystegia* species.

Effects of clonal and population structure on reproductive successes—Fertilization limitation can be due to several factors: autogamy, facilitated selfing, geitonogamy, mating among incompatible phenotypes.

Although in *Calystegia* herkogamy and sequential flowering in a single shoot prevent autogamy and geitonogamy within a shoot, respectively, facilitated selfing and geitonogamy among shoots of one clone are inevitable in large clones. However, fruit and seed sets in natural *Calystegia* populations were not correlated with the flower density, indicating that the clone size did not affect reproductive success. In *Calystegia* species, slender rhizomes intermingle with each other under the ground when two or more clones coexist. Thus, clone size may not affect outcross pollination as in other ‘‘guerilla’’ species.

On the other hand, existence of neighboring clones had great effects on fruit and seed sets in SI *Calystegia* species. The lack of fruit set in *C. soldanella* at site YO1 was perhaps due to there being a single genet at this site. At YO2, only 50 m from YO1, fruit and seed set were relatively high. Although insect species collected from YO1 were not different from other sites, pollinators did not seem to move between sites. Similarly, clones of *C. hederacea* and *C. japonica* that have neighboring conspecific or congeneric clones set more fruits and seeds than isolated clones. At the EG plot two clones produced seeds only when their flowering was synchronized. These findings suggest that seed output in these species is limited by the lack of genetically distinct pollen grains. This is also supported by the fact that open-pollinated flowers of *C. hederacea* and *C. japonica* in our cultivated field, where many clones simultaneously flowered, produced as many fruits and seeds as outcrossed flowers. If the genets are distant from each other, outcrossing occurs infrequently among them (Richards, 1986; Eriksson and Bremer, 1993; Momose, Nagamitsu, and Inoue, in press). In *C. sepium*, however, no effects of clone distribution were found, presumably due to its self-fertility.

Seedling recruitment is rare in most clonal plants (Harper, 1977; Eriksson, 1992; Eriksson and Frøborg, 1996). Seedlings of *Calystegia* species were not found except for a population of *C. soldanella* at HA, suggesting that seedling recruitment in *C. hederacea*, *C. japonica*, and *C. sepium* occurred only when the population was established. In contrast, in *C. soldanella* there was repeated seedling recruitment (see Eriksson, 1989). Repeated recruitment in *C. soldanella* may promote local genet diversity and outcrossing. On the other hand, rare seedling establishment and intensive clonal growth cause patches of the other three *Calystegia* species to be composed of a single genet, as in many clonal plant species (Oinonen, 1967; Anderson and Beare, 1983; Worthen and Stiles, 1986; Murawski and Hamrick, 1990), causing geitonogamy and little outcrossing.

Evolution of self-compatibility in Calystegia sepium—All *Calystegia* species except *C. sepium* are SI in Japan, though *C. sepium* in Europe is SI (A. Ushimaru, personal observation; S. Sakamoto, personal communication, Ryukoku University). Therefore it is possible that in Japan, which is at the margin of the distribution of *C. sepium*, this species has evolved SC from SI without morphological change from herkogamy to autogamy.

Pollinator-limited seed production is often presumed to favor the evolution of SC from SI ancestors (Stebbins, 1957; Levin, 1971; Wyatt, 1983, 1984; Dafni and Bernhardt, 1990; Weisler and Snow, 1992). However, the evolution of SC from SI under pollinator limitation usually occurs together with the change from herkogamy to autogamy (Darwin, 1877; Hogan, 1983; Wyatt, 1984; Inoue, 1988, 1990; Dafni and Bernhardt, 1990). *Calystegia sepium* was visited by many kinds of insects and needs their visits even for selfing, showing that pollinator limitation is not the selective pressure for the evolution of SC.

Calystegia sepium often occurs in isolated patches, as in *C. hederacea* and *C. japonica*. The lack of cross-pollination seems to be the selective pressure favoring the evolution of SC in *C. sepium*. Theoretical work suggests that SC without autogamy can evolve from SI under conditions of fertilization limitation (Ushimaru, Higashi and Kikuzawa, unpublished data). Frequent selfing due to geitonogamy occurs in *Iris versicolor*, *Microtis parviflora*, and *Carex platyphylla*, all of which are SC, nonautogamous (dichogamous and/or herkogamous or monoecious), and clonal (Handel, 1985; Peakall and Beattie, 1989, 1991; Back, Kron, and Stewart, 1996), suggesting that SC without autogamy has evolved and is maintained to reduce the negative effects of geitonogamy in some clonal plant species.

Another possible explanation for the evolution of SC in *C. sepium* is that the loss of genetic diversity in Japan has led to a mixed-mating strategy. Biparental inbreeding due to low genetic diversity has been known to induce the evolution of selfing (Lloyd, 1979; Uyenoyama, 1986). This idea can be examined by comparing the mating systems and genetic diversity of *C. sepium* populations in Japan and in Europe.

From our data, it cannot be explained why only *C. sepium* has evolved SC but *C. hederacea* and *C. japonica*, which often occur as isolated clones, have not. Growth rates of rhizomes and climbing shoots in *C. japonica* and *C. hederacea* are relatively higher than in *C. sepium* (A. Ushimaru, personal observation). This high clonal growth rate may complement infrequent seedling regeneration in *C. japonica* and *C. hederacea*. This is one possible explanation and should be examined in future research.

Our results suggest that a lack of genetically distinct pollen grains caused by the isolation of clones reduces fruit and seed production in three SI *Calystegia* species. Their colonizing habits, rare seeding establishment, and vigorous clonal growth may often decrease local reproductive success. Self-compatibility in *C. sepium* avoids fertilization limitation due to self-pollination by insects and ensures high and stable fecundity.

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APPENDIX 1. Fruit set, seed set, and average number of sound seeds (±SE) in the field for each clone of *Calystegia* in 1993 and 1994. Letter[a] indicates that data was recorded in 1993 and no letter in 1994. — shows no data. Number of flowers is shown in parenthesis at column of fruit set. Number of fruit is shown in parenthesis if $N < 4$. rs means that flowers were randomly sampled in a plot.

Species	Site-	Plot	Plot size (m^2)	No. of flowers	Fruit set (%)	Seed set (%)	No. of seeds per fruit
C. soldanella	MA	—A	1	59[a]	37.3	—	—
		—B	1	180[a]	51.7	—	—
	IM	—A	1	130[a]	24.6	—	—
		—B	1	527[a]	58.4	—	—
			1	355	72.1	—	—
		—C	1	49	67.3	—	—
	YO1		4	352[a]	2.8	—	—
		—A	1	86	0.0	0.0	0.0
		—B	1	83	0.0	0.0	0.0
		—C	1	195	0.0	0.0	0.0
	YO2	—A	1	264[a]	35.2	—	—
				191	49.2	38.1	3.1 ± 0.10
		—B	1	46[a]	41.3	—	—
				98	34.7	23.0	2.6 ± 0.24
		—C	1	77	22.1	15.6	2.8 ± 0.26
	HA	—A	1	221	29.0	15.5	2.3 ± 0.19
		—B	0.25	213	28.6	14.3	2.1 ± 0.18
		—C	1	41	39.0	18.3	1.9 ± 0.40
		—D	1	99	37.3	11.6	1.2 ± 0.26
		—E	1	101	40.6	21.3	2.1 ± 0.24
C. hederacea	EG	—S	4	867[a]	3.1	0.3	0.37 ± 0.12
	EG	—L	4	50[a]	28.0	10.0	1.4 ± 0.39
	PH		2	789[a]	5.2	0.06	0.33 ± 0.21
				253	0.0	0.0	0.0
	BG	—A	1	210[a]	0.8	0.5	0.36 ± 0.15
		—B	0.25	155	0.0	0.0	0.0
	FP		1	53	19.0	5.6	0.50 ± 0.17
	KB3		1	111[a]	7.2	0.5	0.25 ± 0.16
	TU		0.9	49	22.0	5.6	1.0 ± 0.38
	MI		0.25	896	0.1	0.03	1.0 (1)
				100	1.0	0.3	1.0 (1)
C. japonica	YD	—A	2	82[a]	3.7	1.5	1.7 (3)
		—B	2	87[a]	3.4	1.7	2.0 (3)
		—C	2	145[a]	0.0	0.0	0.0
	KA	—A	2	305	4.9	1.6	1.3 ± 0.36
		—B	2	343	7.2	1.7	0.92 ± 0.20
		—C	2	370	5.1	1.4	1.1 ± 0.33
		—D	2	22	13.6	8.0	2.3 (3)
	TA	—A	2 (rs)	133	51.1	27.0	2.0 ± 0.15
		—B	2 (rs)	120	56.7	8.3	0.59 ± 0.13
		—C	2 (rs)	102	52.9	13.0	0.96 ± 0.16
		—D	2 (rs)	135	19.3	2.4	0.50 ± 0.22
		—E	2 (rs)	94	36.2	4.5	0.50 ± 0.14
		—F	2 (rs)	127	15.7	2.4	0.60 ± 0.17
		—G	2 (rs)	185	33.5	7.4	0.89 ± 0.14
C. sepium	KB1		2	232	73.3	48.4	2.6 ± 0.13
	KB2	—A	2	389	66.8	37.0	2.2 ± 0.10
		—B	2	244	63.9	19.2	1.2 ± 0.12
		—C	1	303	50.8	31.5	2.5 ± 0.12
		—D	1	17	76.4	66.2	3.5 ± 0.31
	KB3		2	122	67.2	48.4	2.9 ± 0.15
	KB4	—A	1	63	65.1	44.1	2.7 ± 0.21
		—B	1	136	68.4	49.8	2.9 ± 0.16

APPENDIX 2. Flower visitors collected on *Calystegia* flowers. Species marked with * were caught from flowers of more than two *Calystegia* species.

Order	Family	*C. soldanella*	*C. hederacea*	*C. japonica*	*C. sepium*
HYMENOPTERA	Halictidae		*Nomia* spp. 1*, 2*, 3	*N.* spp. 1*, 2*, 4, 5, 6	*N.* spp. 1*, 7, 8
		Lasioglosum spp. 1*, 2*, 3*	*L. occidens**	*L. exiliceps**	*L. exiliceps**
			L. spp. 2*, 3*, 4, 5, 6	*L.* spp. 1*, 2*, 7	*L. occidens**
					L. apristum
	Megachilidae	*Osmia* spp. 1, 2*		*O.* spp. 2*, 3	
			Megachile spp. 1*, 2, 3, 4	*M.* spp. 1*, 5	
	Anthophoridae	*Tetralonia nipponensis**	*T. nipponensis**		
		*Ceratina falvipes**	*C. japonica**	*C. falvipes**	*C. japonica**
	Apidae	*Apis cerana japonica*	*A. mellifera**	*A. mellifera**	
		*A. mellifera**			
		*Bombus diversus**	*B. hypocrita**		*B. diversus**
		*B. hypocrita**			
	Scolidae	Campsomeris sp. 1			
DIPTERA	Syrphidae		*Epistorophe* sp. 1	Gen. sp. 1	*Epistorophe* sp. 2
			Metasyrphus sp. 1		
			Melanostoma sp. 1		
			Sphegina sp. 1		
	Conopidae		Gen. sp. 1		
	Muscidae		Gen. sp. 1*		Gen. sp. 1*
	Tachinidae				Gen. sp. 1
COLEOPTERA	Scarabaeidae	*Eucetonia pilifera*		*Phyllopertha intermixta*	
	Elateridae	Gen. sp. 1			
	Bruchidae		*Spermophagus rufiventris**	*S. rufiventris**	
	Chrysomelidae	Gen. sp. 1*	Gen. sp. 1*	Gen. spp. 2, 3	Gen. sp. 1*
LEPIDOPTERA	Hesperiidae			*Peloidas mathias oberthueri*	
			*Parnara guttata guttata**	*P. guttata guttata**	*P. guttata guttata**
	Lycaenidae	*Lycaena phaeas damino*	*Pseudzizeeria maha argia*		
HEMIPTERA	Nabidae				Gen. sp. 1
	Lygaeidae	Gen. sp. 1			