ORIGINAL ARTICLE

The effects of inbreeding depression and pollinator visitation on the maintenance of herkogamy in *Oxalis corniculata*, a species derived from a heterostylous ancestor

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Abstract

The evolutionary transition from outcrossing in heterostylous morphs to selfing in homostylous morphs has occurred in many plant lineages. Homostylous variants with a low degree of herkogamy may increase the reproductive advantage of autonomous self-pollination when there is little inbreeding depression and/or low pollinator visitation. *Oxalis corniculata*, a self-compatible perennial herb derived from a tristylous ancestor, is reported to have two floral morphs. Homostyled plants are broadly distributed in Japan, whereas long-styled plants are reported to occur only in coastal areas. We examined the hypothesis that a reproductive advantage in avoiding inbreeding depression and/or frequent pollinator visits enables long-styled plants to live in some areas. Studies on floral variation revealed that the degree of herkogamy varied widely and continuously within and among populations. Homostyled and long-styled morphs were functionally defined based on the ability of autonomous self-pollination. Homostyled plants were widely distributed, whereas long-styled plants were found in and around the two coastal populations and relatively inland populations. Pollination experiments provided no evidence of inbreeding depression in either homostyled or long-styled plants. Pollinator visitation frequency was higher in the two coastal populations than in the other populations. Thus, reproductive disadvantage in long-styled plants lacking autonomous self-pollination has reduced in the two coastal populations, and long-styled plants may be maintained due to a competitive advantage of large seed size. In contrast, we recorded no comparable frequencies of pollinator visitation in other populations containing long-styled plants, indicating that there are other factors contributing to the maintenance of long-styled plants in these areas.

KEYWORDS
herkogamy, heterostyly, homostyled plant, long-styled plant, variation within and among populations

1 | INTRODUCTION

Heterostylous plants possess two (distyly) or three (tristyly) types of flowers (morphs) differing in the relative positions of the stigmas and anthers (Barrett, 1992; Darwin, 1877). Such flowers are generally characterized by heteromorphic incompatibility, which prevents self- and intra-morph mating (Barrett, 1992) and promotes pollinator-mediated cross-pollination between different morphs (Lloyd & Webb, 1992). However, at low population densities and/or low pollinator availability, heterostyly may be disadvantageous, given the low probability of reproductive success, and consequently, floral monomorphism and self-compatibility may evolve (Sakai & Wright, 2008; Yuan et al., 2017). The most common outcome of this type of evolution is the spread or fixation of homostylous variants in heterostylous populations (Barrett, 2019). Homostyly is characterized by a reduction in herkogamy (stigma–anther separation) and loss of self- and intra-morph incompatibility (Yuan et al., 2017; Zhou et al., 2017), consequently facilitating autonomous self-pollination (Haddadchi & Fatemi, 2015).

Some homostylous species derived from heterostylous ancestors still retain the herkogamous trait at various levels of stigma–anther separation, although they are self-compatible (Barrett & Shore, 1987; de Vos et al., 2018; Nakagawa & Naiki, 2014; Sosenski et al., 2017). In the genus *Turnera* (Passifloraceae), homostyly has independently evolved from a distylous ancestor at least three times (Truyens et al., 2005). *Turnera velutina* is a monomorphic herkogamous species that produces a considerably lower number of seeds following autonomous self-pollination than when artificially cross- and self-pollinated (Sosenski et al., 2017). The distylous species *Psychotria carthagenensis* consists of long-styled (pin-)monomorphic populations that may have arisen as a consequence of distyly breakdown (Consolaro et al., 2011). Individuals from long-styled monomorphic populations have been found to be self- and intra-morph-compatible. These findings accordingly indicate that outcrossing phenotypes (e.g., herkogamous individuals) may be favored, even subsequent to the evolution of floral monomorphism and self-compatibility.

Oxalis corniculata L. (Oxalidaceae) is a self-compatible perennial herb distributed throughout the temperate and tropical regions of the world (Amano, 2001; Shibaike et al., 1995; Figure 1a). Members of the *Oxalis* sect. *Corniculatae*, including *O. corniculata*, are suggested to be derived from a tristylous ancestor, and their mating systems have shifted from outcrossing to promote inbreeding (Ornduff, 1972). In the Japanese Archipelago, *O. corniculata* has been reported to have homostyled and long-styled morphs (Shibaike et al., 1995), which has been reported as semi-homostylous (Ornduff, 1972; Figure 1b,c). Flowers have two types of stamens (long and short stamens); the pistil and long stamens are of equal length in homostyled plants, whereas the pistil is longer than the long stamens in long-styled plants (Figure 1b,c). The long stamens contain more pollen grains than the shorter stamens (Shibaike et al., 1995). Therefore, the long stamens are important for pollination by pollinators and autonomous pollination. Note that we use the term "long stamens" to distinguish them from "short stamens" within a flower and do not mean stamens of short-styled morph in typical heterostylous (distylous) systems. The flowers of this species are self-compatible and can be crossed between the two morphs (Shibaike et al., 1995). Homostyled plants are capable of autonomous self-pollination, but long-styled plants require pollinators to set seeds (Fukatsu et al., 2019; Shibaike et al., 1995). Notably, it has been reported that the homo- and long-styled plants seem to have different habitat preference; homostyled populations are common in artificially disturbed habitats and are widely distributed in Japan, whereas long-styled populations are predominant in shady pine forests in coastal areas (Shibaike et al., 1995). The homo- and long-styled plants also differ in seed size, which contributes to their successful establishment at each habitat; larger seeds of long-styled plants may have an advantage for seedling establishment in shady pine forests (Shibaike et al., 1995). The homo- and long-styled plants have been reported to comprise monomorphic populations in different types of habitats (Shibaike et al., 1995). However, in our preliminary observation, we found some populations with a continuous variation from homostyled to long-styled plants.

A possible explanation for the maintenance of herkogamy in long-styled plants is avoidance of inbreeding depression. Under conditions of strong inbreeding depression, self-pollination prevents ovules from making superior outcrossed seed; thus, selection should favor increased herkogamy (Herlihy & Eckert, 2004). Moreover, if the negative effects of inbreeding depression can outweigh the advantages of reproductive assurance provided by autonomous self-pollination, homostyled plants may fail to spread through the population (Boyd et al., 1990). In addition to inbreeding depression, inherent variation in seedling viability among morphs may also play a role. Long-styled plants of *O. corniculata* with larger seed size may be superior in certain competitive environments. Larger initial seed size is expected to lead to larger seedling size and enable better access to light and/or reliable water supply (Westoby et al., 1996). These suggest that long-styled plants may be more advantageous than homostyled plants in certain habitats and maintained in populations if pollinator visitation is sufficient. Thus, both inbreeding depression and the frequency of

FIGURE 1 (a) Flower of *Oxalis corniculata*. (b) Sex organ deployment of homostyled plant. (c) Sex organ deployment of long-styled plants and each floral trait measured: short stamen length (A), long stamen length (B), and pistil length (C). (d–f) The stigmas and anthers come into contact with the bee's head and thorax (d), abdomen (e), and legs (f)
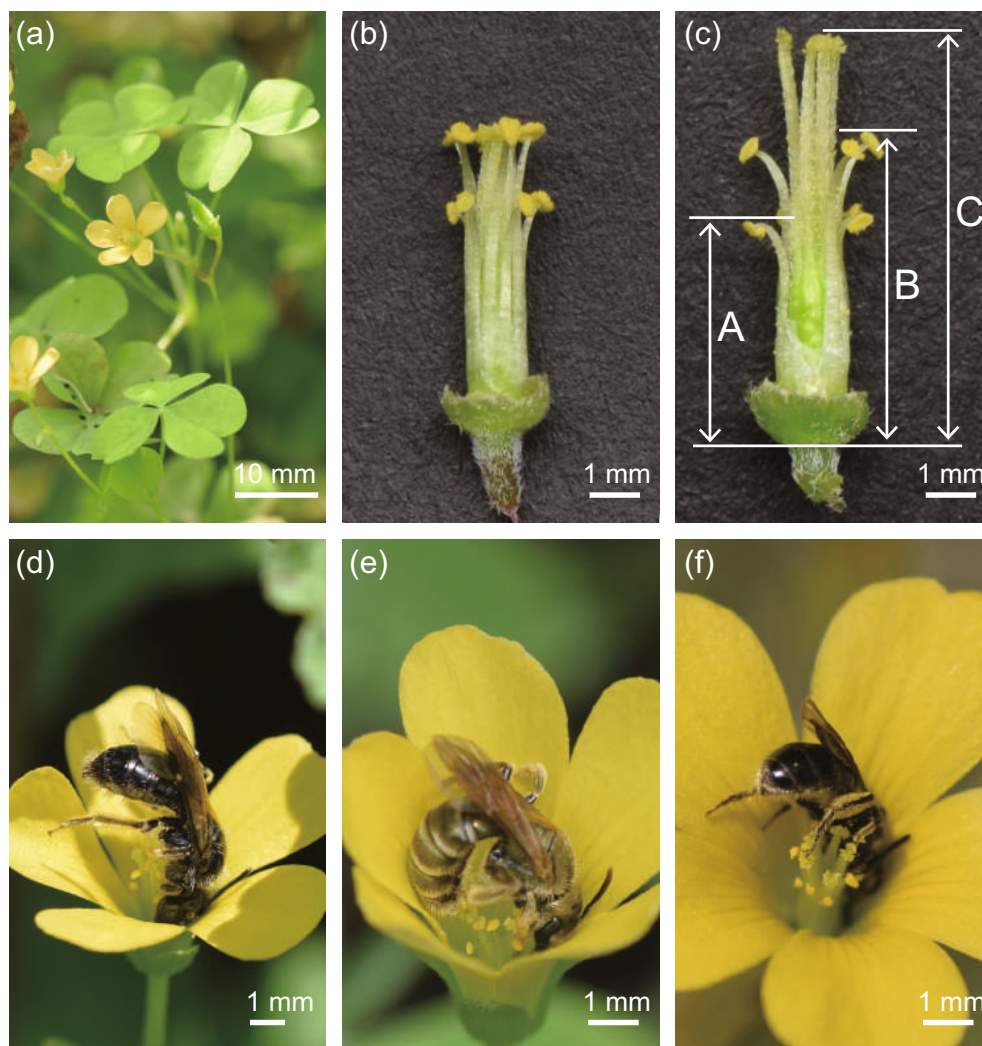
pollinator visitation may be important factors contributing to the maintenance of herkogamy. To date, however, the relationship between these two factors and the degree of herkogamy, that is, the level of separation between stigmas and anthers, in this species has not been sufficiently investigated.

In this regard, we hypothesized that long-styled plants have a reproductive advantage in populations with high inbreeding depression and/or that frequent pollinator visitation maintains long-styled plants in populations, irrespective of the potential disadvantages with respect to reproductive success under some habitat conditions. In this study, we initially confirmed the limited occurrence of long-styled plants in *O. corniculata* populations near Tokyo, Japan (Figure 2) and examined variation in pistil and stamen lengths within and among populations, the degree of inbreeding depression, and the frequency of pollinator visitation. On the basis on these results, we discuss how herkogamy is maintained under self-compatibility and why it is difficult for homostyled plants to invade habitats populated by long-styled plants.

2 | MATERIALS AND METHODS

2.1 | Plants and study sites

Oxalis corniculata is distributed predominantly in disturbed habitats, such as roadsides and cultivated fields. In Japan, *O. corniculata* is believed to be a non-native species that colonized the region during prehistoric times (Doust et al., 1985; Eiten, 1963). The yellow flowers of this species, which open for only a single day, produce nectar and are in bloom from April to October (Fukatsu et al., 2019). In addition to the prolonged flowering period, explosive capsules and sticky seeds have enabled this species to become a successful colonizer and persistent weed (Groom et al., 2019). The most frequent and important pollinators are small bees such as *Halictus* spp. and *Lasioglossum* spp. (Halictidae) (Fukatsu et al., 2019). Unlike the typical heterostylous system in which the two or three floral morphs promote differential placement of pollen onto different parts of the pollinator's body (Keller et al., 2014), the position of the bee's body surface where

the anthers contact may be highly variable because they move around on the flower of *O. corniculata* (Hoshino Y. pers. obs.; Figure 1d–f).

In this study, conducted from 2018 to 2020, we undertook field investigations of a total of 24 populations around Tokyo, central Honshu, Japan (Table 1, Figure 2). We established sites in two areas in Daiba, Tokyo, and in Hayama, Kanagawa (DA and HA, respectively), including DA1 and HA1 facing the sea where the wide variation spanning from homostyled to long-styled plants was found in our preliminary observation. In DA and HA, we established nine and seven populations (DA1–DA9 and HA1–HA7), respectively. These populations were separated by a distance of at least 1 km (except between DA1 and DA2) (Table 1; Figure 2). DA1 and HA1 were in an environment such as a coastal pine forest, where long-styled plants were reported to be dominant in previous studies (Shibaike et al., 1995). In addition, we examined eight populations (IRI, HEB, OTK, AZU, HND, HIN, KOG, and YOG) to achieve an extensive coverage of the area around Tokyo (Table 1, Figure 2). IRI, HEB, OTK,

and AZU were along forest road edges relatively inland (approximately 60 km from the coast), HND was riverbank, and HIN, KOG, and YOG were green lands in urban and suburban areas. These populations were not covered by pine trees.

2.2 | Variation in floral morphology within and among populations

To investigate the variation in flower morphology within and among populations, we collected flowers from all 24 populations (13 to 36 individuals in each population, Table 1, Figure 2). From each of these individual plants, we obtained two to three flowers for the purpose of measuring the lengths of pistils and long and short stamens (Figure 1c). Digital images of floral parts were captured using a Leica MC120 HD camera attached to a stereoscopic microscope, and the above traits were measured using ImageJ software. The values presented for individual plants are averages of the two to three measurements.

2.3 | Effect of herkogamy on autonomous self-pollination

Flowers with long-styled morphs are typically characterized by the avoidance of autonomous self-pollination due to a large distance between stigma and anther (Barrett et al., 2009; Figure 1c). To confirm this characteristic in the long-styled flowers in *O. corniculata*, we investigated the relationship between stigma–anther separation and fruit set after autonomous self-pollination. If long-styled plants avoid autonomous self-pollination, it is predicted that the extent of seed production will decrease with an increasing degree of separation between the stigma and anthers. To this end, we cultivated a total of 37 plants from each of the four populations OTK, KOG, DA1, and HA1 in the laboratory under an 18 h light/6 h dark cycle and at a temperature of approximately 23°C. These four populations were selected with the objective of capturing the natural variation in stigma–anther separation. For each of these cultivated plants, the stigma–anther separation of three flowers was determined as described above (total $N = 111$), and subsequently 1–12 other flowers were selected on each plant, which were covered with bags and marked with tags (total $N = 148$). After a week, we checked whether the fruits had developed. A generalized linear model (GLM; binomial error, logit-link function) was constructed with fruit set (fruit/flower) as the response variable and stigma–anther separation as an explanatory variable.
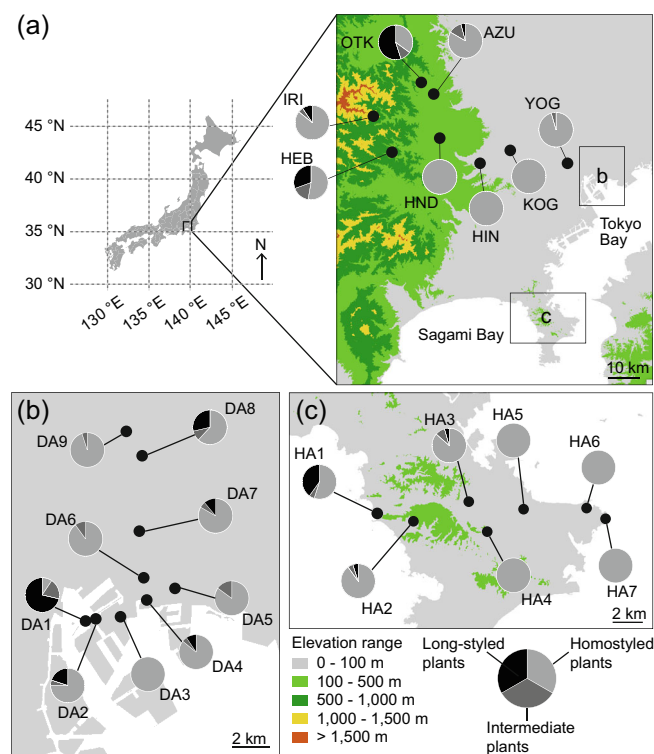

FIGURE 2 The location of 24 populations of *Oxalis corniculata* investigated in this study. Pie charts indicate the frequencies of the long-styled, intermediate, and homostyled plants in each population. Individuals with a stigma–anther separation of <600 μm, 600–800 μm, and >800 μm were classified as homostyled, intermediate, and long-styled morphs, respectively. (a) A map showing the locations of the study sites. (b) An enlarged map of the Daiba district. (c) An enlarged map of the Hayama district

TABLE 1 The location of the 24 sites

Code	Sampling locality	Latitude (°N)	Longitude (°E)	Altitude (m)
DA1	Daiba, Minato Ward, Tokyo Metro.	35.633	139.772	7.5
DA2	Daiba, Minato Ward, Tokyo Metro.	35.633	139.776	1.5
DA3	Ariake, Koto Ward, Tokyo Metro.	35.634	139.797	6.6
DA4	Tatsumi, Koto Ward, Tokyo Metro.	35.641	139.813	2.8
DA5	Yumenoshima, Koto Ward, Tokyo Metro.	35.651	139.826	5.1
DA6	Shiomi, Koto Ward, Tokyo Metro.	35.655	139.808	1.0
DA7	Kiba, Koto Ward, Tokyo Metro.	35.673	139.807	2.5
DA8	Ishihara, Sumida Ward, Tokyo Metro.	35.702	139.808	0.8
DA9	Asakusa, Taito Ward, Tokyo Metro.	35.715	139.803	4.3
HA1	Simoyamaguchi, Hayama Town, Kanagawa Pref.	35.258	139.579	6.3
HA2	Shonan-Kokusaimura, Yokosuka City, Kanagawa Pref.	35.254	139.607	104.2
HA3	Hirasaku, Yokosuka City, Kanagawa Pref.	35.261	139.650	24.3
HA4	Koyabe, Yokosuka City, Kanagawa Pref.	35.249	139.657	102.9
HA5	Otsu-cho, Yokosuka City, Kanagawa Pref.	35.258	139.688	8.8
HA6	Kamoi, Yokosuka City, Kanagawa Pref.	35.258	139.741	2.8
HA7	Kamoi, Yokosuka City, Kanagawa Pref.	35.254	139.746	5.7
YOG	Kamizono-cho, Shibuya Ward, Tokyo Metro.	35.673	139.697	34.7
KOG	Nukuikitamachi, Koganei City, Tokyo Metro.	35.705	139.491	73.3
HIN	Hinohonmachi, Hino City, Tokyo Metro.	35.681	139.401	71.9
HND	Hirai, Hinodemachi, Tokyo Metro.	35.740	139.258	165.0
OTK	Sakaishimachibun, Hanno City, Saitama Pref.	35.903	139.231	243.6
AZU	Nakatoshimogo, Hanno City, Saitama Pref.	35.880	139.258	216.6
IRI	Tozura, Okutama Town, Tokyo Metro.	35.809	139.004	954.9
HEB	Henbori, Hinohara Village, Tokyo Metro.	35.708	139.064	731.8

2.4 | Inbreeding depression

We sought to verify the hypothesis that long-styled plants would have a reproductive advantage in populations with high inbreeding depression by comparing the fruit and seed sets of artificially self-pollinated and cross-pollinated plants. If herkogamy indeed facilitates the avoidance of inbreeding depression, populations containing long-styled plants would be predicted to suffer from inbreeding depression. For this assessment, we cultivated long-styled plants collected from three populations (DA1 [$N = 16$], HA1 [$N = 27$], and OTK [$N = 22$]) and homostyled plants from two populations (KOG [$N = 32$] and OTK [$N = 22$]). Long- and homo-styled plants were defined as those with stigma and anthers separated by intervals of >800 μm and <600 μm, respectively. These criteria were justified, given that autonomous self-pollination rarely occurred in flowers with >800 μm separation, whereas it occurred frequently in those with a separation of <600 μm (see Results for details). Although intermediate plants were also defined as individuals with stigma–anther separation of 600–800 μm (see Results for details), they were not used in this experiment. Using the cultivated individuals, we performed the following two treatments: (1) self-pollination, in which buds and/or flowers were hand-pollinated with their own pollen grains; and (2) cross-pollination, in which buds and/or flowers were hand-pollinated with pollen grains from a different individual of the same morph within the same population. We used pollen grains from longer stamens as pollen donor. Having pollinated the buds/flowers, these were bagged and marked with tags and after 1 week we examined these to establish whether the fruits had developed. Fruit set was measured as the ratio of mature fruits to the total number of treated flowers, whereas seed set was calculated as the number of seeds in a fruit divided by the total number of ovules. Inbreeding depression was estimated by comparing fruit and seed sets of self-pollinated and cross-pollinated plants in each of the two morphs within a population. We constructed a generalized linear mixed model (GLMM; binomial error,

logit-link function) for fruit and seed sets, with pollination treatments and individual identity as explanatory and random variables, respectively.

2.5 | Pollinator visitation frequency

During 2020, we monitored the insects visiting *O. corniculata* flowers in nine populations that included mixed (long-styled and homostyled plants) and monomorphic populations (OTK, AZU, IRI, HEB, YOG, KOG, HIN, DA1, and HA1; see Results). In these populations, we established quadrats (1 m × 1 m) that contained approximately 10–100 flowers. On the day of observation, the total number of open flowers in each quadrat was recorded, as were the size, shape, color, and total number of flowers visited by individual insects entering the quadrat. As reference specimens for identification we collected individuals from among the insect visitors, although not during the observation periods. With the exception of rainy days, observations were undertaken for 15–60 min per day at times randomly selected throughout the day (from 8:00 a.m. to 3:00 p.m.) and the season (from June to October), which is within the flowering period of *O. corniculata*. In total, for each population, we conducted between 4.25 and 12 h of observations over the course of 10–16 days. Visitation frequency was calculated as the number of visits per hour per flower during each observation.

In the nine populations in which we undertook observations of pollinator visitation frequency, we calculated the separation of stigma and anthers as pistil length minus long stamen length to determine the relationship between the degree of herkogamy and pollinator visitation frequency. Stigma–anther separation was compared among populations using GLMs (Gaussian error, identity-link function) and the Tukey–Kramer test for post hoc multiple comparisons.

If herkogamy in *O. corniculata* is maintained by sufficient pollinator visitation, we would anticipate a positive association between visitation frequency and stigma–anther separation or frequency of long-styled plants across the populations examined. A GLM (Gaussian error, identity-link function) was used to examine whether the degree of herkogamy was associated with the frequency of pollinator visitation. In this model, the mean visitation frequency and mean stigma–anther separation were used as explanatory and response variables, respectively. In addition, a GLM (binomial error, logit-link function) was used to examine whether the frequency of long-styled plants in each population was associated with the frequency of pollinator visitation. In this model, the mean visitation frequency and the frequency of long-styled plants were used as explanatory and response variables, respectively. Given
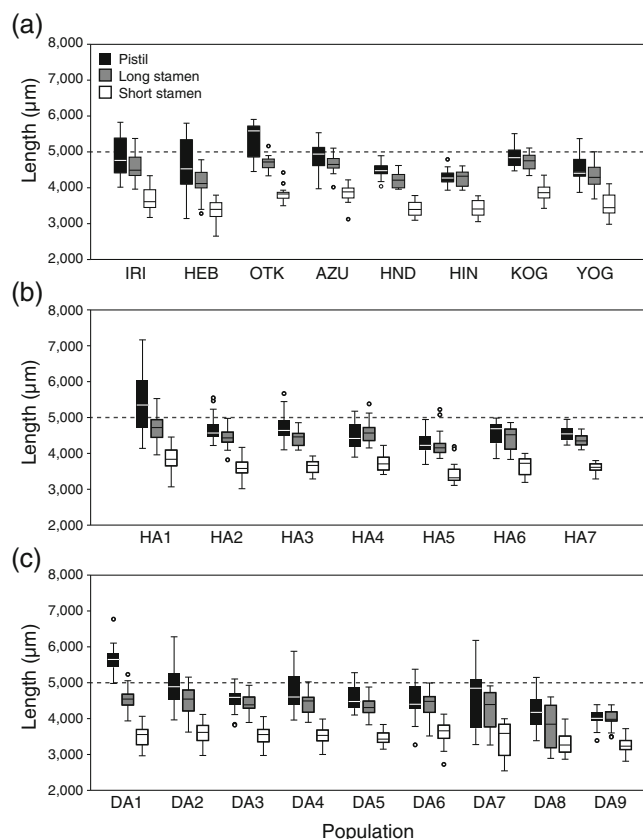

FIGURE 3 Box plots showing lengths of the pistil, long stamen, and short stamen of *Oxalis corniculata* in eight populations (a), seven populations in the Hayama district (b), and nine populations in the Daiba district (c). Boxes, lines in boxes, whiskers, and circles show the quartiles, medians, ranges, and outliers, respectively. Dashed lines were drawn at length = 5000 μm to facilitate comparison

the degree of herkogamy may have a genetic basis (Kulbaba & Worley, 2008), this analysis is designed to examine whether highly herkogamous genetic variants are favored within populations with a relatively high pollination frequency. All statistical analyses were performed using the R statistical package (version 4. 0. 4; R Core Team, 2021).

3 | RESULTS

3.1 | Variation in pistil and stamen lengths within and among populations

We established that there was a certain variation in pistil, long stamen, and short stamen lengths and the relative positions of these three organs among the surveyed populations (Figure 3). The pistil length was almost equal to or longer than the length of the long stamens, and varied continuously across the populations (Figure 3, Figures S1−S3).
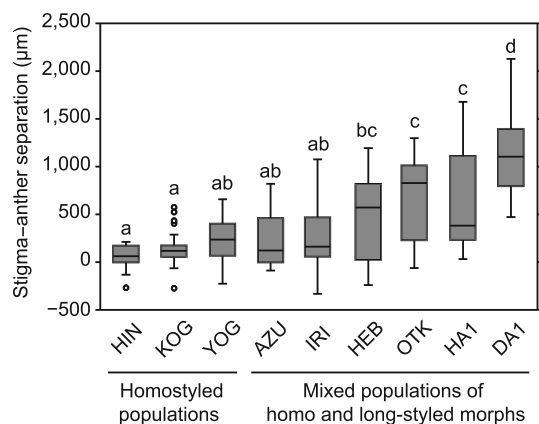
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FIGURE 4 The stigma–anther separation of plants in nine populations of *Oxalis corniculata*. Although YOG contained both homostyled and intermediate morphs, most of the individuals were homostyled morphs (see Figure 2); thus, it was considered as a homostyled population. Boxes, lines in boxes, whiskers, and circles show the quartiles, medians, ranges, and outliers, respectively. Different letters indicate a significant difference (Tukey–Kramer test, $p < 0.05$) among populations

Stigma–anther separation varied significantly among the nine populations (Figure 4), and was found to be greater in flowers of the two coastal populations (DA1 and HA1) than in those of the other populations. At OTK and HEB, a population along forest roads relatively inland, the stigma–anther separation did not differ from that at coastal HA1. At HIN and KOG, which are green lands in urban and suburban areas, the stigma–anther separation tended to be shorter than that in other populations.

In addition to a remarkable variation among populations, stigma–anther separation was observed to vary continuously within population. In the coastal populations, the stigma–anther separation in DA1 ranged from 471.8 to 2129.3 μm and in HA1 it ranged from 32.3 to 1677.6 μm. In populations along the forest road, the stigma–anther separation ranged from −332.1 to 1077.0 μm in IRI, from −240.8 to 1194.2 μm in HEB, from −61.5 to 1298.7 μm in OTK, and from −87.9 to 821.2 μm in AZU. Thus, these populations show continuous variation in the herkogamy and appeared to contain homostyled, intermediate, and long-styled plants (hereafter, mixed population) (Figure 2, see below for definitions of each morph). In contrast, variation in the stigma–anther separation in HIN, KOG, and YOG, which are green lands in urban and suburban areas, tended to be smaller than that observed in the other populations (ranging from −267.7 to 211.4 μm in HIN, from −272.8 to 575.5 μm in KOG, and from −225.3 to 658.2 μm in YOG), and a majority of the individuals in these populations appeared to be homostyled plants (hereafter, homostyled population) (Figure 2, see below).
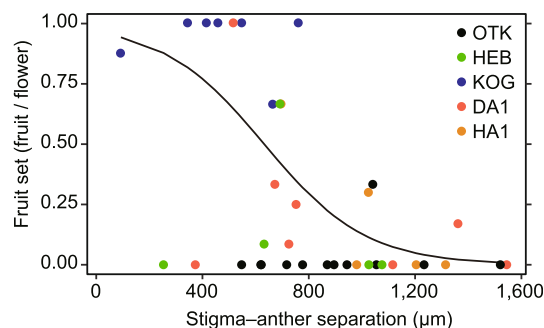

FIGURE 5 The effects of herkogamy on fruit set following autonomous self-pollination examined with individuals in five populations (OTK, HEB, KOG, DA1, and HA1). The fitted curve represents the fruit set estimated based on a generalized linear model analysis

3.2 | Effects of herkogamy on autonomous self-pollination

As predicted based on the hypothesis that herkogamy reduces the extent of autonomous self-pollination, we detected a significant reduction in fruit set with an increase in the distance between stigma and anthers (GLM, $Z = −5.52$, $p < 0.01$, Figure 5). In individuals with a stigma–anther separation of <600 μm, a majority of the flowers formed fruits (26 of 30 flowers). Conversely, when the extent of separation exceeded 800 μm, comparatively few flowers produced fruit (5 of 69 flowers), and the proportion of plants with fruit set was conspicuously low (with 13 of the 16 individuals showing no fruit set). In individuals with a stigma–anther separation ranging from 600 to 800 μm, the percentage fruit set varied from 0% to 100%. On the basis of these observations, we classified individuals with a stigma–anther separation of <600 μm, 600 to 800 μm, and >800 μm as homostyled, intermediate, and long-styled morphs, respectively (Figure 2).

3.3 | Inbreeding depression

In all the populations surveyed, we detected no indication of inbreeding depression with respect to fruit and seed sets in self- and cross-pollinated plants in each of the two morphs (Table 2, Figure 6a–j). In homostyled plants, there was no significant difference in fruit set between treatments (Table 2, Figure 6a,b), although seed set in self-pollinated plants was higher than that in plants that had undergone cross-pollination (Table 2, Figure 6f, g). For long-styled plants, although we detected no significant differences between treatments with respect to fruit set (Table 2, Figure 6c–e), in long-styled plants in the OTK population, seed set following the self-pollination

	Morph	Population	Estimate	Std. error	Z	p
Fruit set	Homostyled	KOG	1.594	1.149	1.387	0.165
		OTK	3.633	4.702	0.773	0.440
	Long-styled	OTK	−0.581	0.497	−1.170	0.242
		DA1	0.336	0.484	0.694	0.488
		HA1	−0.093	0.485	−0.188	0.851
Seed set	Homostyled	KOG	1.149	0.111	10.350	**<0.001**
		OTK	0.401	0.189	2.125	**0.034**
	Long-styled	OTK	1.030	0.141	7.288	**<0.001**
		DA1	−0.022	0.096	−0.226	0.821
		HA1	0.095	0.154	0.615	0.538

TABLE 2 Results of the GLMM comparing fruit and seed sets between the two pollination experiments (self- and cross-pollination) of *Oxalis corniculata*
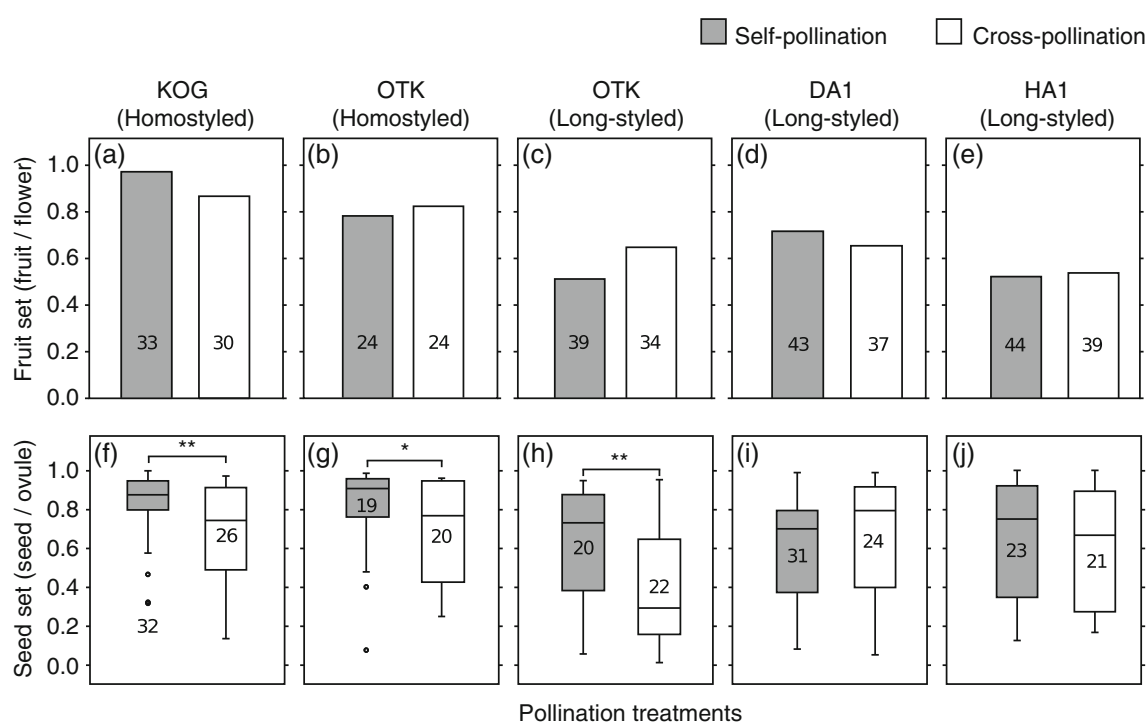
Note: Bold values denote $p < 0.05$.

FIGURE 6 Fruit (a–e) and seed (f–j) sets after artificial pollination of homostyled and/or long-styled plants from four populations (OTK, KOG, DA1, and HA1). Homostyled and long-styled plants are defined as individuals with a stigma–anther separation of <600 μm and >800 μm, respectively. Light gray bars, self-pollination; white bars, cross-pollination. Numbers within and under the bars represent sample sizes. *$p < 0.05$; **$p < 0.001$ (generalized linear mixed model)

treatment was higher than that in the cross-pollinated plants (Table 2, Figure 6h).

3.4 | Pollinator visitation frequency

We recorded a total of 89–646 insect visits among the different populations (Figure 7). These insect visitors were categorized into three main groups, namely, small bees (Andrenidae and Halictidae spp., body length: 4–10 mm),

flies (Syrphidae spp.), and butterflies (Lycaenidae spp.), and two other groups, comprising Diptera (unidentified dipteran species, body length: <5 mm) and other Hymenoptera (*Apis mellifera* and Scoliidae spp.). The small bees were identified as *Lasioglossum apristum* (Halictidae), *Halictus tumulorum ferripennis* (Halictidae), and *Andrena japonica* (Andrenidae), whereas most of the butterflies observed in this study were of a single species, *Pseudozizeeria maha* (Lycaenidae), which is known to utilize *O. corniculata* as a larval host plant.

Insects in these three main visitor groups were observed in all the surveyed populations (Figure 7), in most of which small bees were the predominant visitors (33.7%–85.8% of total flower visits), although visitation frequencies differed among populations (from 0.50 visits flower^{-1} h^{-1} in the mixed population OTK to 4.68 visits flower^{-1} h^{-1} in the mixed population DA1). Flies were, however, the most frequent visitors in the mixed populations IRI and HEB, accounting for 63.0% and 43.2% of the total flower visits, respectively. The frequency of butterfly visits varied from 0.045 visits flower^{-1} h^{-1} in the mixed population IRI to 0.73 visits flower^{-1} h^{-1} in the mixed population HA1. In addition to insects in the three main visitor groups, unidentified dipteran insects were occasionally observed, with the
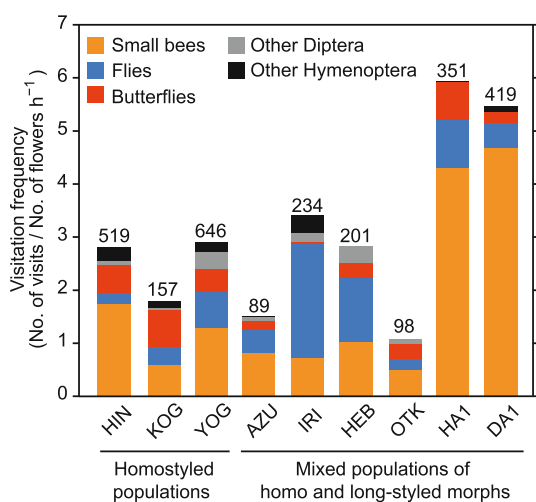
exception of the mixed populations DA1 and HA1 (Figure 7). Furthermore, other Hymenoptera were also occasionally observed in three homostyled (HIN, KOG, and YOG) and three mixed populations (AZU, DA1, and HA1, Figure 7). Collectively, the total visitation frequencies of flower visitors ranged from 1.07 visits flower^{-1} h^{-1} in the mixed population OTK to 5.94 visits flower^{-1} h^{-1} in the mixed population HA1 (Figure 7).

Our GLM analyses indicated that the visitation frequency of small bees was significantly positively associated with both the mean stigma–anther separation and the frequency of long-styled plants (Table 3). Additionally, the total frequency of visits by all flower visitors was significantly positively associated with the frequency of long-styled plants (Table 3). The visitation frequencies of other insect groups were not significantly associated with the mean stigma–anther separation or the frequency of long-styled plants (Table 3).

4 | DISCUSSION

On the basis of our population surveys, we established that there is a significant variation in the herkogamy of *O. corniculata* among the populations around Tokyo. In this regard, the findings of a previous study have indicated that whereas homostyled plants are widely distributed in Japan, long-styled plants are only sporadically observed in coastal areas (Shibaike et al., 1995). Consistent with these findings, we found the extent of flower stigma–anther separation to be higher in plants of the two coastal populations of *O. corniculata* (DA1 and HA1) than in other populations (Figure 4). In addition, we also found the separation of stigma and anthers to be higher in the two relatively inland populations along forest road (HEB and OTK) with values comparable to those recorded in the coastal HA1 population (Figure 4). At the HEB and OTK

FIGURE 7 Variation in the frequency of pollinator visitation among nine populations of *Oxalis corniculata*. Although YOG contained both homostyled and intermediate morphs, most of the individuals were homostyled morphs (see Figure 2); thus, it was considered as a homostyled population. Numbers above the bars represent the total number of visits by observed insects

TABLE 3 Results of the GLM model for the stigma–anther separation and the frequency of long-styled plants in each population of *Oxalis corniculata*

Response variable	Explanatory variable	Estimate	Std. error	t or Z value	p
Mean stigma–anther separation	Visitation frequency for all flower visitors	115.73	62.75	1.84	0.11
	Visitation frequency of small bees	148.34	56.34	2.63	**0.034**
	Visitation frequency of flies	−34.73	204.65	−0.17	0.9
	Visitation frequency of butterflies	−261.4	531.8	−0.49	0.6
Frequency of long-styled plants	Visitation frequency for all flower visitors	0.40	0.11	3.76	**<0.001**
	Visitation frequency of small bees	0.50	0.11	4.73	**<0.001**
	Visitation frequency of flies	−0.23	0.30	−0.75	0.45
	Visitation frequency of butterflies	−1.36	0.80	−1.70	0.091

Note: Bold values denote $p < 0.05$.

sites, some plants are characterized by a stigma–anther separation of >800 μm, and these individuals rarely set fruit following autonomous self-pollination (Figure 4 and 5). Thus, we confirmed that morphologically and functionally long-styled plants are distributed not only in coastal areas but also in relatively inland areas (approximately 60 km from the coast, Figure 2).

Shibaike et al. (1995) suggested that each homostyled and long-styled plant comprises a monomorphic population. Contrary to this, however, this study revealed that all populations including long-styled plants showed continuous variation in the degree of herkogamy spanning from homostyled to highly herkogamous (long-styled) plants (Figures 2–4). This result is significant as it shows that individual variation in reproductive characteristics, especially the capability of autonomous self-pollination, is maintained within populations. Shibaike et al. (1995) have also reported continuous variation in floral morphology within the population; however, they did not strictly classify the two morphs based on the stigma–anther separation and the capability of autonomous self-pollination. The populations considered as monomorphic for homo- or long-styled plants in the previous study were possibly mixed populations of the two morphs when the criteria of this study were applied.

One of our stated hypotheses is that long-styled plants have a reproductive advantage in populations with a high rate of inbreeding depression. However, in neither long-styled nor homostyled *O. corniculata* were we able to detect any evidence for inbreeding depression at the stage of seed production; fruit and seed sets by self-pollination were not lower than those by cross-pollination (Table 2, Figure 6). Generally, homostyled plants are characterized by a high rate of selfing, with self-fertilization contributing to an increase in the homozygosity of recessive deleterious alleles (Boyd et al., 1990; Shibaike et al., 1995). This process is assumed to purge recessive deleterious alleles from such plant populations (Charlesworth & Charlesworth, 1987; Lande & Schemske, 1985), and we speculate that such purging may occur in populations containing homostyled *O. corniculata* plants that show no inbreeding depression. In some cases, we found seed set resulting from self-pollination was higher than that resulting from cross-pollination in homostyled plants. We have no clear explanations for this result, warranting further study. We also found no evidence of inbreeding depression in populations containing long-styled plants (Table 2, Figure 6), which would thus tend to indicate that herkogamy is not necessary to prevent inbreeding depression. As long-styled plants are incapable of autonomous self-pollination, they may be disadvantageous in terms of low pollinator visitation frequency and co-occurrence with homostyled plants. In the present study,

however, we did not examine later-acting mechanisms associated with inbreeding depressions, such as reductions in germination rate, seedling survival rate, pollen viability, and pollen tube growth in selfed progeny (Ishida, 2006; Melser et al., 1999). In addition, inbreeding depression tends to be higher under natural conditions (Ramsey & Vaughton, 1998). Therefore, in order to gain a more comprehensive insight into the occurrence of inbreeding, we would need to estimate the fitness costs of self-pollination at later life stages in the field.

A further hypothesis we sought to verify is that under a high pollinator visitation frequency, the long-styled plants with larger seeds may be more favorable than the homostyled plants due to a competitive advantage in certain environments. In this study, long-styled plants were found in populations in coastal pine forests and relatively inland populations along forest roads (Figure 2). This is consistent with the expectation in previous studies that long-styled plants would have advantages in seedling establishment under shady environments (Shibaike et al., 1995). In addition, we observed that stigma–anther separation and the frequency of long-styled plants were positively associated with the frequency of visitations by small bees (the predominant insect visitors) across populations, and the frequency of long-styled plants was also significantly positively associated with the total frequency of visitation by all insects (Table 3). Contrastingly, we were unable to detect any significant association between herkogamy or the frequency of long-styled plants and the frequency of visits by insects in other groups (Table 3). We speculate that these disparate observations could be attributable to differences in pollination efficiency among pollinator groups, as revealed in *Lonicera japonica* by previous studies (e.g., Miyake & Yahara, 1998). Considering that flies and butterflies are presumed to be inefficient pollinators of *O. corniculata*, further investigations are needed to examine the pollination traits of different groups of insects. We also established that the frequency of small bee visits was higher in the two coastal populations of *O. corniculata* (DA1 and HA1), in which the plants are characterized by a larger stigma–anther separation compared with those in the other surveyed populations (Figure 7). Although autonomous selfing provides reproductive assurance for homostyled plants under low pollinator abundance, homostyled plants have less reproductive advantage in these coastal populations receiving frequent pollinator visits and may experience difficulties invading these populations. However, we were unable to identify a similar trend in the mixed populations HEB and OTK, which also contained plants characterized by a large distance between the stigma and anthers (Figure 7). In these populations, the frequencies of small bee visits were found to be comparable to those recorded in homostyled populations such as KOG

(Figure 7). Thus, a variation in pollinator visitation frequency can only potentially explain the distribution of long-styled plants in some areas but not in others, implying that there are other factors that may influence the distribution of long-styled plants, such as those growing in relatively inland populations such as HEB and OTK.

Here, we propose an additional hypothesis with respect to remarkable variation in the herkogamy of *O. corniculata*, which posits that reproductive interference from coexisting related species selects against the predominant outcrossing of highly herkogamous plants. Given that flowers with a high degree of herkogamy can receive large amounts of outcross pollen (Campbell, 1989), they may also readily receive heterospecific pollen, and as a consequence, suffer from reproductive interference associated with the reception of conspecific pollen and subsequent production of maladaptive hybrids. Thus, under conditions of reproductive interference, flowers with a low degree of herkogamy would have an advantage, in that they have the capacity to minimize reproductive interference by promoting self-pollination (Jiménez-López et al., 2020). In this regard, a previous study has reported that in urban environments *O. corniculata* can coexist with the alien congener *Oxalis dillenii* and that crosses between these two species often give rise to infertile hybrids (Fukatsu et al., 2019). The *O. corniculata* and *O. dillenii* co-occurring in disturbed habitats commonly consist of homostyled plants (Fukatsu et al., 2019), which may imply that these plants are favored in such habitats, as they can minimize the negative effects of reproductive interference via selfing (Fukatsu et al., 2019). Further investigations are needed to reveal the detailed distribution of *O. dillenii* and confirm reproductive interference between long-styled plants of *O. corniculata* and *O. dillenii*.

In conclusion, our examination of variation in the floral morphology of *O. corniculata* within and among populations indicates that long-styled plants occur in both coastal and relatively inland areas. The long-styled plants may, however, be disadvantageous compared with homostyled plants, in that a larger degree of stigma–anther separation in the former tends to be negatively correlated with autonomous selfing, and we found no evidence to indicate that inbreeding depression occurs in either homo- or long-styled plants, at least at the seed production stage. Furthermore, we established that the frequency of pollinator visitation may partially explain the distribution of long-styled plants in coastal populations, although not in relatively inland populations, suggesting that there are other factors that contribute to the variation in herkogamy in this species. Reproductive interference from congeneric alien species is plausibly an additional factor promoting variation in floral morphology within and among populations and accordingly warrants further study.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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