The role of host seed size in mediating a latitudinal body size cline in an introduced bruchid beetle in Japan

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A wide variety of animals show latitudinal cline in body size, which can be caused not only by abiotic factors such as temperature but also by biotic ones such as diet quality. In seed feeding insects, adult body size is affected by seed size. Therefore, seed size may be an important factor to explain the latitudinal cline in body size if the seed size also shows a latitudinal cline. In the present study, we detected a latitudinal cline in body size of an alien bruchid, Acanthoscelides pallidipennis, which was introduced into Japan from North America with its host plant Amorpha fruticosa. In 13 out of 24 populations that we collected in Japan, A. fruticosa seeds were infested with A. pallidipennis. Both body size of A. pallidipennis and host seed weight increased with latitude in the infested populations, but not in the non-infested populations. There was a significant positive correlation between body size and seed weight in both field observation and laboratory experiment. In a common environmental condition, there was no significant difference in body size among three latitudinally different populations. Our results show that the latitudinal cline in adult body size of A. pallidipennis across a non-native range could be explained by the latitudinal cline in seed weight of A. fruticosa, but not by genetic differentiation among populations.

Biological invasion has become an important issue in conservation biology because of large impacts on native species, communities, ecosystems and economies throughout the world (Lodge 1993, Mack et al. 2000, Pimentel et al. 2001, Sakai et al. 2001, Liebhold and Tobin 2008). On the other hand, increasing biological invasion is recognized to be unintentional experiments of evolution (Mooney and Cleland 2001, Yoshida et al. 2007, Suarez and Tsutsui 2008). A variety of organisms have been introduced into a new range far from their native range through human activities (Vitousek et al. 1997). The introduced populations should experience very different biotic and abiotic environmental conditions from native ones. The novel environmental conditions should impose strong selection pressures on the introduced populations, resulting in local adaptations or extinctions in non-native environments.

Range expansion in non-native environments should depend on whether alien species can adapt to geographic heterogeneity. Environmental factors (e.g. temperature) gradually vary with latitude. The latitudinal variation in environmental factors should impose different selection pressures among local populations. Consequently, many insects show a latitudinal cline in traits such as body size (Masaki 1967, Mousseau 1997, Blankenhorn and Demont 2004, Chown and Gaston 2010). Because body size closely correlates with important traits such as fecundity (Honek 1993, Roff 2002), whether body sizes of alien species show a latitudinal cline in non-native ranges is an important subject to understand processes of establishment and adaptation. Nonetheless, such studies have been confined to a few taxonomic groups such as Drosophila (James et al. 1997, Huey et al. 2000, Gilchrist et al. 2001).

Many studies concerning a latitudinal cline in body size have focused on abiotic factors such as temperature, rainfall and season length as selection agents or environmental cues (vanVoorhies 1996, Atkinson and Sibly 1997, Mousseau 1997, Blankenhorn and Demont 2004, Walters and Hassall 2006, Yom-Tov and Geffen 2006, Chown and Gaston 2010). However, biotic factors such as diet quality are also important to explain a latitudinal cline in body size (Arnett and Gotelli 2003, Ho et al. 2010) because diet quality plastically affects insect body size (Nylin and Gotthard 1998). In seed feeding insect such as bruchid, seed size should be an important factor to determine body size because these insects complete larval development inside a seed. If there is a latitudinal cline in seed size, body size of the insect might also show a similar latitudinal cline. In this case, two explanations are possible. One is genetic differentiation among populations resulting from local adaptation to local host seed size. The other is phenotypic plasticity resulting from constraints of local host seed size.

The multivoltine bruchid, Acanthoscelides pallidipennis (Coleoptera; Bruchidae), is native to North America and was introduced to Japan along with Amorpha fruticosa (Leguminosae; Astragalaeae) (Tuda et al. 2001). Because A. fruticosa was first introduced to Japan for control of soil erosion in the late 1940s and was established as early as 1960 (Tuda et al. 2001), A. pallidipennis should also have become established...
in Japan around the same time and is now spread throughout Japan. Acanthoscelides pallidipennis uses exclusively A. fruticosa as a host plant in Japan. Adult females lay eggs on A. fruticosa pods and hatched larvae bore into a seed, develop, and pupate. The entire developmental cycle is completed inside a single seed. Only one adult can emerge from single seed of A. fruticosa because single seed is large enough for one larva only (Szentesi 1999). Therefore, the adult body size of A. pallidipennis might be constrained by seed size of A. fruticosa. Seed size of A. fruticosa may be an important factor to explain the latitudinal cline in body size of A. pallidipennis after its introduction into Japan.

In this study, we asked the following questions. 1) Are there latitudinal clines in both body size and host seed size in Japan? 2) If so, is the constraint of host seed size critical in explaining the latitudinal cline in body size? 3) To what extent does genetic differentiation among populations contribute to the latitudinalcline in body size? To answer these questions, we conducted extensive field sampling in Japan and laboratory experiments.

**Material and methods**

**Field collections**

In 2008, we collected Amorpha fruticosa pods at 24 sites during the period from September through November (Fig. 1). In the period, Acanthoscelides pallidipennis larvae were found inside seeds in the state of fourth instar in the diapause stage. At each site, we haphazardly collected over 1000 pods and these pods were transferred to a laboratory. Fifty pods per site were randomly selected and weighed. We defined pod weight as seed weight because A. fruticosa pods are single-seeded and pod weight is strongly correlated with seed weight (r = 0.93, p < 0.001). Field collected pods were maintained under natural conditions, in terms of daylength and temperature, at Osaka Prefecture Univ. (34°55′N, 135°51′E; Sakai, Osaka, Japan) until overwintered adults emerged the following year. The next spring, the overwintered adults emerged from the pods for 13 out of the 24 sites (Fig. 1). We defined the A. fruticosa populations from which adults did or did not emerge as infested or non-infested populations, respectively.

The emerged adults were immediately sexed and body size was measured. Body size was determined by measuring left elytral lengths using the public domain Image J program (developed at the US Natl Inst. of Health and available at <http://rsb.info.nih.gov/nih-image/> on a Macintosh computer after taking pictures using a digital camera with an attached binocular microscope. The sample size of beetle for body size was 3–70 per site. We averaged body size and seed weight in each population.

**Establishment of laboratory population for experiments**

We used three latitudinally different populations for laboratory experiments. Itakura and Owani populations originated from the adults that emerged from pods collected at sites 13 and 22, respectively (Fig. 1). In addition to these, we collected A. fruticosa pods from Kobe (34°73′N, 135°26′E; Kobe, Hyogo, Japan) in April 2009 and emerged adults were used as a third population. We also collected A. fruticosa pods as oviposition substrate in each A. fruticosa larvae at Kadohara (35°96′N, 136°60′E; Ohno, Fukui, Japan), where the seeds were not infested with beetle larvae.

Newly emerged adults from collected pods were sexed and paired in a petri dish with four compartments (10 cm in diameter and 1.5 cm in height) containing an artificial diet (dry yeast and a cotton ball soaked in sugared water) and 10 dry A. fruticosa pods as oviposition substrate in each population. We established 19, 24 and 16 pairs in Kobe, Itakura and Owani populations, respectively. These petri dishes were maintained at 22°C, L16: D8. The artificial diet was replaced every two days. Once a day, we examined whether eggs had been deposited on the pods. When the pods were found to have eggs, we used them for the following experiments. We defined them as F1 eggs. In Itakura population, we split F1 eggs in two groups, which were used for two different experiments described below.

**Experimental design**

To examine whether host seed size is an important factor to explain the latitudinal cline in body size, we reared the F1 offspring in seeds of a wide variety of sizes until adults emerged. This experiment was conducted using only the Itakura population. The F1 eggs that were laid on pods were carefully peeled and transferred to other pods to rear in seeds.
of a wide range of sizes (2.7–19.4 mg). Although several eggs were laid on a pod in the laboratory, we adjusted the number of eggs to one per pod to remove the effect of larval competition, which might affect adult body size. The pods with an F1 egg were individually weighed and maintained at 22°C, L16: D8. We used 24 females, which laid one to 42 eggs. In total, we used 539 eggs in this experiment. We checked whether adults emerged from pods every day. When adults emerged, we determined developmental time from oviposition to adult emergence and measured adult body size using the method described above. The individuals that did not emerge by the 100th day after oviposition were regarded as dead because mean developmental time was about 55.5 days.

Next, to examine the extent to which genetic variation among populations contributes to the latitudinal cline in body size, we conducted a common garden rearing experiments using offspring originated from Kobe (34°73’N, 135°26’E), Itakura (36°21’N, 139°66’E) and Owani populations (40°51’N, 140°61’E). Seeds with F1 eggs were maintained at 22°C, L16: D8 until adult emergence. F2 eggs were obtained in a manner similar to that for the F1 eggs described above. In Itakura population, F2 eggs were obtained from another F1 group unused for the above experiment. The F2 eggs laid on pods were carefully peeled and transferred to the pods that exceeded 14 mg in weight, which is an appropriate size to detect genetic variation among populations because it was heavier than the mean seed weight infested with *A. pallidipennis* in the field (preliminary observation). The pods with F2 eggs from each population were maintained in a common environmental condition of 22°C, L16: D8. We used 27, 34 and 29 females in Owani, Itakura and Kobe, respectively. Each female laid one to 11 eggs. We checked whether adults emerged from pods every day. When adults emerged, we recorded sex, developmental time from oviposition to adult emergence and adult body size using the method described above.

**Statistical analysis**

We used R ver. 2.10.0 (R Development Core Team) in all analyses. For data from field sampling, both body size and seed weights were estimated in terms of relationships with latitude using general linear models. In the analysis of body size, latitude, sex and their interaction were incorporated into the model. In the analysis of seed weight, latitude, infestation (whether *A. pallidipennis* did or did not infest the population) and their interaction were incorporated into the model.

General linear model was used to estimate the effects of seed weight and other abiotic factors on body size. Abiotic data were obtained from Japan Meteorological Agency (<www.jma.go.jp/jma/index.html>) for 21 years period (1979–2000). To estimate the abiotic conditions for each location, we collected data from weather station nearest to each population. Ten abiotic factors were collected for each population: average annual mean temperature, average annual minimum temperature, average annual maximum temperature, average annual relative humidity, annual amount of precipitation and those factors during season where beetles most active (from April to October). These abiotic factors were divided into two groups. One group was factors related to temperature, such as annual mean temperature, annual minimum temperature, annual maximum temperature, active season mean temperature, active season minimum temperature and active season maximum temperature, and the other one was factors related to moisture, such as annual mean relative humidity, annual amount of precipitation, active season mean relative humidity and active season amount of precipitation. Each group was estimated by principal component analysis and PC1 in temperature and moisture groups accounted for 97.2% and 92.4% of the variation, respectively. These two PC1 variables and seed weight were examined using general linear model. In this analysis, we also included a quadratic term of seed weight because we predicted nonlinear relationship between body size and seed weight.

To estimate the relationship between seed weight and body size in both field and laboratory, we conducted nonlinear regression analysis because we predicted a negative exponential relationship between body size and seed weight. This assumes that body size should be constrained by seed size and increase with seed weight until leveling off at a certain seed weight. For modeling purposes, the function $B_o = B_M [1 - \exp (\frac{S_o}{R})]$ was used, where $B_o$ and $S_o$ are adult body size and seed weight observed, respectively. $B_M$ is asymptote, where the function tends to stabilize in y-axis (i.e. the maximum potential body size). $R$ is the exponent of the function. For the laboratory experiment, we included family as random effect. We estimated the effect of seed size on survival and developmental time from the data of laboratory experiment. We defined the individuals that did or did not successfully emerge as survived or dead, respectively. The difference in probability of survival and developmental time were estimated using generalized linear mixed model (GLMM) with binomial (logit–link) and Poisson (log–link) distribution of error variance. We included family as random effect into the models.

To estimate genetic variation among three latitudinally different populations, differences in body size and developmental time were analyzed using general linear mixed model and GLMM (log–link, Poisson distribution), respectively. In both models, we included population, sex and their interaction as fixed effect, and family as random effect.

**Results**

**Latitudinal cline**

In body size, there were no significant effects of sex ($F_{1,22} = 0.01$, $p = 0.92$) and interaction between latitude and sex ($F_{1,22} = 0.08$, $p = 0.78$). Therefore, we pooled the data of sexes in the following analyses. Body size increased with latitude ($R^2 = 0.51$, $p < 0.01$; Fig. 2a). Average body size in each population ranged from 1.17 to 1.51 mm. *Acanthoscelides pallidipennis* had a larger body size at higher latitudes. In seed weight, there was no significant effect of latitude ($F_{1,20} = 3.55$, $p = 0.07$) and infestation ($F_{1,20} = 0.14$, $p = 0.71$), but marginally significant effect of their interaction ($F_{1,20} = 3.93$, $p = 0.06$). Therefore, we divided populations into two subsets that were either infested or not infested with *A. pallidipennis* and conducted regression analyses in each subset. Seed weight increased with
latitude in the infested populations ($R^2 = 0.66, p < 0.001$), but not in the non-infested populations ($p = 0.95$; Fig. 2b). Average seed weight in each population ranged from 8.67 to 13.67 and from 8.08 to 14.82 mg in the infested and the non-infested populations, respectively. We conducted the nonlinear regression analysis between body size and seed weight. Because three apparent outliers were present (Fig. 3), we analyzed in both before and after exclusion of those. After the exclusion, the exponent of function $R$ was not significant and $B_m$ (i.e. maximum potential body size) was evidently large compared to observed values possibly because of small sample size (Table 1). Before the exclusion, negative exponential relationship was detected between body size and seed weight as we predicted. Body size increased with increasing seed weight (Fig. 3).

In the analysis used the model included two abiotic variables and seed weight, there was no significant effect on body size (in all variables, $p > 0.1$). However, when we excluded the three outliers, the latitudinal cline in body size was significantly explained by seed weight ($\hat{\beta} = 0.90 \pm 0.25$, $t = 3.57, p < 0.05$), quadratic term of seed weight ($\hat{\beta} = -0.038 \pm 0.011$, $t = -3.51, p < 0.05$), and temperature ($\hat{\beta} = -0.038 \pm 0.011$, $t = -3.51, p < 0.05$), but not moisture ($\hat{\beta} = -0.00002 \pm 0.000006$, $t = -3.33, p = 0.76$). Significance of quadratic term of seed weight indicated the nonlinear relationship between body size and seed weight. Body size nonlinearly increased with increasing seed weight and decreasing temperature.

### Phenotypic plasticity by seed size constraints

In 278 (51.6%) out of the 539 eggs that we obtained, insects successfully emerged as adults. Seed weight had a significant effect on the probability of survival. Estimated probability of survival increased when larvae exploited larger seeds (GLMM: intercept, $\hat{\beta} = -1.80 \pm 0.34$, $z = -5.25, p < 0.001$; seed weight, $\hat{\beta} = 0.17 \pm 0.03$, $z = 5.62, p < 0.001$). Emerged adults ranged from 0.862 to 1.640 mm in body size. As predicted, a negative exponential relationship was detected between body size and seed weight (Fig. 4a, Table 1).
Although body size steeply increased with seed weight, it gradually leveled off as seed weight increased. Developmental time elongated with decreasing seed weight (Fig. 4b; GLMM: intercept, $\beta = 4.10 \pm 0.03$, $z = 122.24$, $p < 0.001$; seed weight, $\beta = -0.0075 \pm 0.0028$, $z = -2.67$, $p < 0.01$).

**Genetic variation**

When we reared three populations in a common environmental condition, adults did not show significant variation in body size among populations (Table 2). There were no significant effects of population ($F_{3,287} = 1.59$, $p = 0.21$), sex ($F_{1,492} = 0.64$, $p = 0.42$) and their interaction ($F_{2,492} = 0.64$, $p = 0.53$). To examine whether the difference between populations in the field disappeared in a common garden, we compared body size between Itakura and Owani populations using multiple comparison tests adjusted by Bonferroni method ($\alpha = 0.0125$). In this analysis, we pooled the data of sexes because there was not significant effect of sex in both field and laboratory.

**Table 2.** Average body size and developmental time in Kobe, Itakura and Owani populations of *A. pallidipennis* in field and laboratory, which were reared on seeds that exceeded 14 mg at 22°C, L16: D8.

<table>
<thead>
<tr>
<th>Population</th>
<th>Latitude (°N)</th>
<th>Body size (mm)</th>
<th>Developmental time (days)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kobe</td>
<td>34°53′16″</td>
<td>1.503 (0.006)</td>
<td>152.6 (0.3)</td>
<td>99</td>
</tr>
<tr>
<td>Itakura</td>
<td>36°21′33″</td>
<td>1.537 (0.008)</td>
<td>152.4 (0.3)</td>
<td>30</td>
</tr>
<tr>
<td>Owani</td>
<td>40°51′15″</td>
<td>1.505 (0.010)</td>
<td>152.0 (0.2)</td>
<td>30</td>
</tr>
</tbody>
</table>

Standard error of means is shown in parentheses. Body size significantly varied between Itakura and Owani in field ($t = 5.25$, $DF = 117.54$, $p < 0.001$), but not in laboratory ($t = 1.94$, $DF = 364.9$, $p = 0.05$). Body size also varied between field and laboratory in Itakura ($t = 10.54$, $DF = 73.12$, $p < 0.001$) but not in Owani ($t = 2.18$, $DF = 81.41$, $p = 0.03$).
In the field, body size significantly varied between Itakura and Owani (\(t = 5.75, DF = 117.54, p < 0.001\)), but not in laboratory (\(t = -1.94, DF = 364.9, p = 0.05\); Table 1). Body size significantly varied between field and laboratory in Itakura (\(t = 10.54, DF = 73.12, p < 0.001\)) but not in Owani (\(t = -2.18, DF = 81.41, p = 0.03\)). For developmental time, there were no significant effects of population and sex and their interaction (GLMM: in all variables, \(p > 0.3\); Table 2). Developmental time correlated negatively with body size only in Itakura (Pearson product moment correlation, \(r = -0.42\)).

\[ t_{i} = \frac{\text{growth rate} \times \text{seed size}\%}{\text{seed size} \times \text{growth rate}} \]

\[ r_{i} = \frac{\text{growth rate} \times \text{seed size}\%}{\text{seed size} \times \text{growth rate}} \]

\[ DF = \frac{n(n-1)}{2} \]

\[ b = \frac{\sum (x_{i} - \bar{x})(y_{i} - \bar{y})}{\sum (x_{i} - \bar{x})^{2}} \]

\[ a = \bar{y} - b \bar{x} \]

\[ y_{i} = a + bx_{i} \]

\[ \text{Pearson product moment correlation} = \frac{\sum (x_{i} - \bar{x})(y_{i} - \bar{y})}{\sqrt{\sum (x_{i} - \bar{x})^{2}\sum (y_{i} - \bar{y})^{2}}} \]

\[ r = \frac{\sum (x_{i} - \bar{x})(y_{i} - \bar{y})}{\sqrt{\sum (x_{i} - \bar{x})^{2}\sum (y_{i} - \bar{y})^{2}}} \]

\[ t = \frac{\text{test statistic}}{\text{standard error}} \]

\[ p = 1 - \Phi(t) \]

\[ \Phi(t) = \frac{1}{2} \left[ 1 + \frac{t}{\sqrt{1 + t^2}} \right] \]

\[ \text{GLMM: in all variables, } p > 0.3; \text{ Table 2}. \]

\[ \text{Developmental time correlated negatively with body size only in Itakura (Pearson product moment correlation, } r = -0.42). \]

Discussion

Host size-dependent latitudinal cline

Our study indicated that, in alien bruchid Acanthoscelides pallidipennis, host seed size was an important factor to explain the latitudinal cline in body size and probably affected the success of establishment in Japan.

In our field research, body size increased with latitude (Fig. 2a), which followed Bergmann’s rule that has been shown for a variety of animals (Ashton et al. 2000, Ashton 2002a, b, 2004, Ashton and Feldman 2003, Meiri and Dayan 2003). In the host populations that A. pallidipennis infested, seed weight also increased with latitude (Fig. 2b) and correlated with adult body size (Fig. 3), suggesting that host seed size affect the latitudinal cline in adult body size. The model analysis included two abiotic variables and seed weight also indicated that the latitudinal cline in body size was caused by clines in seed size and temperature.

In the laboratory experiment, body size was correlated with the host seed size that larvae exploited (Fig. 4). In addition, there was no significant difference in adult body size among the three latitudinally different populations when we reared them in a common environmental condition; where A. pallidipennis were derived from Amorpha fruticosa populations with small seeds and they could attain a similar body size to those from the populations with large seeds (Table 1). The difference in body size detected in the field disappeared when they were reared in a common garden. The experimental results indicated that the latitudinal cline in adult body size was plastically determined by the latitudinal cline in host seed size and is not genetically based.

In insect studies, latitudinal clines in body size have reported across native (Masaki 1967, Mousseau and Roff 1989, Mousseau 1997, Blankenhorn and Demont 2004) and non-native ranges (James et al. 1997, Huey et al. 2000, Gilchrist et al. 2001), which was based on genetic differentiation among populations. For example, latitudinal variation in season length caused genetic variation in body size due to the time constraint on development. In higher latitudes, because the favorable season for development is limited to a shorter period, insects should require faster development, resulting in a smaller body size (Masaki 1967, Roff 1980, Mousseau and Roff 1989, Mousseau 1997, Blankenhorn and Demont 2004). However, in A. pallidipennis, phenotypic plasticity largely contributes to the formation of the latitudinal cline in body size in the non-native range. In another bruchid, Stator limbatus, the latitudinal cline in body size was partly explained by host seed size (Stillwell et al. 2007). However, this latitudinal variation was partly determined genetically because S. limbatus from smaller seed hosts showed a smaller body size in a common environmental condition (Amarillo-Suarez and Fox 2006).

Although seed size importantly contributed to the latitudinal cline in body size, we could not reject the effect of temperature on it because temperature also showed a latitudinal cline. Many insects follow the temperature-size rule, in which body size negatively correlates with rearing temperature (Atkinson 1994, 1996). The temperature–size rule would partly contribute the latitudinal cline in body size. Seed size and temperature act on body size in the same direction. For example, in high latitudes, larvae can utilize large seed and grow in low temperature, resulting in large adult body size. Consequently, there is a large difference in body size between low and high latitudes. However, because we did not test the effect of temperature on body size in the laboratory experiment, we could not argue the relative importance and interaction effect of seed size and temperature on the latitudinal cline in body size. Therefore, we need to test the effects of temperature and seed size on body size in future studies.

Host size-dependent success of establishment

It is important to note that seed weight showed a latitudinal cline in the infested populations, but not in the non-infested populations (Fig. 2b). The latitudinal cline in seed weight may be caused by larval weight within seeds. However, the percentages of seeds that infested by larvae were less than 10% in most population, especially less than 5% in latitudinally high populations (> 38°N) and adult weight is about 1–2 mg (unpubl.). Therefore, larval body weight has little effect on population mean of seed weight.

Our prediction to this intriguing pattern is that, in high latitudes, A. pallidipennis infested host populations with a small seed size failed to establish there owing to small body size and low survival rate. In high latitudes, environmental conditions are severe, e.g. low temperature and long winter period. In several insect species, large body size has benefits in strengthening cold tolerance (Saunders et al. 1999, Renault et al. 2003), and is correlated with energy reserves (Reim et al. 2006, Blankenhorn et al. 2007), which are important for diapause maintenance because energy reserves gradually decrease during diapause (Ellers and van Alphen 2002). The depletion of energy reserves during diapause negatively affects post-diapause traits (Ishihara and Shimada 1995, Kroon and Veenendaal 1998, Saunders 2000, Ellers and van Alphen 2002, Ito 2007). In addition, developmental time negatively correlated with seed weight in our laboratory experiment. Because of the short season length available for development at high latitudes, long developmental time due to small seed size might be disadvantageous. Thus, A. pallidipennis populations that exploited small seeds might be hard to establish in high latitudes, resulting in the latitudinal cline in seed size of A. fruticosa only in the infested populations.
In contrast to our prediction, there was one non-infested population with large seed in low latitudes (Fig. 2b). This population could be an outlier in our analysis. However, reanalysis excluding the outlier did not affect overall result (latitude, t = 0.99, p = 0.33; infestation, t = -1.65, p = 0.12; interaction, t = 1.68, p = 0.11), indicating that the absence of latitudinal cline in non-infested populations was not responsible for the outlier. This host population may not have been invaded by chance. However, this study could not offer sufficient data to reach the conclusion. Therefore, we need to examine our prediction in near further studies.

Several studies have reported that the establishment of alien species is accelerated by certain original traits of the alien species such as competition ability (Lodge 1993, Sakai et al. 2001, Suarez and Tsutsui 2008). Present study suggested that the traits in other species like hosts that are closely related to the target species and environmental conditions are important for the successful establishment of the alien species in a non-native environment.

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