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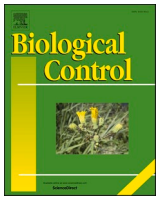

Genetic matching and the identification of a promising biocontrol agent validates a decision to survey natural enemies of *Urena lobata* in Malaysia

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HIGHLIGHTS

- The native range of *Urena lobata* is uncertain but likely includes Asia.
- We surveyed in Malaysia and prioritized a tingid *Haedus vicarius* for testing.
- *H. vicarius* proved to be host specific.
- *U. lobata* plants in Malaysia are a good match to plants in Vanuatu.
- Our selection of survey sites was validated.

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ABSTRACT

Urena lobata is a major introduced pasture weed in Vanuatu where a biocontrol program to mitigate its impacts commenced in 2018. There was considerable uncertainty regarding the native range of *U. lobata*, although published literature, coupled with very simple climate matching suggested that Southeast Asia should be the most promising region in which to survey for natural enemies. We, therefore, conducted surveys for candidate biocontrol agents in Malaysia and conducted genetic matching to compare plants growing in Vanuatu, Malaysia, and other regions in the invaded and purportedly native range of *U. lobata.* Surveys in Malaysia prioritized a tingid bug *Haedus vicarius* as a promising candidate agent for the biocontrol of *U. lobata* and subsequent host specificity testing confirmed it is sufficiently host specific to be released in Vanuatu. Genetic matching indicated that plants growing in Malaysia are a good match to plants present in Vanuatu, validating our selection of survey sites.

1. Introduction

Urena lobata L. (Malvaceae) is an erect, woody perennial herb or small shrub, usually around 1.5 m tall, commonly known as Caesar weed or Congo jute in many countries, and hibiscus bur in Vanuatu. It is a pantropical weed in pastures, sugarcane fields, coffee plantations, rice plantations, and perennial crop plantations (CABI, 2013). In Vanuatu, *U. lobata* is listed as a target for biological control (DEPC, 2014) due to its serious impacts on pastures affecting the cattle industry. A biocontrol program targeting *U. lobata* in Vanuatu, commenced in 2018.

Host-specific, coevolved natural enemies are most likely to be found on the target weed within its native range (e.g., Goolsby et al., 2006a). Further, some biocontrol agents are adapted to only attack certain biotypes of the host plant, for example, the Chromolaena stem gall fly *Cecidochares connexa* (Macquart) (Paterson and Zachariades, 2013); some eriophyid mites (Mukwevho et al., 2017; Smith et al., 2010); and

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several plant pathogens, including the Chondrilla rust, *Puccinia chondrillina* (Bubak & Syd.) (e.g., Burdon et al., 1981). Consequently, the success of a biocontrol program may depend on identifying regions within the native range of a weed where plants belong to the same biotype as those which occur in the invaded range where biocontrol is required. This can be achieved using genetic techniques to compare genotypes which can potentially identify and match native and exotic populations (e.g., Gaskin et al., 2011; Goolsby et al., 2006b; Prentis et al., 2009).

There has been considerable uncertainty regarding the original native range of *U. lobata*. Austin (1999) concluded that because the species was first found (in the late 1600 s) in Asia, where there are close relatives endemic to the region, and was often absent from the earliest records of floras outside Asia, the limited data support the assumption that *U. lobata* is native to Asia. Moreover, insect diversity is positively correlated with evolutionary history (Strong et al., 1984) and a literature review conducted by Paynter (2024, submitted for publication) supported Austin's (1999) conclusion, finding that the highest diversity of potentially host-specific natural enemies reported to attack *U. lobata* occurs in Asia. Climate matching further refined the potential search area to parts of Southeast Asia (Indonesia, Malaysia, the Philippines, parts of Thailand and Sri Lanka) that have a similar climate to Vanuatu.

In this study, we describe how samples were sourced during fieldwork associated with the biocontrol program and from international herbaria to determine if there is a good genetic match between *U. lobata* plants growing in Southeast Asia and in Vanuatu. We also document the identification and specificity testing of a candidate biocontrol agent for *U. lobata* that was found during surveys in Malaysia.

2. Materials and methods

2.1. Molecular work to narrow down the native range of invasive U. lobata populations

Ten microsatellite markers were designed for *U. lobata* (see file 1, supplementary files), using sequencing information from a sample from the island of Efate, Vanuatu, and were screened against material from Vanuatu, China, Sri Lanka, and Thailand. These markers were used to amplify 121 Vanuatu samples from across 5 key islands, 110 samples from across eight regions in Malaysia, eight Australian samples, one Tongan sample, two Fijian samples and three samples from the Dominican Republic. In addition, a range of samples across the suspected original range were obtained from various herbaria (see file 2, supplementary files, for all sample details). The same PCR conditions, thermal cycling parameters and allele scoring techniques were used as per the development procedure.

Samples with missing data for more than two makers were removed from the data set. Clonal samples were identified with the multilocus matches function in GenAlEx v6.501 (Peakall and Smouse, 2006), and samples that were identical to others within the same location were removed. A highly clonal data set will violate the assumptions of various clustering algorithms and does not add value to the phylogenetic tree pattern; therefore, most clones were removed for these subsequent analyses. To visualize the relationship between the samples, a simple matching genetic distance matrix was generated from the raw data using GenAlEx, followed by a Neighbor-net tree (Fig. 1) being constructed from these distances as implemented in SplitsTree v4.19.2 (Huson and Bryant, 2006).

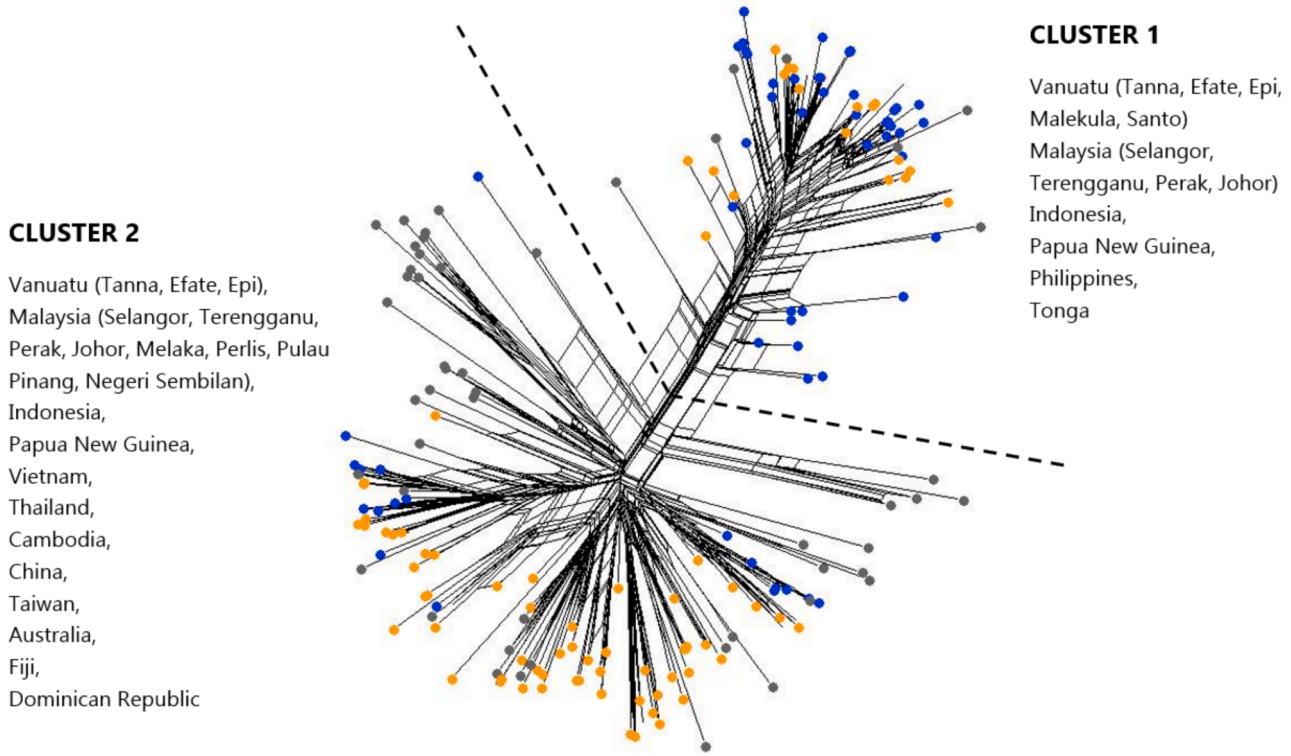

Fig. 1. Neighbor-net tree drawn in SplitsTree from a simple matching genetic distance matrix created in GenAlEx. Samples from Vanuatu are colored blue, samples from Malaysia are colored orange and other locations are colored grey. The clusters identified as 1 and 2 were determined with Structure and Structure Harvester. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Structure v2.3.4 (Pritchard et al., 2000) analysis was conducted to find the appropriate number of clusters that best explain the genotypic distribution of our data set, by implementing a Bayesian algorithm to estimate the likelihood of each individual belonging to a particular cluster. Model parameters used with Structure include the potential number of clusters (K) set from 1 to 12, 6 iterations/independent runs performed per K, no admixture, alleles correlated, a burn-in length of 20,000 and a run length of 200,000 MCMC cycles. Iteration consistency was checked with Clumpak (Kopelman et al., 2015). The Structure output was assessed with the (Evanno et al., 2005) method implemented in the Structure Harvester command line scripts (Earl and VonHoldt, 2012), where the greatest rate of change between the likelihood values with respect to K (represented as the highest Delta K value) was selected as the most appropriate K. To identify any additional underlying substructure, the data set was then divided into two based on the clustering pattern from the first run and a subsequent Structure run was performed for each subset, using the same parameters as above (except the maximum K was set to 10). A plot of the Delta K output for all three structure runs (Fig. 2) was drawn in R v4.1.2 (R Core Team, 2021), and the clustering pattern from the optimal K of the first run was added to the UPGMA tree (Fig. 1) to compare the output for both types of analyses.

2.2. Surveys for candidate agents

Malaysia was selected as a suitable locality to conduct the initial surveys of *U. lobata,* on the basis that: (1) the literature review conducted by Paynter (Paynter, 2024, submitted for publication) indicated that potentially host-specific natural enemies are present in Malaysia; (2) Regions where *U. lobata* is present in Malaysia have an identical Köppen-Geiger climate classification to Vanuatu (Peel et al., 2007); (3), for the practical reason that research contacts had been established in Malaysia, facilitating, for example, obtaining research permits and permits to export candidate agents, which has become increasingly difficult in some jurisdictions since the implementation of the Nagoya Protocol (Cock et al., 2010).

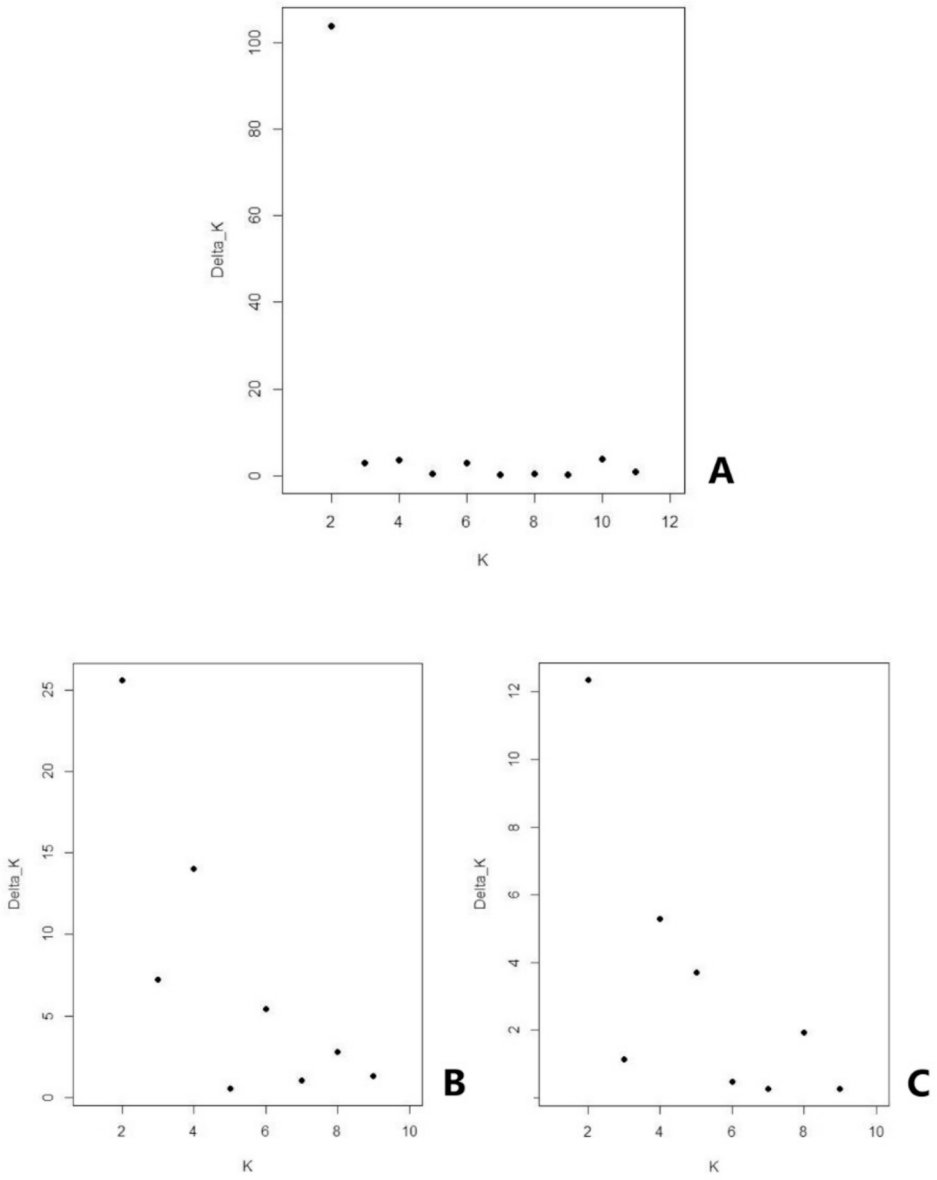

Fig. 2. The Delta K output for the three Structure runs as determined by Structure Harvester. Plot A is from the first run, indicating two is the most appropriate number of clusters for the whole data set. Plot B and plot C are subsets of the data set based on the output from the first run, with an unconvincing number of clusters determined with these runs.

Preliminary surveys of *U. lobata* were first undertaken in West Malaysia (Selangor, the Federal Territory of Putrajaya, and the Federal Territory of Kuala Lumpur), in March and September 2019. Survey work was subsequently severely restricted by the COVID-19 pandemic (resulting in border closures and associated inability to travel between countries and in periodic lockdowns and restrictions on travel within countries). Nevertheless, additional *U. lobata* surveys were conducted in Malaysia between July 2020 and March 2021. Overall, 15 localities in the states of Negeri Sembilan and Selangor, and the Federal Territories of Putrajaya and Kuala Lumpur were visited between March 2019 and March 2021, all located within c. 120 km of Kuala Lumpur (Table S1, supplementary files).

Plants were visually inspected for arthropods attacking *U. lobata*, or for arthropod feeding damage, such as leaf-mines from which herbivorous arthropods could be reared. Immature stages of arthropods found feeding on *U. lobata* were collected and reared to the adult stage in Malaysia so that they could subsequently be identified. Time spent searching for agents varied from a few minutes to 1 h depending on the number of plants present. Arthropod specimens were identified by taxonomists based at the Malaysian Agricultural Research and Development Institute (MARDI) and Manaaki Whenua – Landcare Research (MWLR) or by DNA barcoding (Ratnasingham and Hebert, 2007).

2.3. Agent shortlisting and specificity testing

Agent prioritization for specificity testing followed the system described by Paynter (2023). This prioritized candidate agents according to: (1) likely host specificity (based on expert opinion, the literature review, and field observations); (2) potential to cause damage to the target weed (based on the literature review and field observations); (3) an assessment of whether any agent life stage was likely to be severely attacked by natural enemies in Vanuatu, for example due to the presence of native ecological analogues (Paynter et al., 2018; Paynter et al., 2010). Consideration was also given to whether the agent was easy to collect, culture, host range test, ship and potentially release in Vanuatu.

Test plant species were selected following the centrifugal phylogenetic method (Briese and Walker, 2002). For *U. lobata*, the most recent plant phylogeny that includes *U. lobata* (Pfeil and Crisp, 2005) was cross-referenced against the first modern checklist of the flora of Vanuatu using data from the Vanuatu National Herbarium (PVNH) and online databases (Plunkett et al., 2022). We consulted this checklist to build a list of Malvaceae present in Vanuatu and identify the most closely related species to *U. lobata* that are present in Vanuatu.

2.3.1. Haedus vicarius testing in New Zealand

A starter culture of a tingid bug *Haedus vicarius* (Drake) was shipped from the Selangor region of Malaysia to New Zealand in February 2021. Subsequent generations were used to conduct host-specificity tests between September 2021 and June 2023. All tests were done in a containment glasshouse in the Beever Plant Pathogen Containment Facility, in Auckland, New Zealand under natural daylength. The glasshouse temperature varied between c. 20 °C at night and 25 °C to 30 °C during the day.

No-choice testing to assess the ability of *H. vicarius* to oviposit and for nymphs to develop on a plant species followed a methodology similar to that described by Zhang et al. (2016). For each replicate, five mature adults were caged within a fine mesh polyester sleeve (10 × 20 cm) placed over a few leaves of each potted test plant; each open end was tied closed with a wire twist-tie. After one week the adults were removed because preliminary investigations on *U. lobata* indicated that one week was sufficient time for oviposition to occur and allow nymphs to develop without adult feeding damaging the leaves to an extent that reduced the subsequent survival of any developing nymphs. Although the main aim of the test was to assess oviposition/development, it was noted whether the adults used to inoculate the plants were alive or dead after seven days, so that adult survival could be quantified, and the presence or absence of any visible feeding damage (chlorotic spots) was also noted. Sex determination (based on the shape of the terminal sternite) requires using a binocular microscope and manipulating the lace bugs and was done after the adults were removed from the sleeves to avoid damaging them before the tests. Replicates containing only males were discarded, and new replicates were set up. Tests with only females present were included in subsequent analyses because all control replicates on *U. lobata* with only females present resulted in nymphs developing, indicating that at least one of the females used in each replicate must have already mated and the presence of males within the cages was unnecessary to guarantee fertile eggs being laid. Pale, freshly molted adult females were commonly observed mating in containment – indicating that even very young adult females were likely to have mated prior to the tests being set up.

After the adults were removed, the sleeves were then reattached using the twist-tie, so that if oviposition had occurred, any developing nymphs would be contained. Each cage was checked regularly, and the presence of developing nymphs was noted. Any nymphs that reached the adult stage were counted and removed until all surviving nymphs had completed development (after 4–6 weeks). For most test plant species, two replicates were set up per plant on five plants, so that ten replicates were performed. Replicates in which leaf abscission occurred before the test was completed, potentially preventing nymphs from developing, were discarded. If there were sufficient healthy leaves and adult *H. vicarius* available, new replicates were set up, but this was not always possible so that the final number of replicates per test plant species was inconsistent. Plant species could not all be tested simultaneously, because of space limitations, so plants were tested sequentially. Positive controls (*U. lobata*) were always included in each trial, resulting in many more replicates being performed on *U. lobata*, compared to the test plants.

For *Malvaviscus arboreus* Cav., where some development to adult occurred, additional replicates were set up to improve the estimation of relative performance as a means of assessing the risk of *M. arboreus* being a field host (Paynter et al., 2015). F1 offspring from these tests were used to set up tests to assess *H. vicarius* performance on *M. arboreus* over multiple generations of the agent (sometimes referred to as continuation trials; Day, 1999).

Two *Malus* species were also tested to investigate the dubious record of *H. vicarius* attacking *Malus sylvestris* (L.) Mill. (Rosaceae) in Vietnam (Stusak, 1984). *M. sylvestris* is apparently absent from Vietnam (POWO, 2019), so a similar crab apple of Asian origin was selected: *Malus floribunda* Siebold ex Van Houtte, as well as *Malus domestica* Borkh., which is the most commercially valuable *Malus* and is genetically more closely related to *Malus sylvestris* than to its Central Asian progenitor, *M. sieversii* (Cornille et al., 2012). There were two replicates of each *Malus* species.

2.3.2. Haedus vicarius testing in Malaysia

Additional testing was set up in Malaysia, using durian, *Durio zibethinus* L., which belongs to the Malvaceae subfamily Helicteroideae. Two cages (a control and a test cage) were set up. One potted *U. lobata* plant was placed in the control cage, and one potted *D. zibethinus* plant in the other cage. Twenty adult *H. vicarius* were released in each cage. The number of dead adults on the floor and surviving adults on the plant were recorded daily for 8 days. The plants were checked for the presence of *H. vicarius* nymphs at the end of the test and the plants were then removed from the cages. Subsequent replicates were performed sequentially with new plants and new batches of 20*H. vicarius*. Each plant species was tested 5 times between 13 June and 31 August.

2.4. Analysis of specificity testing

All analyses were performed using Genstat (VSN International Ltd). For the no-choice development tests, the proportion of replicates where

nymphs developed to adult was analyzed by selecting Generalized Linear Models, binomial errors, and a logit link. The response variable was whether nymphs developed to adult in each replicate (1 = yes; 0 = no), the binomial totals were set to 1. A similar analysis was performed for no-choice adult feeding observations where the response variable was the number of replicates where adult feeding was recorded, and the binomial totals were set to 1, and for adult survival where the response variable was the final number of adults surviving in each replicate and the binomial totals were the initial numbers used in each replicate (i.e., 5). In all analyses the model fitted was "Species" treated as a factor with levels corresponding to the plant species used in each test.

3. Results

3.1. Molecular work to narrow down the native range of invasive U. lobata populations

Due to variable DNA quality, the microsatellite markers successfully amplified DNA in approximately 35 % of the herbaria samples. A high rate of clonality was found overall, with 37 % of the samples being identified as clones. These samples were found across Vanuatu, Malaysia, and Australia, with the highest rates occurring in Vanuatu (80 % of the samples from Malekula, 70 % of the samples from Epi and 51 % of the samples from Santo). The clonal samples from Malekula and Epi were exclusively from the same clonal group which also included two samples from Malaysia (Selangor), while Santo formed its own clonal group. Clonal samples within Malaysia only occur within a sampling site.

The Neighbor-net tree (Fig. 1) displays two main clusters, with no geographic pattern to this split. Samples from across multiple islands in Vanuatu (Epi, Efate, Tanna), multiple sites in Malaysia (Selangor, Johor, Perak, Terengganu) and those from Indonesia and Papua New Guinea are present in both clusters. Samples from the remaining locations are present in only one of the clusters.

The optimal number of clusters for the first Structure run was two (Fig. 2 A), with a Delta K value of 104. The division of samples into these two clusters correlated well with the pattern on the tree (Fig. 1). The subsequent Structure run with samples from Cluster 1 only to determine any sub-structure revealed an optimal K of two (Fig. 2 B), however the Delta K value (25.64) is not much higher than other K in this run and the placement of samples into two groups does not correlate with the pattern on the tree. This suggests any real and convincing sub-structure is not present. The Structure run with samples from Cluster 2 only revealed an optimal K of two (Fig. 2 C), with a Delta K value of 12.36. Similarly, this Delta K value is not much higher than the other K in the run and the sample assignment to the two groups also does not correlate with the pattern on the tree. These results support a total of two genotypes being present within the data we have looked at.

The *U. lobata* plants from Vanuatu that were used for host-specificity testing represented both genotypes. No genotype specific differences in insect preference were identified during agent testing.

3.2. Surveys for candidate agents

Only two potentially monophagous arthropod species were recorded during the surveys in Malaysia: *Haedus vicarius* and *Phyllonorycter conista* (Meyrick). *H. vicarius* was occasionally abundant and damaging, causing conspicuous "bleaching" of the foliage. In contrast, *P. conista* leaf mines were only found in 2019. In addition, symptoms consistent with the fungal pathogen *Oidium urenae* Yen were seen at one locality in September 2019 but did not appear particularly damaging. All other species recorded were either polyphagous e.g., *Clethrogyna turbata* Butler, or oligophagous species with host ranges that are too broad for them to be considered as biocontrol agents for *U. lobata* in Vanuatu (Table 1).

Table 1
List of arthropod herbivores found feeding on *Urena lobata* in Malaysia. Potential specificity is based on published literature records.

Arthropod	Localities	Potential specificity
HEMIPTERA		
Cicadellidae		
Amrasca biguttula Ishida	Paya Indah Wetlands, Dengkil	Polyphagous (Kamble and Sathe, 2015).
Bothrogonia ferruginea (F.)	Kg. Tanjung, Beranang Paya Indah Wetlands, Dengkil	Polyphagous (Viraktamath, 1989).
Cicadellidae sp.	Paya Indah Wetlands, Dengkil	?
Kolla paulula (Walker)	Jalan Sungai 2, Rinching	Polyphagous (Tuan et al., 2017).
Neodartus acocephaloides Melichar	Paya Indah Wetlands, Dengkil	Polyphagous (Rao, 1993).
Tingidae		
Haedus vicarius (Drake)	Paya Indah Wetlands, Dengkil Kg. Tanjung, Beranang Kg. Rinching Hilir, Rinching	See specificity testing in this manuscript.
Pyrrhocoridae		
Dysdercus cingulatus (Fabricius)	Paya Indah Wetlands, Dengkil Kg. Tanjung, Beranang	Oligophagous (Gupta et al., 2018).
Scutelleridae		
Hotea curculionoides (Herrich-Schäffer)	Jln Pam Air, Tampin Kg. Baru Pantai, Seremban Kg. Gebok, Seremban Kg. Tanjung, Beranang	Polyphagous (https://www.ndsu.edu/pubweb/~rider/Pentatomoidea/Hosts/plant_Scutelleridae.htm).
COLEOPTERA		
Coccinellidae		
Henosepilachna pusillanima (Mulsant)	Paya Indah Wetlands, Dengkil.	Reported to feed on Cucurbitaceae (Behere et al., 2015), may not have been feeding on *U. lobata*.
Chrysomelidae		
Aspidimorpha miliaris (F.)	Jalan Sungai 2, Rinching	Host plants normally Convolvulaceae, may not have been feeding on *U. lobata*.
Nisotra orbiculata Motschulsky	Paya Indah Wetlands, Dengkil.	Oligophagous (Pandit, 1998).
Curculionidae		
Metapocyrtus adspersus Waterhouse	Jalan Sungai 2, Rinching Kg. Gebok, Seremban	Polyphagous (Genka and Yoshitake, 2018).
LEPIDOPTERA		
Gracillariidae		
Phyllonorycter conista (Meyrick)	Paya Indah Wetlands, Dengkil Kg. Rinching Hilir, Rinching	Potentially adequately specific: only reported host – additional records from *Triumfetta neglecta* Wight & Arn. are very doubtful, (Kumata, 1993, 1995).
Tortricidae		
Adoxophyes privatana (Walker)	Kg. Tanjung, Beranang	Polyphagous (Robinson et al., 2010).
Ophiorrhabda sp.	Kg. Tanjung, Beranang	?
Limacodidae		
Limacodidae sp.	Kg. Tanjung, Beranang	?

(*continued on next page*)

Table 1 (*continued*)

Arthropod	Localities	Potential specificity
Hesperiidae		
Odontoptilum angulata (Felder)	Paya Indah Wetlands, Dengkil Kg. Rinching Hilir, Rinching	Oligophagous (Robinson et al., 2010).
Crambidae		
Haritalodes derogata (Fabricius)	Paya Indah Wetlands, Dengkil Kg. Genting Malek, Batang Kali Kg. Tanjung, Beranang Kg. Rinching Hilir, Rinching Wetland, Putrajaya Jalan Pinang Sebatang, Sepang	Polyphagous (Robinson et al., 2010).
Erebidae		
Anomis fulvida (Guenée)	Kg. Tanjung, Beranang	Polyphagous (Robinson et al., 2010).
Clethrogyna turbata Butler	Kg. Tanjung, Beranan Kg. Rinching Hilir, Rinchin	Polyphagous (Robinson et al., 2010).
Orgyia sp.	Kg. Juntai, Jelebu Kg. Tanjung, Beranang	Likely to be polyphagous.
Nolidae		
Earias cupreoviridis (Walker)	Kg. Tanjung, Beranan Jalan Sungai 2, Rinching Kg. Labohan Dagang, Banting	Oligophagous (Robinson et al., 2010).
Noctuidae		
Amyna natalis (Walker)	Kg. Tanjung, Beranang	Oligophagous (Robinson et al., 2010).
Xanthodes transversa Guenée	Not noted	Oligophagous (Robinson et al., 2010).

3.3. Agent shortlisting and testing

Agent prioritization identified *H. vicarius* as the most promising candidate agent. Literature records indicated it is potentially host specific and it is damaging in the native range: Tigvattnanont (1991) noted that *H. vicarius* seriously damages *U. lobata* in Thailand. Further, surveys on Efate, Vanuatu (Q. Paynter, C. McGrannachan, unpublished data), found no native ecological analogues (*sensu* Paynter et al., 2010) of *H. vicarius*.

3.3.1. Haedus vicarius testing in New Zealand

A summary of the testing results is provided in Table 2. Development varied significantly between species (deviance ratio = 16.84, d.f. = 14, $P < 0.001$). Only one test plant species, *M. arboreus,* supported *H. vicarius* development. Development to the adult state occurred in 75 out of 77 replicates on *U. lobata* (97.4 %) but only 5 of 19 replicates on *M. arboreus* (26.3 %). An additional analysis was conducted to compare the number of *H. vicarius* reared in each replicate, corrected for the inconstant number of females by using the variable "adults per female" (i.e., the total number of adult *H. vicarius* reared, divided by the number of *H. vicarius* females initially used to inoculate each replicate). As this analysis investigated the numbers reared (rather than the proportion of replicates where development occurred), an analysis of variance (ANOVA) should have been appropriate. However, the data failed to meet the assumptions of an ANOVA, even when transformed, so a non-parametric Kruskal-Wallis one-way analysis of variance was performed. This analysis indicated that significantly more *H. vicarius* were reared per female on *U. lobata* compared to *M. arboreus* (c. 5.83 vs 0.32, respectively; Kruskal-Wallis one-way analysis of variance, $H = 27.31$, d. f. = 1; $P < 0.001$), resulting in a relative performance risk score (i.e., development on *M. arboreus* divided by development on *U. lobata*; Paynter et al., 2015) of 0.054 (i.e., the number of *H. vicarius* adults that were reared on *M. arboreus* was only 5.45 % of the number reared on *U. lobata.*).

Sufficient F1 *H. vicarius* adults were reared from *M. arboreus* to set up only 3 replicates of a continuation test, which was conducted with 10 concurrent replicates on *U. lobata*. All 10 *U. lobata* replicates resulted in nymphs that developed to adult. In contrast, none of the *M. arboreus* replicates resulted in nymphs that developed to adult (deviance ratio = 15.01, d.f. = 1, $P < 0.001$). This indicates that *H. vicarius* cannot persist on *M. arboreus* for more than one generation.

Adult *H. vicarius* feeding also varied significantly between plant species (deviance ratio = 17.96, d.f. = 14, $P < 0.001$). A few chlorotic spots were noted in 3 out of 10 *A. esculentus* replicates, but only four species showed conspicuous evidence of adult feeding: *U. lobata, Hibiscus tiliaceus* L., *M. arboreus,* and *Sida rhombifolia* L. Examination of the Least Significant Differences indicated that adult feeding was significantly ($P < 0.05$) higher on *U. lobata* compared to all test plants except *H. tiliaceus, M. arboreus,* and *S. rhombifolia*.

Adult survival also varied significantly between plant species (deviance ratio = 37.88, d.f. = 14, $P < 0.001$). Survival was highest (>90 %) on *U. lobata* and was also relatively high on plant species where adult feeding was recorded in most replicates: *H. tiliaceus* (87 %), *M. arboreus* (65 %), and *S. rhombifolia* (64 %). However, survival was significantly lower on all test plants, compared to *U. lobata*, except for *H. tiliaceus* (Least Significant Difference, $P < 0.05$). Adult survival was generally very low on species where feeding was not observed (e.g., <10 % of adults survived for a week when confined on *Malus* spp., *Theobroma cacao*, and *Thespesia populnea*).

3.3.2. Haedus vicarius testing in Malaysia

H. vicarius nymphs were found on all five replicates on *U. lobata* and none on *D. zibethinus* (deviance ratio = 13.86, d.f. = 1, $P < 0.001$). No *H. vicarius* adults survived beyond 8 days on *D. zibethinus*. In contrast, *H. vicarius* survived in all five replicates on *U. lobata* (mean proportion surviving 0.81; deviance ratio = 172.75, d.f. = 1, $P < 0.001$).

4. Discussion

4.1. Molecular work to narrow down the native range of invasive *U. lobata* populations

The lack of geographic structure may not be surprising considering this plant has been intentionally spread globally from the eighteenth century for a variety of reasons, particularly for its use as a fiber crop and for its useful medicinal properties (CABI, 2013; Langeland et al., 2008). Its widespread dispersal is also encouraged via the seed, as barbed spines that cover the fruits readily attach to animal fur and feathers and to people's clothing (CABI, 2013).

While *U. lobata* is not known to reproduce vegetatively, it does exhibit a reproductive strategy of both allogamy (cross-fertilization) and autogamy (self-fertilization) (Clément et al., 2022), where the latter would result in genetically very similar individuals. This could explain why clonal samples were overrepresented. The large numbers of clonal samples identified across Santo, Malekula and Epi could point to only one introduction source containing material of limited diversity, combined with high rates of autogamy, to retain such a low level of diversity in these locations. Reproductive systems of invasive plants are more likely to be self-compatible (Van Kleunen and Johnson, 2007) because when introduced plant populations become established in new

Table 2
Summary of the results of no-choice host specificity testing conducted on *Haedus vicarius*.

Species	Family	Subfamily	Tribe	Clade/subordinate clade	No. replicates	% Replicates with adult feeding/leaf chlorosis	Adult survival (%)	% Replicates with development to adult
Urena lobata L.	Malvaceae	Malvoideae	Hibisceae	Phylloglandula/ Urena	77	100	91	97
Hibiscus sabdariffa L.	Malvaceae	Malvoideae	Hibisceae	Phylloglandula/ Furcaria	7	0	37	0
Hibiscus diversifolius Jacq.	Malvaceae	Malvoideae	Hibisceae	Phylloglandula/ Furcaria	10	0	50	0
Hibiscus tiliaceus L.	Malvaceae	Malvoideae	Hibisceae	Phylloglandula/ Azanzae(1)	9	100	87	0
Malvaviscus arboreus Cav.	Malvaceae	Malvoideae	Hibisceae	Trionum/ Malvaviscus	19	74	65	26
Abelmoschus moschatus Medik.	Malvaceae	Malvoideae	Hibisceae	Trionum/ Abelmoschus	7	0	37	0
Abelmoschus esculentus (L.) Moench	Malvaceae	Malvoideae	Hibisceae	Trionum/ Abelmoschus	10	30	16	0
Abelmoschus manihot (L.) Medik.	Malvaceae	Malvoideae	Hibisceae	Trionum/ Abelmoschus	6	0	20	0
Hibiscus rosa-sinensis L.	Malvaceae	Malvoideae	Hibisceae	Euhibiscus	8	0	48	0
Thespesia populnea (L.) Sol. ex Corrêa	Malvaceae	Malvoideae	Gossypieae		10	0	6	0
Gossypium hirsutum L.	Malvaceae	Malvoideae	Gossypieae		10	0	52	0
Abutilon indicum (L.) Sweet	Malvaceae	Malvoideae	Malveae		8	0	2	0
Sida rhombifolia L.	Malvaceae	Malvoideae	Malveae		9	100	64	0
Durio zibethinus L.	Malvaceae	Helicteroideae	Durioneae		5	0	0	0
Theobroma cacao L.	Malvaceae	Byttnerioideae			11	0	2	0
Malus spp.	Rosaceae				4	0	5	0

environments, they are influenced by factors such as lack of available mates, suitable pollinators, or other altered ecological conditions. The summary diversity statistics for each Malaysian and Vanuatuan population presented in file 1 (supplementary files) support this pattern of Santo, Malekula and Epi having the lowest diversity and highest inbreeding rates, represented by the lowest number of alleles and heterozygosities and the greatest distance between expected and observed heterozygosities. Efate and Tanna in contrast may have had multiple introduction events or one event containing material with a greater level of diversity as their diversity statistics and inbreeding rates are comparable to the Malaysian populations.

The Malaysian material from four regions (Selangor, Johor, Perak, Terengganu) is a good fit for what is present in Vanuatu, as both genotypes are found amongst samples in these Malaysian regions as well as on all five Vanuatuan islands. Biocontrol agents sourced from these locations are likely to be compatible with plants in Vanuatu. Indonesia and Papua New Guinea (across a range of locations) also indicate material from there is a good match to what is present Vanuatu, as they also are represented in both genotypes. For locations where we do not have many samples, it is possible we may not have sampled from areas containing the second genotype, but for those where we do have sufficient samples (i.e., China) this suggests a less appropriate match.

4.2. Surveys / specificity testing of Haedus vicarius in New Zealand and Malaysia

Only a subset of the potentially host-specific natural enemies identified in the literature review (Paynter, 2024, submitted for publication) was located during limited native range surveys in Malaysia. For example, the potentially host specific bruchid beetles *Spermophagus drak* Borowiec, and *S. niger* Motschulsky, which are known to occur in Malaysia (Delobel and Anton, 2011), were not recorded. Conversely, *H. vicarius* was not known to occur in Malaysia prior to the current study (Shohaimi et al., 2022). It would have been desirable to perform additional surveys in Malaysia, and in other countries, but this was not possible during the COVID-19 pandemic. Nevertheless, *H. vicarius* appears to be an excellent prospect for biocontrol of *U. lobata*: it is reportedly very damaging to *U. lobata* (Tigvattnanont, 1991) and poses little risk even to plants that are most closely related to *U. lobata*, according to the most recent molecular phylogeny which considered *Hibiscus* spp. within the Phylloglandula clade of the Tribe Hibisceae to be the closest relatives to *Urena* (Pfeil and Crisp, 2005) and an older phylogenetic analysis of "core" Malvales based on morphological, anatomical, palynological, and chemical features which indicated *Malvaviscus* is the closest genus to *Urena* (Judd and Manchester, 1997). Although development occurred on *M. arboreus* in no-choice testing, the low relative performance risk score (see Paynter et al. (2015), and the subsequent continuation test both indicated that *M. arboreus* will not support sustained populations of *H. vicarius*. Further, *M. arboreus* is native to the Neotropics (its native range extends from Mexico and Central America to northern South America; POWO, 2019) and is widely grown as a garden ornamental, particularly in tropical and subtropical regions of the world. It has escaped from cultivation and become naturalized principally in open, disturbed areas, including in Vanuatu and is considered invasive on some Pacific islands, including the Galapagos Islands, New Caledonia and New Zealand (CABI, 2015). Consequently, the very low risk of spillover damage to *M. arboreus* should not prevent the introduction of *H. vicarius* in Vanuatu.

The lack of development of immature stages on test plants that belong to more distantly related tribes Gossypieae and Malveae and subfamilies Helicteroideae and Byttnerioideae provides further reassurance that *H. vicarius* is not a threat to other potential non target species. Moreover, we found no literature records of *H. vicarius* feeding on any crops that belong to the Malvaceae that are commonly grown in Asia and South-east Asia, such as okra *Abelmoschus esculentus* (L.) Moench; durian, *D. zibethinus*; kenaf *Hibiscus cannabinus* L.; cotton *Gossypium* spp.; cacao *Theobroma cacao*, or on related ornamental species, such as *Hibiscus syriacus* L (e.g. Azhar, 1995; Ketsa et al., 2020; Kim et al., 2013; Matthews and Tunstall, 1994; Selvaraj et al., 2016).

The lack of adult feeding or development of immature stages on two *Malus* spp., indicates that Stusak's (1984) host record on *Malus* was erroneous. Indeed, Figs. 1-3 in Stusak's (1984) paper show *H. vicarius* eggs on the underside of a '*Malus sylvestris*' leaf that depicts stellate trichomes that closely resemble the trichomes on *U. lobata* leaves

illustrated by Chavan et al. (2014) and bear little resemblance to the long hair-like trichomes on *Malus sylvestris* depicted by Abo-bakr and Ali (2005) and Koçyiğit et al. (2015). An application to release *H. vicarius* in Vanuatu was approved by the Department of Environmental Protection and Conservation (DEPC) in April 2024.

5. Conclusions

At the start of the biocontrol program in 2018, there was considerable uncertainty regarding the original native range of *U. lobata* and consequently where to search for candidate biocontrol agents. A literature review (Paynter, 2024, submitted for publication) indicated that the highest diversity of host specific natural enemies of *U. lobata* occur in Asia. Climate matching indicated that the search area could be further narrowed to include only parts of Southeast Asia that are climatically matched to Vanuatu, including Malaysia. Subsequent work described above identified *H. vicarius* as a promising candidate biocontrol agent that is sufficiently host specific to be used as a biocontrol agent in Vanuatu. Moreover, *U. lobata* populations growing in Malaysia are a good genetic match to invasive populations in Vanuatu. This confirms that Malaysia was a suitable place to search for agents, as predicted by Paynter (2024, submitted for publication) and indicates that literature searches to determine the diversity of natural enemies can assist with targeting the survey stage of biocontrol programs against novel weed biocontrol targets of uncertain geographic origin.

CRediT authorship contribution statement

Caroline M. Mitchell: Visualization, Validation, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Quentin Paynter:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Stephanie Morton:** Writing – review & editing, Methodology, Investigation. **Chris M. McGrannachan:** Writing – review & editing, Methodology, Investigation, Data curation. **Zane McGrath:** Methodology, Investigation, Formal analysis, Data curation. **Michael D. Day:** Methodology, Investigation. **Mohamad Shahidan Mohamed Shohaimi:** Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Nurin Izzati Mohd Zulkifli:** Writing – review & editing, Supervision, Investigation, Data curation. **Azimah Abd Kadir:** Writing – review & editing, Methodology, Investigation, Data curation. **Nor Asiah Ismail:** Writing – review & editing, Methodology, Investigation, Data curation. **Saiful Zaimi Jamil:** Writing – review & editing, Resources, Investigation, Data curation. **Mohd Masri Saranum:** Methodology, Investigation, Data curation. **Farah Farhanah Haron:** Writing – review & editing, Project administration, Investigation, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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