EXPERIMENTAL FIELD RELEASES AND THE EFFECTS OF HYBRIDIZATION ON THE WEED BIOLOGICAL CONTROL AGENT *APHALARA ITADORI* (HEMIPTERA: APHALARIDAE)

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ABSTRACT

This thesis focuses on a recently approved biological control agent, the psyllid *Aphalara itadori* for biological control of invasive knotweeds. Two different populations of *A. itadori* are available for field releases that have differential performance on three knotweed species and have distinct climate adaptations. A southern population from the island of Kyushu in Japan is recommended for release on Japanese and Bohemian knotweeds (*Fallopia* spp.) while the northern population from Hokkaido develops best on giant knotweed. In the first chapter, different release approaches are evaluated including the importance of release frequency and the genetic composition of released populations for establishment success in southern Michigan. Kyushu psyllids were released on Japanese knotweeds and a mix of Kyushu and Hokkaido psyllids were released on Bohemian knotweeds either as part of a single release or two introduction events. Signs of a phenological mismatch were seen for Kyushu psyllids and limited overwintering success. Release frequency appeared to be more important at smaller release sizes.

In the second chapter the effects of hybridization between Kyushu and Hokkaido psyllids are investigated on host preference and performance. Parental and reciprocal hybrid psyllids showed largely indiscriminate host choice behavior and laid eggs on all three knotweed species offered. This behavior was maladaptive for Hokkaido psyllids that were not able to develop on Japanese and Bohemian knotweeds. Hybridization had a neutral effect on reproductive potential and a neutral to positive effect on development success. These results indicate that intentional hybridization may help to increase establishment success and control of different knotweed species in different climatic regions.

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Introduction

Classical biological control, the introduction of host-specific natural enemies from the native range of an exotic species, can provide long-term sustainable control of invasive species (Hajek et al., 2007; Heimpel and Mills, 2017; Van Driesche et al., 2009). One of the milestones of a successful biological control program is the establishment of permanent field populations of the introduced biological control agents. Currently, only about 63% of releases against invasive weeds lead to establishment (Schwarzländer et al., 2018) and there is little data from biological control on how various release methods may affect establishment success (Day et al., 2004; Fowler et al., 2008; Grevstad, 1999a; Grevstad, 1999b; Memmott et al., 1998). It is known from invasion biology that propagule pressure, the size and number of introductions together, is the most consistent predictor of establishment success across a wide range of taxa (Blackburn et al., 2016; Lockwood et al., 2005; Simberloff, 2009). Additionally, outcomes of weed biocontrol releases can be influenced by abiotic factors, most notably mismatch between the climates of the collection and release areas can limit establishment success (Harms et al., 2020; Mukherjee et al., 2014; Reddy et al., 2019).

The few manipulative experimental studies conducted with weed biocontrol agents in the field show a positive relationship between release size and establishment success (Grevstad, 1999b; Grevstad, 2006; Memmott et al., 2005; Memmott et al., 1998; Williams et al., 2021) at least in the early stages of establishment (Clerck-Floate and Wikeem, 2009). However, the number of discrete introduction events can be just as important as release size for establishment success (Grevstad et al., 2011; Grevstad, 1999a; Koontz et al., 2018; MEMMOTTLZ et al., 1996). A review of 74 weed biocontrol agent introductions in Oregon found that the number of release events was more important for establishment success than release size, especially if the biocontrol agent is classified as ‘difficult to establish’ (Grevstad et al., 2011). For gorse thrips (*Sericothrips staphylinus* Haliday) released against gorse (*Ulex europaeus*) in New Zealand, multiple smaller releases were recommended instead of a single larger release to reduce the probability of a chance event driving the population extinct (Memmott et al., 1998). In an insect model system that used *Tribolium* flour beetles, several small introductions were found to work best to establish populations both in a novel environment with fluctuating environmental quality and in a stable benign environment (Koontz et al., 2018). Modeling studies that incorporate release frequency recommend multiple smaller introductions within a variable environment, but favor a single large release when Allee effects are present in a homogenous environment (Grevstad, 1999a). In cases when the target host is established only at a few sites, a few large releases are predicted to yield success (Shea and Possingham, 2000). Given the scarcity of manipulative field studies and the contrasting recommendations of models and laboratory experiments, there are no clear guidelines on how to release biological control agents against invasive weeds.

For invasive weeds with a broad distribution, there can be geographic variation in control success if natural enemies have a narrower habitable climate range than their plant hosts (Harms et al., 2020). Temperature, especially cold winters in temperate regions, is often found to limit establishment and control success (Cowie et al., 2016; Harms et al., 2020; Harms and Shearer, 2017). For example, the alligatorweed flea beetle, *Agasicles hygrophila* has a larger impact in southern Louisiana where it is able to overwinter than in northern Louisiana and in Mississippi where alligatorweed infestations need to be recolonized each summer due to overwintering mortality (Harms and Shearer 2017). For the biocontrol of water hyacinth (*Eichhornia crassipes*) that is native to South America, biological control using the weevil species *Neochetina eichhorniae* is limited in colder and temperate regions (Reddy et al., 2019). A recent review found that the variable success rates of weed biocontrol programs are likely due to climate mismatch in at least 13% of cases (Harms et al.).

In this study invasive knotweeds (*Fallopia* spp.) are used and the recently approved knotweed psyllid *Aphalara itadori* (Shinji) (Hemiptera: Aphalaridae) to assess how various release approaches may impact establishment success in southern Michigan. As part of a classical biological control program *A. itadori* was approved for field release in the USA in 2020 (Grevstad et al., 2022). This species had been released as a biocontrol agent against knotweeds since 2010 in the United Kingdom (Shaw et al., 2011), since 2014 in Canada (Clements et al., 2016), and since 2020 in the Netherlands (Camargo et al.). To date there have been no reports of its long-term establishment at any locations (Grevstad et al.). Multiple reasons have been proposed to explain the lack of establishment including predation, climate maladaptation, and low fitness due to long-term laboratory rearing (Andersen and Elkinton, 2022; Grevstad et al., 2022; Jones et al., 2021; Jones et al., 2020).

Two populations of the knotweed psyllid have been used for biological control of knotweeds. One population of *A. itadori* was founded from individuals originating from the island of Hokkaido, which lies at a latitude of 43.2°N and has a humid continental climate. The second population was collected on the southern island of Kyushu which is located at 32.6°N and is categorized as having a subtropical climate (MacLeod and Korycinska, 2019; Peel et al., 2007). Previous studies have shown that these two populations appear to be locally adapted to climate based on different responses to temperature and photoperiod (Grevstad et al., 2022). As such, the northern population should be a better climate match for southern Michigan, which lies at 42.7°N and has a humid continental climate like Hokkaido.

The two *A. itadori* populations also differ in their performance on different knotweed species. The northern population has higher reproduction and larger impact on giant knotweed (*Fallopia sachalinensis*) while the southern population performs better on Japanese knotweed (*F. japonica*) and Bohemian knotweed (*F. x bohemica*) (Grevstad et al.). However, in southern Michigan the dominant species are Japanese and Bohemian knotweeds, that are the optimal hosts for the southern Kyushu population of *A. itadori*. In this situation the ideal *A. itadori* population would carry cold-adaptation traits from the Hokkaido population along with host-adaptation traits from the Kyushu population. One method of producing such intermediate phenotypes is intentional hybridization between the above described two populations.

In insects, most life history traits including those related to climate adaptations are under polygenic control and upon crossing of distinct populations, the resulting hybrid offspring often express intermediate characters or traits that more closely resemble the maternal parent (He et al., 2021; Reznik et al., 2022; Szűcs et al., 2012; Tauber et al., 1986). In addition, intra-specific hybridization of herbivorous insects that have divergent host adaptations can alter the preference and performance of the hybrid individuals on their natal and novel host plants (Bitume et al., 2017; Hoffmann et al., 2002; Mathenge et al., 2010). Thus, it is possible that hybridization between the northern and southern populations of *A. itadori* may produce individuals that are better suited to the climate in southern Michigan and are also able to develop on Japanese and Bohemian knotweeds that are present in the region.

Experimental releases of *A. itadori* were conducted on Japanese and Bohemian knotweed infestations in southern Michigan using the southern (Kyushu) population of *A. itadori* on

Japanese knotweed infestations and both the southern and the northern (Hokkaido) populations on Bohemian knotweeds. On both knotweed species the effect of release frequency was tested on seasonal abundance and establishment success. The predictions were that multiple releases within a season would increase establishment success and that the presence of an *A. itadori* population with a better climate match or the opportunity for hybridization would result in higher seasonal abundance of psyllids on Bohemian knotweeds. In addition, we used a field cage experiment to assess the overwintering ability of the northern and southern populations and their reciprocal hybrids in southern Michigan. The expectation was that the northern population and the hybrids would have higher overwintering success than the southern population.

Materials and Methods

Study system

Knotweeds that are herbaceous perennial plants are listed among the world's worst invasive species because of their quick growth, aggressive spreading habits via rhizomes, and difficulty of control with conventional methods (Ahrens, 1975; McHugh and West Haven, 2006; Soll, 2004). Invasive knotweeds comprise three species in North America that can interbreed with each other. The three species are: Japanese knotweed (*Fallopia japonica* [Houttuyn] Ronse Decraene), giant knotweed (*F. sachalinensis* Schmidt ex Maxim) and their hybrid, Bohemian knotweed (*F. x bohemica* [Chrtek and Chrtkova] JP Bailey). Japanese and giant knotweeds are native to East Asia (Barney, 2006) and Bohemian knotweed is a result of both intentional and natural crossing. Knotweeds were introduced to North America in the 1800s as ornamentals and for erosion control, but soon escaped cultivation and have spread across 42 states in the USA, and have become particularly problematic in the northeast and Pacific Northwest (Weston et al., 2005). Large populations of Japanese and Bohemian knotweeds are present in southern Michigan where this study took place, and a few infestations of giant knotweed are found in the north (Upper Peninsula of Michigan, misin.msu.edu). Knotweeds are distinguishable based on morphological features of the leaves and stems, although Bohemian knotweeds can be difficult to identify as they inhabit the full spectrum of intermediate phenotypes as a hybrid species that is capable of backcrossing with either parental species (Gillies et al., 2016).

Knotweeds are quick-growing plants that form hollow bamboo-like stems that can grow up to 4cm in a single day (Kidd, 2000). Invasive populations of knotweeds have been shown to reproduce primarily through vegetative regeneration from cuttings and rhizomes (Brock and Wade, 1992; Sásik and Pavol Jr, 2006). As stem and rhizome fragments are often dispersed through runoff into waterways, many populations tend to form dense monocultures along riverbanks and roadsides which are difficult to control once they have become established. Chemical, physical and cultural methods can take years to have an effect on knotweed infestations due to the constant regrowth from their extensive rhizome systems (Drazan et al., 2021; Grevstad et al., 2018; Parepa et al., 2014).

The knotweed psyllid (*Aphalara itadori*) is a tiny (~2 mm) phloem-feeding insect that was identified as a highly-specialized herbivore of knotweeds that effectively depletes plant resources leading to a reduction in stem growth and even plant death at high enough densities (Grevstad et al., 2013; Shaw et al., 2009). Knotweed psyllids undergo five juvenile nymph stages which are largely sessile before emerging as adults, and therefore dispersal is mainly attributed to adult movement. Adults reach sexual maturity after about 4 days and females can lay up to 700 eggs over the course of their adult lifespan, which is approximately 46 days under laboratory conditions (20°C, 50-70% RH) (Myint et al., 2012). Females lay their eggs on the leaf surface of young plant growth, and development time from egg to adult is about 33 days at 23°C (Shaw et al., 2009) meaning that this species is multivoltine and capable of producing multiple generations each year. There are no known morphological characteristics that can be used to distinguish between the Hokkaido and Kyushu populations. The knotweed psyllid overwinters as adults, and in their native range, *A. itadori* has been observed using conifer trees as an overwintering host, although it is unknown whether psyllids use these hosts as a food source (Miyatake, 1973).

Psyllid rearing

All three species of knotweeds that were used to rear the different populations of *A. itadori* have been grown year-round at the Michigan State University (MSU) greenhouse complex. Each knotweed species has been grown from rhizomes collected from a single location in Michigan (Japanese knotweed from-83.495170, 42.466906, Bohemian knotweed from -83.7908, 42.6317, and giant knotweed from -88.479681, 46.6442711) in April 2021. Rhizomes were cut into 10-15 cm pieces and transplanted into 5.7 liter plastic pots using a peat and perlite potting mix (Suremix, Michigan Grower Products). Plants have been fertilized with Osmocote Plus (N:P:K = 15:9:12, ICL fertilizers, USA) once every 2 months. To increase the number of plants for rearing and experiments either new rhizome collections were conducted at the field sites or stem cuttings were taken from plants that reached > 50 cm in size in the greenhouse. The cuttings were approximately 5 cm long and contained at least one node where new roots could be formed. Cuttings were planted and grown similarly as the rhizomes described above.

The southern population of *A. itadori* was founded from collections made in 2004 and 2015 within Kumamoto prefecture on the Japanese island of Kyushu on Japanese knotweed. The northern population was collected in 2007 from the Lake Toya area on the island of Hokkaido on giant knotweed (Grevstad et al., 2013). The *A. itadori* populations used for experiments and field releases were founded via shipments of 480 and 400 Kyushu (southern population) adults in April and June, respectively, and 600 Hokkaido (northern population) adults in June 2021 from Oregon State University. Additional 350 Kyushu individuals were shipped by the Phillip Alampi Beneficial Insect Rearing Laboratory (New Jersey Department of Agriculture) in August 2021.

We reared the southern population on Japanese knotweed and the northern population on giant knotweeds as recommended by Grevstad et al. (2013). To initiate rearing, 100 adults were placed for 14 days onto potted knotweed plants in 40 x 40 x 60 cm mesh cages (Restcloud, Chengdu, China). The adults emerging in 4-5 weeks were kept for an additional two weeks in cages to mate before the next generation was started as described above. Insect rearing took place in two different laboratory rooms maintained at room temperature (22 °C ± 5, RH 40% ± 10, 16L: 8D).

To create reciprocal crosses between the southern (Kyushu) and northern (Hokkaido) populations, rearing cages that contained a large proportion of 5th instar nymphs were cleared of any adult stages. Newly emerging individuals were collected from these cages every 24 hours, sexed, and paired with individuals of the opposite sex from a different population. Both reciprocal hybrid crosses, the one using Kyushu females paired with Hokkaido males (FemKYU), and the one using Hokkaido females paired with Kyushu males (FemHOK) were reared on Bohemian knotweeds as described above. There was no backcrossing as rearing proceeded to the second and third generations.

Field releases

Sites for field releases were located using observations reported to the Midwest Invasive Species Information Network (misin.msu.edu). All chosen sites were in southeastern Michigan at least 2 km apart (Table 1.1, Fig. 1.1). The knotweed populations ranged in size from < 100 to >500 m^2 and usually formed linear monocultures of at least 11 m in length along roadways (Table 1.1). Identification of *Fallopia* species at each location was conducted using morphological characteristics according to Wilson (Wilson).

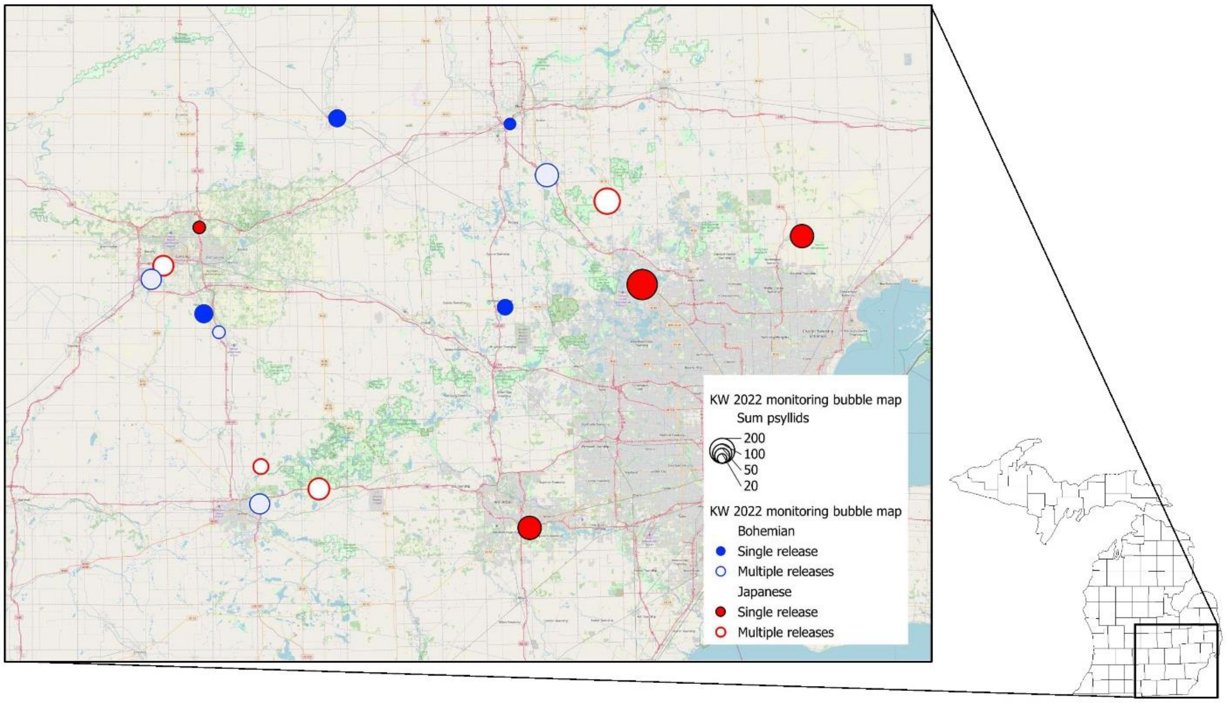

Figure 1.1. Knotweed infestations across southeast Michigan that were used for *A. itadori* field releases. Each location is colored based on the knotweed species identified (red = Japanese knotweed, blue = Bohemian knotweed). Two release treatments were used for each knotweed species: single release (solid fill) or multiple (two) release (no fill). The size of each point represents the total number of *A. itadori* adults and juveniles observed over the course of the 2022 monitoring season. For more information on the sites, release sizes and *A. itadori* population used see Table 1.1.

Table 1.1: Summary of *A. itadori* releases conducted in Michigan during 2021 and 2022. Psyllid releases were conducted in two consecutive years on Japanese knotweed sites. The top numbers indicate release sizes in 2021 and the bottom numbers are release sizes for 2022. In both years on both knotweed species two release treatments were applied, releasing either all psyllids as part of a single release or multiple (two) releases.

Site	Longitude	Latitude	Knotweed Species	Infestation size	Release dates	Psyllid population	Release size
1	-83.4628	42.8377	Japanese	200-400m^2	June+July 2021 May+June 2022	Kyushu	110+110 500+500
2	-83.5166	42.7662	Japanese	<100m^2	June 2021	Kyushu	220
3	-83.3727	42.6795	Japanese	<100m^2	June 2021 May 2022	Kyushu	220 1000
4	-83.0136	42.6437	Japanese	<100m^2	June+July 2021	Kyushu	110+110
5	-84.6053	42.7154	Japanese	200-400m^2	June+July 2021 May+June 2022	Kyushu	110+110 500+500
6	-84.5128	42.7881	Japanese	100-200m^2	May 2022	Kyushu	500+500
7	-82.9613	42.7717	Japanese	100-200m^2	June 2021 May 2022	Kyushu	220 1000
8	-84.2050	42.2922	Japanese	>400m^2	June+July 2021 May+June 2022	Kyushu	110+110 500+500
9	-83.6625	42.2185	Japanese	<100m^2	June 2021 May 2022	Kyushu	220 1000
10	-84.3543	42.3344	Japanese	<100m^2	May+June 2022	Kyushu	500+500
11	-84.1577	42.9937	Bohemian	100-200m^2	May 2022	Kyu+Hok	2000
12	-84.4623	42.5893	Bohemian	>400m^2	May+June 2022	Kyu+Hok	1000+1000
13	-84.5012	42.6243	Bohemian	>400m^2	May 2022	Kyu+Hok	2000
14	-84.6364	42.6894	Bohemian	>400m^2	May+June 2022	Kyu+Hok	1000+1000
15	-83.7130	42.9831	Bohemian	<100m^2	May 2022	Kyu+Hok	2000
16	-83.7255	42.6366	Bohemian	200-400m^2	May 2022	Kyu+Hok	2000
17	-84.3572	42.2641	Bohemian	100-200m^2	May+June 2022	Kyu+Hok	1000+1000
18	-83.6179	42.8866	Bohemian	100-200m^2	May+June 2022	Kyu+Hok	1000+1000

In 2021, eight Japanese knotweed sites were identified, of which four were randomly assigned to receive a single release while the remainder received two *A. itadori* releases of the Kyushu population 1 month apart (Table 1.1). At the single-release sites, 220 adults were released on 3-4 July 2021. At the multiple-release sites 110 adults were released on 3-4 July 2021 and 5-7 August 2021.

In 2022, eight Japanese and eight Bohemian knotweed infestations were selected for field releases. Two of the 2021 Japanese knotweed sites had to be excluded; one due to construction work and the other because of misidentification of the knotweed species that was exposed based on flowering morphology late in the 2021 season. To replace these sites, two additional Japanese knotweed infestations were located in the vicinity. The other six Japanese knotweed sites were reused for releases in 2022. Eight new Bohemian knotweed sites were identified in the same region of Michigan where the Japanese knotweed release sites were located (Fig. 1.1).

Similar to 2021, the eight field sites of both knotweed species were randomly assigned to two release treatments: single release or multiple releases. Only Kyushu psyllids were used for release on Japanese knotweeds while on Bohemian knotweeds half the psyllids released were of the Kyushu and the other half of the Hokkaido population (Table 1.1). The Japanese knotweed infestations received either a single release of 1,000 on 23-24 May 2022 or two separate releases of 500 psyllids each on 23-24 May and 24-25 June 2022. On four of the Bohemian knotweed sites 2,000 psyllids (1,000 Kyushu and 1,000 Hokkaido) were released on 2-3 June 2022. On the other four sites 1,000 psyllids (500 Kyushu and 500 Hokkaido) were released on 2-3 June and another 1,000 on 28 June 2022. In both years, to conduct the releases, the appropriate number of psyllids were placed on potted knotweed plants in rearing cages the day prior to release. The cages were transported to the field sites where the inoculated plants were carefully removed from the cage and placed in the center of the infestation. Any leftover adults were shaken from the cages onto the potted plant which was left out at the field site for one week to allow for adult psyllids to naturally disperse.

Monitoring

In 2021, we implemented a pruning treatment at each of the eight Japanese knotweed release sites to test whether providing fresh plant growth to the psyllids would increase population persistence and establishment success (Jones et al.). To implement this treatment, we pruned a 3 m wide section once in June, a separate 3 m wide section in July and left the rest of the infestation unpruned. The pruned and unpruned strips were assigned randomly within each site. Between the pruned and unpruned strips there was a 1m wide section that was pruned each month and served as a buffer between the different pruning treatments. For the pruning a weed eater was used with a brush cutter attachment (Ryobi) to cut back all plants within the strip to a height of about 10 cm. All cut stems were removed from the sites and incinerated.

The presence of psyllids was monitored every two weeks, starting one week after release by choosing a random stem within a pruning treatment and counting the number of eggs, nymphs and adults on the four closest neighboring plant stems. This strategy was repeated until 20 stems were searched in each pruning treatment, resulting in data for 60 stems at each site. Psyllid monitoring continued until 9 October 2021.

For the monitoring in 2022 we did not implement the pruning treatment. Stem heights of 10 randomly chosen knotweed stems were measured at each site at the beginning (23 May) and end of the season (26 September) to establish some baseline metrics that may be affected by biocontrol in the long term (Table S1.1). Monitoring of psyllid populations started 1 week after the first releases and continued every 2 weeks until 4 October 2022. Psyllid monitoring proceeded as described for 2021 by choosing one random stem and its four closest neighbors to count the number of eggs, nymphs, and adult psyllids. This was repeated until 20 stems were monitored at each site. In addition, a 15-minute visual search was conducted for psyllids throughout each site.

Testing overwintering success of Aphalara itadori *populations*

A common garden experiment was set up to test the overwintering ability of the two parental populations (Kyushu and Hokkaido) and their third-generation reciprocal hybrid crosses (F3 FemKYU and F3 FemHOK). Sixteen adult psyllids were placed on potted knotweed plants and placed outdoors for the winter. The Hokkaido psyllids were placed on giant knotweed plants, the Kyushu psyllids on Japanese knotweed and the two hybrid populations on Bohemian knotweed. The number of replications for each treatment differed because of differing availability of psyllids. There were 19 replicates for the Kyushu, 12 for the Hokkaido, 5 for the F3 FemKYU and 4 for the F3 FemHOK populations.

Psyllids assume a darker coloration as they are exposed to shorter daylengths and prepare for diapause (Grevstad et al.). To get psyllids of this so-called winter morph about half of the rearing cages were moved outdoors to the MSU Entomology farm (-84.497063, 42.691867, East Lansing) at the beginning of August 2022 where they were exposed to natural temperature and photoperiod conditions. Simultaneously, another portion of the rearing was conducted under modified laboratory conditions with reduced photoperiod (12L:12D) to induce diapause (22 °C ± 5, RH 40% ± 10) (Grevstad et al., 2022).

All three knotweed species used for the experiment were grown from cuttings in 5.7 L plant pots under greenhouse conditions and fertilized with slow-release Osmocote Plus (N:P:K = 15:9:12) until they were at approximately 12 weeks old. Plants were covered with a mesh sleeve (1 m tall x 40 cm wide) that was secured around the rim of the pot with an elastic cord and the winter morph psyllids were released onto the plants on 16 October 2022. Potted plants with psyllids were dug into the ground up to the rim at the MSU Entomology farm in two rows using a completely randomized design to assign treatments. Upon the first frost in mid-November, all plant material was cut down and left within the sleeve. This caused cages to collapse close to ground level, and thus allowed natural snow accumulation over the potted plants during the winter. Potted plants were dug up on 21 April 2022, placed in a greenhouse and monitored until 31 May 2022 for the presence of any adults.

Statistical methods

Statistical analyses were only conducted for the 2022 monitoring season due to the low number of psyllid observations in 2021. An Analysis of Variance (Bashtanova et al.) were used to compare the number of psyllid detections between the release treatments. Separate models were constructed for the sites with Japanese and Bohemian knotweeds given that psyllid release sizes differed (1,000 vs. 2,000) and because different *A. itadori* populations were used for releases (Kyushu only vs. Kyushu and Hokkaido combined). The detections on the 20 random knotweed stems and from the 15-minute timed searches were combined. Data were summed across monitoring weeks to create a grand total of egg, nymph, and adult detections for each site over the season. The number of adults and total number of all three developmental stages detected were compared between treatments. The release treatment (single vs. multiple release) was the fixed effect. Histograms and goodness of fit tests were used to assess data distribution. Counts were square root transformed to improve normality of the data.

A generalized linear model (*glm*) with a binomial distribution was used to compare overwintering success among the four *A. itadori* populations. Overwintering success was coded as ‘1’ if at least one psyllid was found on a replication in spring 2022 and it was coded as ‘0’ when no psyllids were found. All analyses were conducted in R v.4.2.3 using the *lme4* package (R core team 2022).

Results

Releases in 2021 on Japanese knotweed

Combining all the observed life stages, including eggs, nymphs, and adults, 144 psyllids were found in total during the 12 weeks of post-release monitoring across the eight Japanese knotweed field sites where a total 1,760 adult psyllids were released. Most of the observations were eggs (n = 72), followed by adults (n = 44) and the nymphal stage was the least commonly observed (n = 28). Sixty one percent of adults, 75% of eggs and 100% of nymphs were found at single release sites (Table 1.2). Eggs were mostly found in the first two weeks of monitoring and a few eggs were seen five to eight weeks into the monitoring indicating the initiation of a second generation in the field (Fig. 1.2). There was no apparent positive effect of knotweed trimming on psyllid numbers since more eggs (72%), more nymphs (100%), and more adults (77%) were found on untrimmed knotweeds compared to trimmed knotweeds through the season (Fig.1.2, Table 1.2). Most egg and adult detections occurred within 1-2 weeks after release indicating the presence and oviposition activity of the released adults. The observation of nymphal and adult stages until October indicates that a second generation may have been completed before winter. Overwintering success was found at two of the eight release sites in mid-May 2022 where the multiple release treatment had been implemented a year prior. A single adult was found with a 15-minute timed search at one of the sites and three 4th instar nymphs were found at one other site prior to the new psyllid releases in 2022.

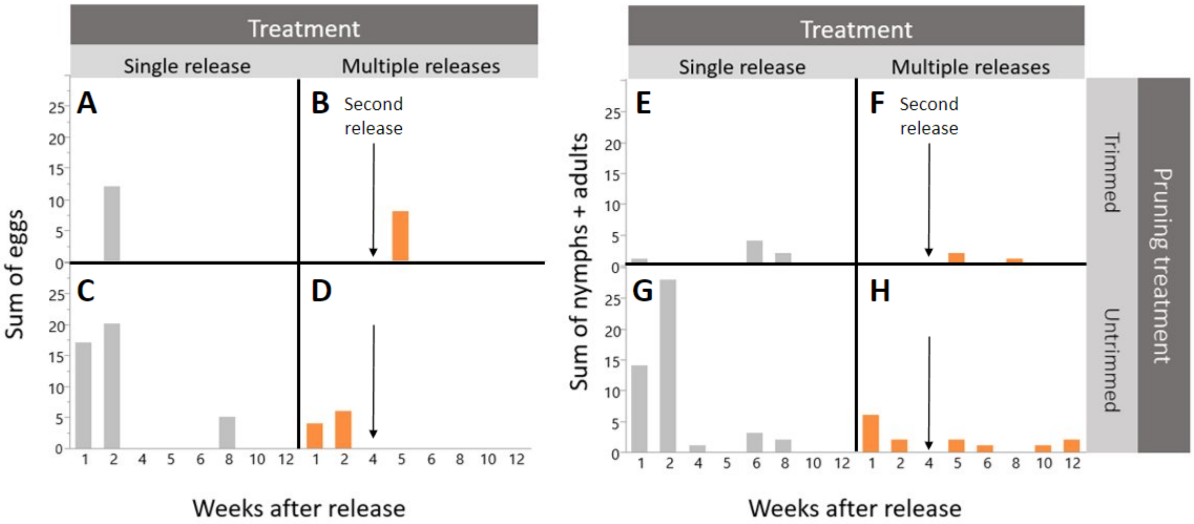

Figure 1.2. Total number of eggs (A, B, C, D) and the combined number of nymphs and adults (E, F, G, H) observed during 12 weeks of visual monitoring of leaves and stems within Japanese knotweed field sites in 2021, where 220 *A. itadori* had been released either as part of a single or multiple (two) release treatment. The arrow indicates when the second release was made, 4 weeks after the initial releases. Knotweed trimming was performed at each site, and the number of psyllids found on strips that were cut back in June and July are combined because of low observations (A, B, E, F).

Table 1.2. The total number of different developmental stages of *A. itadori* observed over a 12-week monitoring period on Japanese knotweed. In parentheses the percentage of the observations represented compared to the total observations over the different release and trimming treatments are shown. At each site 220 *A. itadori* of the Kyushu population were released. The numbers are sums across the four replicated sites for each release treatment. Trimming was performed at each site, and the June and July trimming results are combined because of low observations.

		Life stages observed in the field		
Treatment	**Total released**	**Egg**	**Nymph**	**Adult**
Single Release	880	54 (75%)	28 (100%)	27 (61%)
Multiple Release	880	18 (25%)	0 (0%)	17 (39%)
Trimmed		20 (28%)	0 (0%)	10 (23%)
Untrimmed		52 (72%)	28 (100%)	34 (77%)

Releases in 2022 on Japanese and Bohemian knotweeds

There were no differences in the number of adult psyllid detections over the season between the single (n = 446) and multiple release (n = 488) treatments either on Japanese ($F_{1,6}$ = 0.57, P = 0.5659) or on Bohemian knotweeds (n = 248 and 220, respectively) ($F_{1,6}$ = 2.04, P = 0.1721) (Fig. 1.3). The results were similar when all the detected life stages (egg, nymph, and adult) were summed up into a single metric both on Japanese ($F_{1,6}$ = 0.38, P = 0.5613) and Bohemian knotweeds ($F_{1,6}$ = 1.55, P = 0.2589) (Fig. 1.3). There were 818 total psyllids of all stages found at single release sites and 707 at multiple release sites on Japanese knotweed (Table 1.3). The total detections across life stages were somewhat lower at both the single (n = 393) and multiple release sites (n = 487) on Bohemian knotweed compared to Japanese knotweed (Fig. 1, Table 1.3).

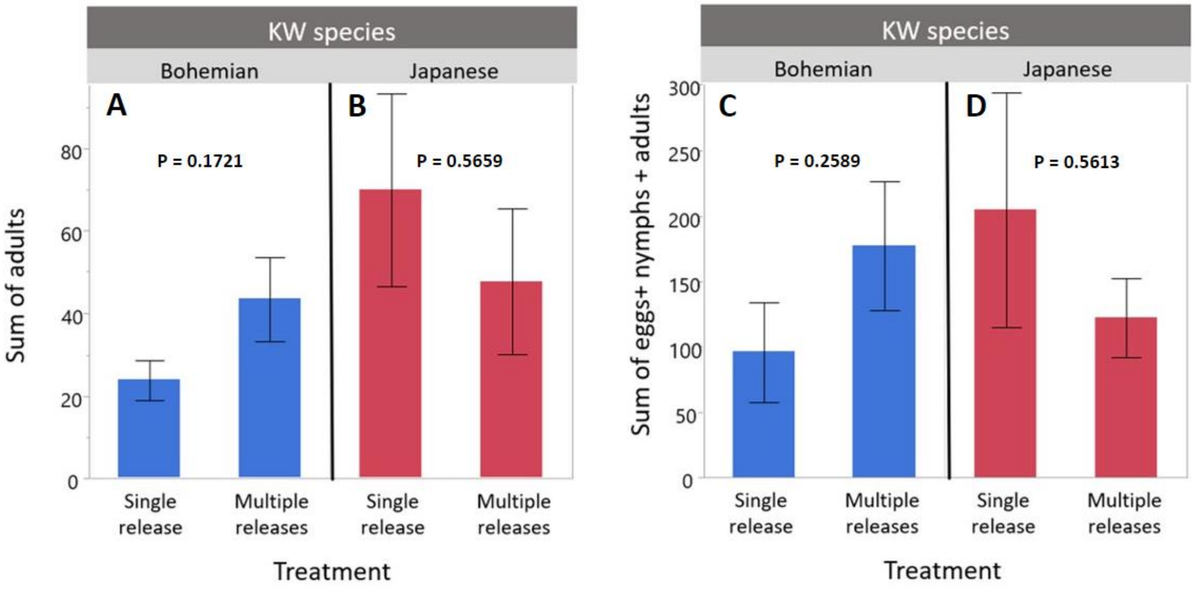

Figure 1.3. Mean (± SE) number of adults (left) and the combined number of eggs, nymphs, and adults (right) observed over the course of the 2022 monitoring season following the release of 1,000 (Kyushu) *A. itadori* on Japanese knotweed sites and 2,000 (1,000 Kyushu + 1,000 Hokkaido) *A. itadori* on Bohemian knotweed sites either as part of a single or multiple (two) release treatment.

Table 1.3. The total number of different developmental stages of *A. itadori* observed across four replicate sites for each release treatment over a 15-week monitoring period on Japanese knotweed and Bohemian knotweed. In parentheses the percentage of observations compared to the total observations over the different release and knotweed species are shown. There were four replicated sites for each of the two release treatments for both knotweed species.

Knotweed species	**Psyllid population**	**Release Treatment**	**Total released**	**Life stages observed in the field**		
				Egg	**Nymph**	**Adult**
Japanese	Kyushu	Single release	4000	446 (48%)	92 (67%)	280 (32%)
Japanese	Kyushu	Multiple releases	4000	488 (52%)	45 (33%)	174 (38%)
Bohemian	Kyu + Hok	Single release	8000	248 (53%)	40 (34%)	95 (33%)
Bohemian	Kyu + Hok	Multiple releases	8000	220 (47%)	76 (66%)	191 (67%)

Psyllids were found at all sites post-release with most stages being present until the middle of summer. Adult *A. itadori* were observed until 12 September in Japanese knotweed infestations and until 19 August within Bohemian knotweeds (Figs. 1.1, 1.4). Most psyllid detections occurred within the first month after the initial release and then decreased gradually, likely due to dispersal and/or mortality. Eggs were observed throughout the season until 9 September on Japanese knotweed and until 22 July on Bohemian knotweed. For both knotweed species, the multiple-release plots appear to have more consistent observations across sites throughout the monitoring season than the single-release treatments (Figs. 1.1, 1.4).

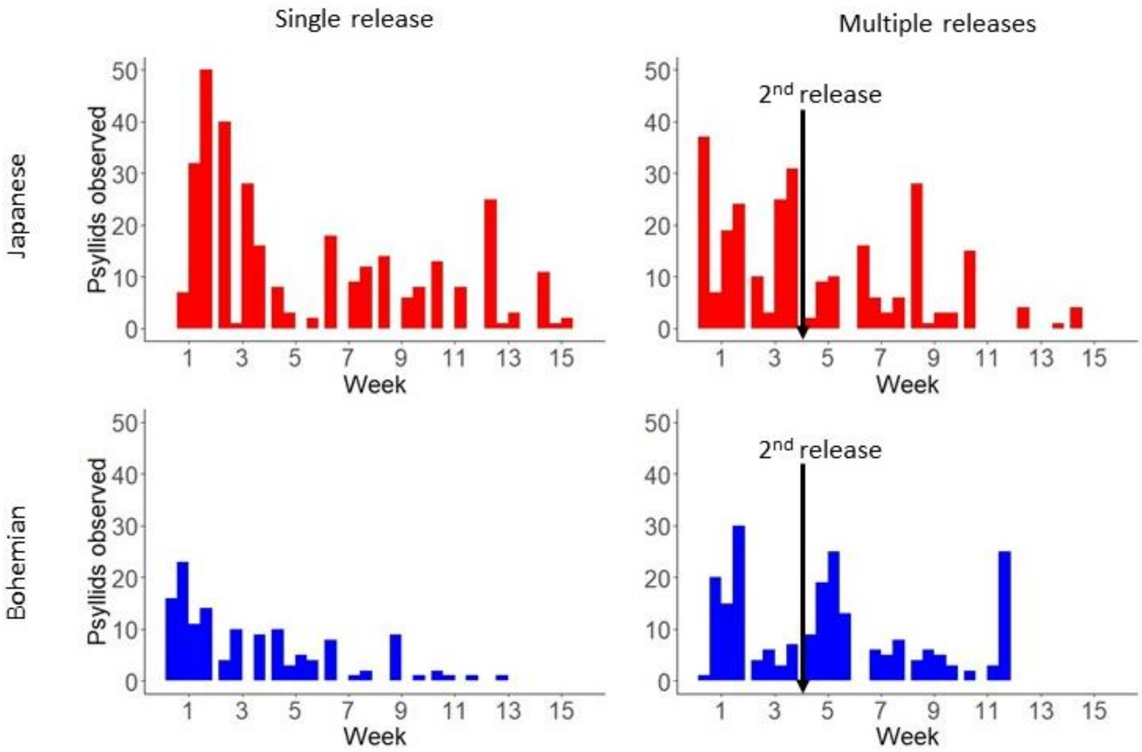

Figure 1.4. Grouped bar chart show the number of *A. itadori* adults and nymphs found at each site (individual bars for each monitoring week) over 15 weeks (30th May – 15th September) of post-release monitoring in 2022. Top graphs (red) show the number of psyllids at Japanese knotweed sites where 1,000 *A. itadori* of the Kyushu population were released either as a single (n = 4 sites) or multiple release (n = 4 sites) treatment. Bottom graphs (blue) show the number of psyllids at Bohemian knotweed sites where 2,000 *A. itadori* of the Kyushu (n = 1,000) and Hokkaido (n = 1,0000) populations were released either as a single (n = 4 sites) or multiple (two) release (n = 4 sites) treatment.

Overwintering success

There was no difference in overwintering success between the parental and hybrid populations of *A. itadori* ($\chi = 0.075$, df = 3, P = 0.9947). Out of the 40 plants that received psyllids in fall 2021 overwintering success was observed in 14 replications (potted plants). Most of these 14 plants (n = 13) yielded only a single surviving *A. itadori*. The only treatment where no overwintering survival was observed was the hybrid FemHOK population, but this population only had four replications, which was the lowest of the four *A. itadori* populations. Thirty seven percent of the Kyushu, 42% of the Hokkaido, and 40% of the FemKYU replications yielded at least one surviving adult.

Discussion

We conducted experimental field releases of the knotweed biological control agent *A. itadori* in Michigan in 2021 and 2022 to test how release frequency and the genetic composition of psyllids may influence establishment success. Psyllid detections were very low within the release years, which made any assessment regarding the effect of release frequency on seasonal abundance difficult. We did not find evidence that *A. itadori* would prefer fresh knotweed growth for oviposition or feeding in the field. We found that overwintering survival of the Kyushu population is possible in Michigan despite likely climatic and phenological mismatch. However, overwintering data is needed for sites that received either pure Kyushu or mixed Kyushu and Hokkaido *A. itadori* populations to evaluate how host match, climate match or genetic mixing may influence long-term persistence.

The field releases in 2021 used relatively few psyllids at each site (n = 220) and within season monitoring detected few psyllids (n = 144) of any developmental stages across sites. However, most of the psyllid detections (76%) occurred at sites where the 220 psyllids were released all at once compared to sites where two releases of 110 psyllids each took place a few weeks apart. The following year release sizes of *A. itadori* were much higher, 1,000 or 2,000 adults per site, and for those the different approaches of single versus multiple releases had no influence on psyllid numbers for the rest of the season. These findings suggest that when fewer individuals are available for release a single larger release may be more appropriate than multiple smaller releases for *A. itadori*. However, it is quite possible that psyllid detections within the year of releases are not the best predictor of long-term establishment success. The two sites where overwintering success was observed both were part of the multiple-release treatment where very few psyllids had been observed during the release year. The release sizes we used are comparable to other studies that released psyllids or similarly small-bodied insects at 20-1200 individuals per site (Kuniata, 1994; Memmott et al., 2005; Memmott et al., 1998). Even though establishment success of the psyllid *Arytainilla spartiophila* against broom (*Cytisus scoparius*) positively correlated with release size, most releases that used 100 psyllids or more did establish (Memmott et al., 2005). Similarly, release sizes of 270 individuals of gorse thrips resulted in 100% establishment (Memmott et al., 1998). Thus, it is likely that both the smaller 2021 and larger 2022 releases are within the range of what may be needed for establishment of *A. itadori* were it not for the possible climate or host mismatch.

We found all life stages of *A. itadori* through the season indicating reproductive activity and the production of a second generation in the field. However, it is unclear whether the timing of reproduction is within the optimal range for certain psyllid populations. Kyushu psyllids laid eggs until mid-September that is probably too late for the completion of a full generation before inclement weather. These field observations are in line with a modeling study predicting phenological mismatch of the Kyushu population at most locations in southern Michigan (Grevstad et al., 2022). This is due to differences in the critical photoperiod for diapause induction between the Kyushu and Hokkaido populations that evolved in response to local daylight conditions in their area of origin (Danilevskii, 1965). As southern populations, such as Kyushu, are moved to higher latitudes they will tend to diapause too late in the season when their critical daylight is reached and are likely to attempt more generations than what they are able to complete (Grevstad et al., 2022). On the other hand, the phenology of the Hokkaido population appears to be a good match because they were collected at a similar latitude as southern Michigan (Grevstad et al., 2013; Grevstad et al., 2022).

We found overwintering Kyushu individuals in the field so, clearly, there is variation within the population for diapause induction and other life history traits that will allow some individuals to successfully complete development. Critical photoperiod for diapause induction can also evolve quickly, such as in *Diorhabda carinulata* beetles that were released against *Tamarix* spp. and started exhibiting adaptive diapause responses within 7 years of introduction (Bean et al., 2012). This evolution in diapause timing took place during southward range expansion in *Diorhabda* beetles from already established large populations that were located at the right latitudes for minimal phenological mismatch (Bean et al., 2012). However, the Kyushu population of *A. itadori* faces a phenological mismatch immediately upon release that can result in sharp declines in population size. To account for this, higher release sizes may be recommended and even the 1000-2000 individuals we released at each site may be insufficient for long-term establishment. In fact, in Canada where up to 30,000 Kyushu psyllids were released locally long-term establishment has been elusive and sustained populations of this psyllid population have not been reported at any location to date (Grevstad et al., 2018). Thus, any inference we may make from one season and after 1-year post-release monitoring may be irrelevant if there is no long-term establishment.

We released the Hokkaido population of *A. itadori* in southern Michigan even though its optimal host plant, giant knotweed does not occur here. Given the phenological mismatch of the Kyushu population and its general lack of establishment elsewhere alternative release approaches need to be explored. One such approach may be to use intentional hybridization as a tool to increase the adaptive potential and generate novel genotypes of biocontrol agents (Szűcs et al., 2019). The Hokkaido population appears to be a good climate and phenological match and there is evidence from other studies that hybridization can alter climate adaptations, preference, and performance on different hosts in herbivorous insects and in other biocontrol systems (Bitume et al., 2017; Dingle et al., 1982; Hoffmann et al., 2002; Mathenge et al., 2010; Szűcs et al., 2012; Tauber et al., 1986). We released equal numbers of Kyushu and Hokkaido psyllids on Bohemian knotweeds and only Kyushu psyllids on Japanese knotweeds. The prediction is that by providing opportunities for hybridization, the long-term establishment of the mixed population may be better than when the Kyushu population is released alone. Observations within the release year provide little insight of whether this approach improves establishment success. Qualitative comparisons of psyllid detections suggest that slightly more eggs and adults were present at Japanese compared to Bohemian knotweed sites which would go against our expectations. It is possible that the slightly higher psyllid detection on Japanese knotweeds reflects more widespread establishment success from the 2021 releases than what we were able to observe prior to the second set of psyllid releases in 2022. Alternatively, it is possible that most Hokkaido psyllids died shortly after release because of low developmental success on Bohemian knotweeds and did not contribute to any late-season detections (Grevstad et al., 2013). Multi-year observational data combined with molecular analyses will be needed to assess the genetic composition of any surviving psyllids on the Bohemian knotweed releases to assess whether hybridization occurred and if it confers any advantage for long-term establishment and population growth.

We implemented a pruning treatment in 2021 since it has been suggested that it may increase survival of *A. itadori* (Jones et al., 2020). Since this treatment was applied to all release sites we cannot assess if it resulted in increased survival between sites, but we did not find evidence that psyllids would seek out plants with fresh growth. We found fewer eggs, nymphs and adults on plants that were cut back. The pruned strips were uniformly lower within a 3-m wide strip than the surrounding knotweed plants and it is possible that while providing more favorable conditions for feeding and development, psyllids could have been more apparent to potential predators such as pirate bugs and ladybugs as a result of fewer hiding places. Predation is one of the factors that may limit establishment and population growth of psyllids (Jones et al., 2020), and therefore it is possible that dispersing adults avoided the pruned plants.

With this study we have begun testing different release approaches to establish *A. itadori* for the biological control of invasive knotweeds in Michigan. The two years of monitoring we performed to date are inadequate to assess the importance of release frequency or the effects of possible hybridization in the field between populations on establishment. However, the data discussed above may provide some insights regarding the establishment capacity of a newly-introduced insect, and how long-term establishment of this species may be influenced by abiotic factors.

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CHAPTER 2: COULD HYBRIDIZATION INCREASE ESTABLISHMENT SUCCESS OF THE BIOLOGICAL CONTROL AGENT *APHALARA ITADORI* (HEMIPTERA: APHALARIDAE) AGAINST INVASIVE KNOTWEEDS?

Introduction

Classical biological control aims to introduce and establish exotic natural enemies to reduce populations of invasive species. It can provide long-term and sustainable control of invasive species; however, low establishment success and variable or low impact of the introduced agents are ongoing problems during the implementation of biocontrol programs (Cock et al., 2016; Schwarzländer et al., 2018; Van Driesche et al., 2020). This could be due to a variety of factors, including reduced genetic diversity because of long-term laboratory rearing that can limit the ability of the biocontrol agents to adapt to their new environment (Szűcs et al., 2019). The use of intentional intraspecific hybridization as a tool, and in general, a better integration of evolutionary principles into the practice of biological control are emerging new directions to enhance the fitness and adaptive ability of biocontrol agents (Leung et al., 2020; Moffat et al.; Sentis et al., 2022; Szűcs et al., 2019).

Hybridization can promote rapid evolutionary change and facilitate adaptations, since it can increase genetic variation, which serves as the raw material for evolution (Dlugosch et al., 2016; Schierenbeck and Ellstrand, 2009; Stebbins, 1959). Inter- and intraspecific hybridization can also create novel genotypes that may exhibit unique traits and adaptations that are not found in the parental populations (Arnold, 1997; Rieseberg and Willis, 2007; Stebbins, 1959). Early-generation hybrids can exhibit hybrid vigor, or heterosis, whereby the fitness of hybrids is higher than that of the parents (Edmands, 2007; Lynch, 1991). However, recombination in later-generation hybrids can erase heterosis, disrupt co-adapted gene complexes (hybrid breakdown), and outbreeding depression may ensue that can result in lower fitness of hybrids than their parents (Edmands, 2007; Lynch, 1991).

In the context of biological control, promoting rapid adaptation to novel and changing climates is desirable, but there are concerns that hybridization may lead to rapid evolutionary changes in the host range of agents. Upon hybridization many traits may show intermediate values compared to the parents, which may be due to the high additive genetic variance that governs many life history traits (Danilevskii, 1965; Dingle et al., 1982; Hoy, 1975; Tauber et al., 1986). However, there can also be sex-linkage and maternal effects in the expression of traits in hybrids, which can result in resemblance towards either parent, but more often the maternal parent (Hard et al., 1993; Mousseau and Dingle, 1991; Tauber et al., 1986). From the few studies that investigated the effects of hybridization on host use it appears that hybridization can have immediate effects on host-specificity of herbivorous insects. When the strains or species that are crossed have different host preferences the hybrids may exhibit specificity towards either of the parental host species and preference can change as hybridization progresses in later generations (Bitume et al., 2017; Hoffmann et al., 2002; Mathenge et al., 2010). However, when the parental strains that are crossed have similar host ranges hybridization do not necessarily alter the preference or performance of hybrids on suboptimal non-target species (Szűcs et al., 2021). Little is known about how intraspecific hybridization may influence host and climate adaptations in herbivorous insects used as biocontrol agents and without a better understanding of these basic processes, we cannot integrate evolutionary principles into biocontrol to improve the outcomes of programs.

The current study investigates whether intraspecific hybridization could be used as a tool to improve the establishment and the impact of biological control agents against invasive knotweeds. Three species of invasive knotweeds, Japanese knotweed (*Fallopia japonica*), giant knotweed (*F. sachalinensis*) and their hybrid Bohemian knotweeds (*F. x bohemica*) have been targeted for biological control by the psyllid *Aphalara itadori* (Hemiptera: Aphalaridae) for over a decade in the United Kingdom, since 2014 in Canada and since 2020 in the USA and the Netherlands (Camargo et al., 2022; Grevstad et al., 2022). Despite large scale, repeated introductions of *A. itadori* using thousands of individuals locally, long-term establishment, population growth and control of knotweeds have not been successful at any locations to date (Fung et al., 2020; Grevstad et al., 2018; Grevstad et al., 2022; Jones et al., 2021). The lack of establishment can be due to multiple factors, including climate mismatch, predation, or low fitness of the agents because of long term laboratory rearing (Andersen and Elkinton, 2022; Grevstad et al., 2022; Jones et al., 2021; Jones et al., 2020). Given that currently two populations of *A. itadori* are available for introduction in Michigan which are specific to different knotweed species and that the populations have distinct climate adaptations, intraspecific hybridization between them could increase genetic diversity, improve fitness, adaptive potential to different climates, and alter host preference. These outcomes would be desirable in Michigan where only the long-term laboratory-reared populations are available for introduction, and where releases face the problem of matching either the most fitting population on existing knotweed infestations or the best climate match of *A. itadori*.

In southern Michigan, where a humid continental climate prevails, large populations of Japanese and Bohemian knotweeds are present (misin.msu.edu). The southern population of *A. itadori* which was collected on the island of Kyushu in Japan has the best performance on these two knotweed species (Grevstad et al., 2013). However, Kyushu has a subtropical climate. The northern population of *A. itadori* was collected from a similar climate as southern Michigan, on the island of Hokkaido in Japan, but they have the best performance on giant knotweeds and low fitness on Japanese and Bohemian knotweeds (Grevstad et al., 2013). Thus, there appears to be no optimal release approach in southern Michigan using either populations of *A. itadori*.

Hence, we explored the effects of hybridization on fitness, host choice and developmental success of *A. itadori* to evaluate the biocontrol potential of hybrids. We created reciprocal hybrids between the southern and northern populations and compared their fecundity and their host choices between the three knotweed species with those of the parental populations in multiple choice tests. We also assessed developmental success of the hybrid and parental populations on the three knotweed species. We hypothesized that hybrids would show intermediate traits between the parental populations regarding host choice and developmental success on the different knotweed species. In addition, we predicted that hybridization would lead to heterosis, possibly increasing fecundity of either or both reciprocal hybrid crosses.

Materials and Methods

Study system

Knotweeds were introduced in the 1800s as ornamentals and for erosion control but escaped cultivation and have now invaded 42 states in the USA, becoming particularly problematic in the northeast and Pacific Northwest (Grevstad et al., 2018). Knotweeds are herbaceous perennial plants with leathery leaves and hollow, bamboo-like stems that can grow 1-3 meters tall. They primarily reproduce vegetatively by cuttings and rhizomes (Grevstad et al., 2018). Knotweeds often create dense monocultures in forests, riverbanks, floodplains, and roadsides. They are difficult to control by chemical, physical and cultural methods because of constant regrowth from their extensive root systems (Grevstad et al., 2018).

The knotweed psyllid, *A. itadori* feeds on the sap of knotweeds using their piercing mouthparts. This feeding can weaken plants and at high enough psyllid densities may kill them (Grevstad et al.). Psyllids undergo incomplete metamorphosis with eggs hatching into nymphs that undergo five stages that resemble adults more with each successive stage (Hodkinson, 1974). Nymphs are largely sessile, and the development time from egg to adult is about 33 days at 23°C to develop (Shaw et al., 2009). Adult knotweed psyllids are about 2 mm long and reach sexual maturity approximately 5 days after adult eclosion, but are capable of mating as early as 48 hours after adult emergence. Females reach peak fecundity at 20 days old, and a single individual can lay up to 700 eggs over the course of its lifetime. Adult lifespan averages about 55 days for females and 38 days for males under ambient laboratory conditions (20°C, 50-70% RH) (Myint et al., 2012).

Growing knotweeds

Rhizomes of each of the three knotweed species were collected from a single location in Michigan (Japanese knotweed:-83.495170, 42.466906, Bohemian knotweed:-83.7908, 42.6317, and giant knotweed:-88.479681, 46.6442711) in April 2021. Rhizomes were cut to 10-15 cm pieces and transplanted into 2.5 l plastic pots using a peat and perlite potting mix (Suremix, Michigan Grower Products). Plants were fertilized with Osmocote Plus (N:P:K = 15:9:12, ICL fertilizers, USA) once every 2 months and grown at the Michigan State University (MSU) greenhouse facilities. To propagate knotweeds, 5 cm stem cuttings that contained at least one node were taken from plants of >50 cm size. Cuttings were planted similarly as the rhizomes described above and plants grown from these cuttings were used for experiments. The height of knotweed plants that were used for this experiment ranged from 20 cm to 35 cm.

Psyllid rearing and hybridization

The original Kyushu colonies were founded by individuals from collections made in 2004 and 2015 within Kumamoto prefecture on the Japanese island of Kyushu, while the source population of the Hokkaido colony originated from individuals collected in 2007 from the Lake Toya area on the island of Hokkaido (Grevstad et al., 2013). The *A. itadori* populations at MSU were founded via shipments of 480 and 400 Kyushu (southern population) adults in April and June, respectively, and 600 Hokkaido (northern population) adults in June 2021 from colonies maintained at Oregon State University. Additional 350 Kyushu individuals were shipped from the Phillip Alampi Beneficial Insect Rearing Laboratory (New Jersey Department of Agriculture) in August 2021.

We reared the Kyushu population on Japanese knotweed and the Hokkaido population on giant knotweeds (Grevstad et al.) by placing 100 adults into a 40 x 40 x 60 cm mesh cage (Restcloud, Chengdu, China) containing a single potted knotweed plant for 14 days. The adults emerging over the course of 4-5 weeks were kept for an additional 2 weeks in cages to mate and then the next generation was started as described above. The rearing took place in a laboratory at room temperature (22 °C ± 5, RH 40% ± 10, 16L: 8D); under these conditions, psyllids developed from egg to adult in 35 days.

To create the reciprocal crosses knotweed plants infested by either Kyushu or Hokkaido 5th-instar nymphs were cleared of all adults and monitored every 24 hours. Newly-emerging adults were collected, sorted by sex based on the identification of genitalia (Hall, 2008) and paired with similarly collected virgin individuals of the opposite sex and population. Both reciprocal hybrid crosses, the one using Kyushu females paired with Hokkaido males (FemKYU), and the one using Hokkaido females paired with Kyushu males (FemHOK) were reared on Bohemian knotweeds by placing 100 psyllids (50♀ x 50♂) in a cage containing a potted knotweed plant. Adults were kept in the cage for 2 weeks to mate and lay eggs and then removed. Developing psyllids were kept together in the same cage for 8 more weeks that allowed for adult emergence, sexual maturation, and mating. These first-generation (F1) adults were used to start the second generation (F2) without any additional crossing or back-crossing.

Figure 2.1. ***Aphalara itadori*** multiple-choice tests with the three knotweed species (Japanese, Bohemian and giant knotweed) arranged in a cage. Each cage represented a replication and received 25 pairs of Hokkaido, Kyushu or reciprocal hybrid *A. itadori* (F2 FemKYU and F2 FemHOK) that had 72 hours to lay eggs.

Host choice experiment

One knotweed plant of each species was placed into a rearing cage equidistant from each other in a randomly assigned position in each cage (Fig. 1). Each cage was randomly assigned to receive one of four psyllid treatments (Hokkaido, Kyushu, F2 FemKYU, or F2 FemHOK), with six replicate cages set up for each treatment for a total of 24 cages. Using a manual aspirator, 25 females and 25 males about 14 days old of the corresponding psyllid population were collected in a 33 mL polystyrene vial and then released at the center of the cage. The inoculation period for this experiment was 72 hours, after which all adult psyllids were removed from the cage. The knotweed plants were also removed, and the number of eggs on each plant was counted by visually inspecting the surface of the leaves using a 10x magnification hand lens. Once the eggs were counted each plant was covered individually by a mesh cage (1 m tall x 40 cm wide) that was secured to the rim of the pot by an elastic band and labeled with the treatment and replication number. Individually covered plants were placed in the greenhouse (21 °C ± 5, RH 50% ± 10, 16L: 8D) where knotweeds were grown. After 50 days, once almost all of the nymphs had emerged as adults but prior to substantial adult mortality, the number of emerged adults from each plant was recorded.

Statistical analyses

Fecundity was calculated as the sum of eggs laid within each mesh cage by the 25 females released on the three knotweed plants within each of the six replicated cages. Fecundity data were square root transformed to meet the assumptions of normal distribution. An Analysis of Variance (Bashtanova et al.) model was used with psyllid populations (Kyushu, FemKYU, FemHOK, and Hokkaido) as a fixed effect to compare fecundity of the different populations.

To evaluate oviposition preference the number of eggs laid on each plant species by each psyllid population were compared using a linear mixed model (*lmer*) where psyllid population, knotweed species (Japanese, Bohemian, and giant) and their interactions were the fixed effects. Cage was included as a random effect to account for possible differences among plants used in the different replicates. Egg count data were square root transformed to improve normality.

A general linear mixed model (*glmer*) was used to compare development success of the different psyllid populations on the three knotweed species. Given that the survival data is bounded between 0 and 1, a binomial distribution was used with a logit link function. Psyllid population, knotweed species and their interaction were the fixed effects and cage was the random effect in the model. Wald chi-square test was used to obtain test statistics for this model.

For the oviposition preference and survival analyses post-hoc pairwise comparisons were performed using the *emmeans* package with Tukey-adjustment (Lenth et al., 2019). All analyses were conducted in R v.4.2.3 using the *lme4* package (R core team 2022).

Results

Host choice experiment

There was no difference in the fecundity of the parental and hybrid *A. itadori* populations ($F_{3, 20}$ = 1.03, P = 0.3988). The 25 females released within each cage (replicate) of the Kyushu parent laid 292 ± 57 (mean ± SE), the Hokkaido parent 447 ± 70, the FemKYU hybrid 396 ± 66, and the FemHOK hybrid 378 ± 65 eggs on average across the three knotweed species offered. The different psyllid populations tended to lay similar number of eggs ($F_{3,20}$ = 1.02, P = 0.4052) on the three knotweed species ($F_{2,40}$ = 2.56, P = 0.0902) except for the Hokkaido population (psyllid population*plant species: $F_{6,40}$ = 2.79, P = 0.0233) (Fig. 2). Hokkaido females laid significantly more eggs on Bohemian knotweeds (206.4 ± 38.7) than on Japanese knotweeds (74.9 ± 23.3) while the Kyushu parent and the hybrids did not discriminate among knotweed species.

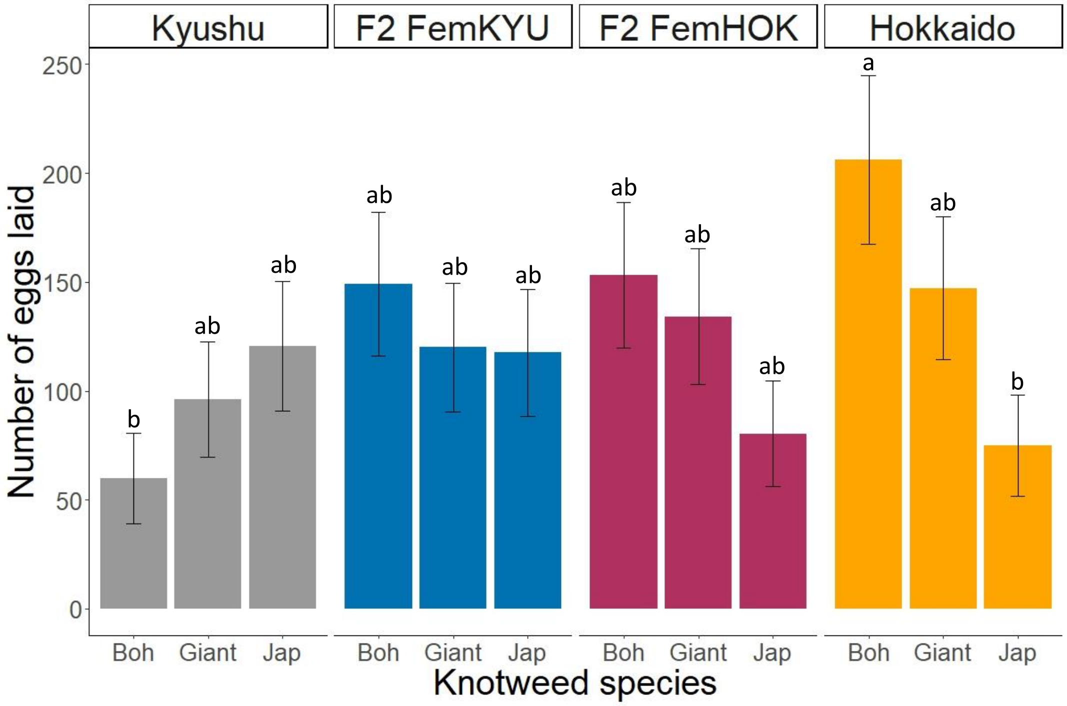

Figure 2.2. Mean ± SE of eggs laid by 25 females originating from four *A. itadori* populations (Hokkaido, Kyushu and their reciprocal hybrids: FemKYU and FemHOK) on three knotweed species (Japanese, Bohemian, and giant) over the course of 72 hours in a multiple-choice experiment. Letters above the bars indicate significant differences at ($\alpha < 0.05$) across population treatments based on post-hoc pairwise comparisons using Tukey adjustment.

There were significant differences in development success of nymphs from the four psyllid populations on the three knotweed species (psyllid population*plant species interaction: χ = 833.87, df = 6, $P < 0.001$) (Fig. 2.3). Nymphs of the FemHOK hybrid population had similar levels of developmental success across all knotweed species. The Hokkaido and Kyushu parent had the highest survival on their optimal hosts, while the FemKYU hybrid performed best on Bohemian knotweed.

In the cross where the female is from the Hokkaido population and the male is from Kyushu (FemHOK) hybridization significantly increased survival on Bohemian (36%) and Japanese knotweeds (45%) compared to the Hokkaido parent (< 7%). However, hybridization also led to lower development success on giant knotweeds (37%) compared to the maternal parent (63%). For the other type of cross where the female was from Kyushu (FemKYU) there was some decline in survival on Bohemian knotweeds (52%) compared to the maternal parent (63%) and a larger reduction on Japanese knotweed (21%) compared to the Kyushu parent (61%). On giant knotweed both reciprocal crosses had similar performance as the Kyushu parent.

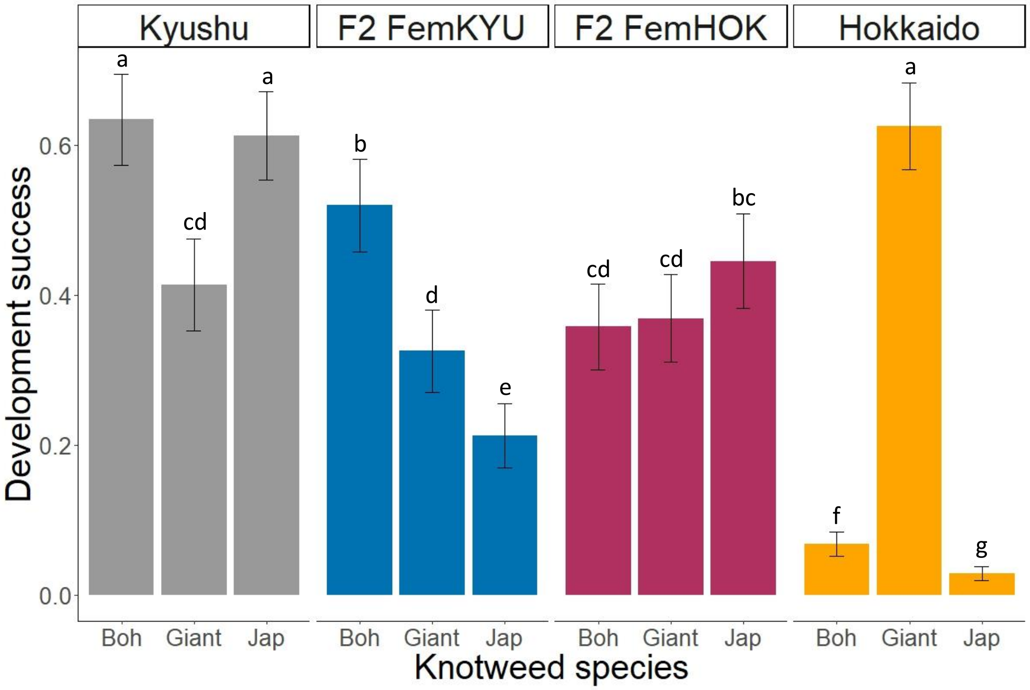

Figure 2.3. Development success (mean ± SE) of four populations (Hokkaido, Kyushu and their reciprocal hybrids: FemKYU and FemHOK) of *A. itadori* on three knotweed species (Japanese, Bohemian, and giant). Letters above the bars indicate significant differences ($\alpha < 0.05$) across treatments based on post-hoc pairwise comparisons using Tukey adjustment.

Discussion

We found that the effects of intraspecific hybridization were neutral in terms of fecundity and host acceptance. The reciprocal hybrids and the parental populations laid eggs on all knotweeds showing little preference towards any of the three species except for the Hokkaido parent that preferred to lay a greater number of eggs on Bohemian knotweed than Japanese knotweed. However, this host choice was maladaptive because of low survival of eggs to adulthood. On the other hand, the survival of the hybrid crosses was either intermediate between the parental populations or higher than the survival of at least one of the parents on given host plants. Thus, the sum effects of hybridization appear to be either neutral or positive in this system.

We did not find evidence for hybrid vigor with regards to fecundity since the reciprocal hybrid populations and the parental populations laid similar number of eggs when summed up across knotweed species. Heterosis is usually strongest in the first generation, and we used second generation hybrids in our experiments where the effects may be less pronounced. Yet, given that both parental populations have undergone long-term laboratory rearing some increase in fitness was expected upon hybridization, and second and later generation intraspecific hybrids showed higher performance than their parents in other insects as well (Hufbauer et al., 2013; Szűcs et al., 2012; Szűcs et al., 2017). It is possible that because of the small size, high fecundity, and relative ease of rearing, *A. itadori* have been kept at large enough population sizes that buffered them from genetic problems, such as inbreeding or drift that hybridization could have alleviated.

When presented with a choice between three knotweed species, both the parental and hybrid *A. itadori* populations accepted all three species for oviposition (Fig. 2). This indiscriminate egg laying behavior was also observed in a recently collected distinct population (Murakami) of *A. itadori* in paired-choice tests (Camargo et al., 2022). Similarly, all three knotweed species were accepted for oviposition in no-choice tests by both the Kyushu and Hokkaido populations during host range testing (Grevstad et al., 2013). All the above experiments were conducted in different geographical regions, the one using the Murakami population in the Netherlands (Camargo et al., 2022), the host range testing in Oregon and the

United Kingdom (Grevstad et al., 2013), and this study in Michigan, and they all used local knotweed populations for the experiments. Thus, it appears that a variety of genotypes of the three knotweed species are all recognized as potential hosts by *A. itadori*. This can be beneficial for field releases when mixed stands of different knotweed species co-occur, however, some of these choices do not reflect survival probability of psyllids.

The host choices of the Hokkaido population appear maladaptive since females from this population lay most of their eggs on Bohemian knotweed but can only develop on giant knotweed, which may be an artifact of over a decade of laboratory rearing and lack of exposure to different knotweed species. Its developmental success on Bohemian and Japanese knotweeds were under 1% (Fig. 3). Such suboptimal host choice behavior has been observed in many other herbivorous insects (Alred, 2021; Badenes-Perez et al., 2006; Berenbaum, 1981; Davis and Cipollini, 2014; Faldyn et al., 2018; Ries and Fagan, 2003; Schlaepfer et al., 2005). Notably, monarch butterflies (*Danaus plexippus*) may lay up to a quarter of their eggs on invasive swallow-wort vines (*Vincetoxicum* spp.) that are related to their milkweed hosts but do not support larval development (Alred, 2021; Casagrande and Dacey, 2014). The Kyushu population does not discriminate among knotweed species for oviposition, but it has relatively high development success (40-62%) on all three species. Our results for survival of both the Kyushu and Hokkaido populations on the different knotweed species are in line with findings during host-specificity testing (Grevstad et al., 2013).

In the same way that hybridization can have a limited effect on host preference towards suboptimal hosts when species share a similar host range (Szűcs et al., 2021), it is not surprising that the reciprocal hybrid populations exhibit the same indiscriminate oviposition behavior as was exhibited by both parental populations. Additionally, our development success results align with one other study by Fung et al. (Fung et al.) that compared the survival of the F4 FemHOK cross and the Kyushu parent on Japanese knotweed and found lower performance of the hybrids. The conclusion made by Fung et al. was that hybridization would not be beneficial for biological control in this system. However, we can place the effects of hybridization of *A. itadori* in a better context since we tested both reciprocal hybrids and compared their performance to both parental populations on all three knotweed species. We found that the survival of the reciprocal hybrids is somewhat intermediate between those of the parental populations on all three knotweed species (Fig. 3), which is a common outcome of intraspecific hybridization (Dingle et al., 1982; He et al., 2021; Szűcs et al., 2012; Tauber et al., 1986). This means that overall, hybridization improved performance compared to one parent and decreased performance compared to another parent. However, an intermediate developmental success is better than no development at all (see Hokkaido psyllids on Japanese and Bohemian knotweeds).

Considering developmental success alone we concur with Grevstad et al. (2013) that the Kyushu population is best suited for release on Bohemian and Japanese knotweeds and the Hokkaido population should only be used for releases on giant knotweed. However, the Kyushu population could also be used on giant knotweeds if the Hokkaido population is not available since they have relatively high developmental success on this species. In cases where cold adaptation traits from the Hokkaido population may be desirable, such as in Michigan, the release of hybrids might increase chances of overwintering success, and should be considered as a viable alternative to either parental population.

An additional climate factor is photoperiod, which combined with temperature is used by most insects, including *A. itadori* to decide when to enter diapause (Danilevskii, 1965; Grevstad et al., 2022). Native species are locally adapted to use the cues from shortening daylength to prepare for winter and they will switch from a reproductive phase to a non-reproductive phase at a critical photoperiod (Danilevskii, 1965; Masaki, 1999). The Hokkaido population that was derived from collections made at 42.6° latitude enters its non-reproductive stage at a longer critical photoperiod than the Kyushu psyllids originating from a latitude of 32.8° N, meaning that field populations will diapause earlier than their Kyushu counterparts (Grevstad et al., 2022). This appears to be a desirable trait in southern Michigan that is located at the same latitude as Hokkaido. However, the Hokkaido population will have low survival on the prevailing Japanese and Bohemian knotweeds in southern Michigan. On the other hand, because of their shorter critical photoperiod, Kyushu psyllids may start a new generation later in the season that they cannot complete before cold temperatures set in.

In sum, based on mismatches in temperature, photoperiod, and host plant availability neither the Kyushu nor the Hokkaido population may be ideal for releases in southern Michigan. Given that the hybrids between these two populations are able to develop and lay eggs on any of the three knotweed species and that they will have a mix of genotypes from both parental populations it is likely that there will be individuals with traits that can confer better survival and performance than those of either parent. Hybrids could also adapt faster to altered climates and photoperiod regimes because of their likely higher genetic diversity. We know from other weed biocontrol systems that critical daylength can evolve rapidly and that hybridization can alter the timing of diapause (Bean et al., 2012; Szűcs et al., 2012). For example, rapid evolution of the critical photoperiod was found in *Diorhabda carinulata* that allowed the southward expansion of this agent used to control *Tamarix* spp. In the USA (Bean et al., 2012). In ragwort flea beetles (*Longitarsus jacobaeae*) used against the invasive *Jacobaea vulgaris* intraspecific hybridization altered the summer diapause response (Szűcs et al., 2012) and increased the biocontrol potential of hybrids in the field (Szűcs et al., 2019).

Additionally, as a hybrid species, Bohemian knotweeds can possess a greater degree of genetic diversity, which has been proposed as a main characteristic in this species' invasive potential (Gillies et al., 2016; Parepa et al., 2014). In such a case where genotypes of a target species are diverse, hybrids may be well suited in that they maintain a greater degree of diversity of their own compared to the parental populations, potentially benefitting establishment probability through the increased likelihood of adaptive evolution (Szűcs et al., 2017).

The release program of *A. itadori* in North America is still in its early stages, so monitoring of initial Kyushu and Hokkaido field populations will be essential to assess the establishment success and control potential of the Kyushu and Hokkaido populations. In addition, a new population of *A. itadori* was collected in Niigata prefecture in Japan on the island of Honshu in 2019, called the Murakami population (Camargo et al., 2022). This population performs best on Bohemian knotweed and based on collection location its climate adaptation may be somewhat intermediate between the Kyushu and Hokkaido populations (Camargo et al., 2022). The first releases of the Murakami population were conducted in the Netherlands in 2020 (Camargo et al.). However, it may take years before this population is approved for field release in the United States, therefore, releases for the foreseeable future has to focus on the populations that are currently available.

The effort to combat the spread of knotweed through the use of biological control has been a long and challenging process while producing limited success in establishing long-term field populations of the agent. In order to avoid the pitfalls encountered in previous efforts, it is apparent that there need to be modifications made to current release protocols. Hybridization between two populations already approved for biocontrol release might prove to be a relatively simple and efficient method of increasing rates of establishment.

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APPENDIX 1: RECORD OF DEPOSITION OF VOUCHER SPECIMENS

The specimens listed on the following sheet(s) have been deposited in the named museum(s) as samples of those species or other taxa which were used in this research. Voucher recognition labels bearing the Voucher No. have been attached or included in fluid-preserved specimens.

Voucher No.: 2023-04

Title of thesis or dissertation (or other research projects):

Experimental field releases and the effects of hybridization on the weed biological control agent *Aphalara itadori* (Hemiptera: Aphalaridae)

Museum(s) where deposited and abbreviations for table on following sheets:
Entomology Museum, Michigan State University (MSU)
Other Museums:

Investigator's Name(s) (typed)

Andrew Yoshimoto

Date 3/17/20

*Reference: Yoshimoto, C. M. 1978. Voucher Specimens for Entomology in North America. Bull. Entomol. Soc. Amer. 24: 141-42.

Deposit as follows:

Original: Include as Appendix 1 in ribbon copy of thesis or dissertation.

Copies: Include as Appendix 1 in copies of thesis or dissertation. Museum(s) files. Research project files.

This form is available from and the Voucher No. is assigned by the Curator, Michigan State University Entomology Museum.

APPENDIX 2: SUPPLEMENTARY TABLES

Table S1. Knotweed heights and stem densities at eight Bohemian and eight Japanese knotweed sites prior to field releases of *A. itadori* and at the season in 2022.

		Stem height (mean[cm]±SE)		**Stem density (mean±SE)**	
Knotweed spp.	**Release treatment**	**Pre-release**	**End of season**	**Pre-release**	**End of season**
Japanese	Single	79.1 ±15.41	184.25±18.04	19.5±3.0	25.3±5.6
	Multiple	96.8±20.64	158.48± 19.29	29.5±16.7	23.5±11.2
Bohemian	Single	105.5±14.74	217.75±16.69	22.5±7.3	29±3.7
	Multiple	128.8±20.64	204.23± 25.70	17.5± 7.6	19.5±7.3