Fitness and morphology support genetic differentiation across different scales in a native insect utilising native vs invasive host plants

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Abstract

Native species can evolve rapidly in response to utilising invasive species as novel resources. We investigated the genetic and trait differentiation of the Australian soapberry bug *Leptocoris tagalicus* across three biotypes: those feeding on invasive *Cardiospermum grandiflorum* in New South Wales (NSW) and Queensland (Qld), invasive *C. halicacabum* in the Northern Territory (NT), and on the native host *Alectryon tomentosus* (in Qld). Genetic analyses revealed moderate differentiation between NT insects and those from NSW and Qld. Conversely, insects from NSW and Qld had low genetic differentiation, irrespective of their host plant associations. Field data indicated ongoing adaptation in proboscis length in insects feeding on the two invasive host plant species, likely in response to the sizes of their fruits. Multi-generation hybridisation experiments demonstrated high heritability in insect proboscis length and body size. Crosses involving F1 hybrids of insect biotypes generally outperformed inter-biotype and control crosses. Taken together, these findings support ongoing genetic differentiation among *L. tagalicus* biotypes across different spatial scales, even instances of high gene flow.

Keywords hybridisation, invasive species, plant-insect interactions, rapid evolution

Introduction

Invasive alien species are significant drivers of global change, impacting biodiversity across all levels of organisation, from genes to ecosystems (IPBES, 2023). While invasive species often outcompete and displace native species, some natives may co-exist with them. Native species may tolerate, evade, or utilise invasives through behavioural plasticity (Ghalambor et al., 2007) or rapid evolutionary responses (Carroll et al., 2011). For example, in response to consuming toxic invasive cane toads (*Rhinella marinus*), some Australian snake species evolved smaller heads, reducing their gape sizes to limit predation of larger cane toads that are more toxic than smaller cane toads (Phillips and Shine, 2004).

The strength of the selection imposed by invasive species on native species depends partly on their shared eco-evolutionary experience (EEE), which pertains to adaptations to biotic interactions over evolutionary timescales (Saul et al., 2013). In the context of biological invasions, EEE relates to the role of pre-adaptations in determining the ease by which new interactions are formed between natives and invaders, i.e. ecological integration into novel communities (Saul et al. 2013, Saul and Jeschke 2015). Invasive species will exert strong selection on native species that they frequently interact with and that lack EEE with them, but with which they share high EEE, such as the cane toad and snake example mentioned above. A case of high EEE between frequently interacting partners native and invasive species involves soapberry bugs (true bugs in the genus *Leptocoris*), which are obligate seed predators of species in the soapberry family Sapindaceae. Soapberry bugs have colonised invasive soapberry plants in many parts of the world (Carroll and Loye, 2012) and in some instances these new associations have led to rapid evolutionary responses in the insects (e.g. Carroll et al. 2005a; Foster et al. 2019).

In Australia, the soapberry bug *Leptocoris tagalicus* has two subspecies, subsp. *tagalicus* and subsp. *vulgaris* (Gross 1960). *Leptocoris tagalicus* subsp.

tagalicus is larger-bodied, occurring in eastern and northern wet and dry rainforests, while *L. tagalicus* subsp. *vulgaris* is smaller-bodied and typically inhabits central desert and northern savanna areas. Both *L. tagalicus* subspecies have colonised invasive *Cardiospermum* balloon vines in Australia as new food resources (Carroll and Loye, 2012). These vines are native to the tropical and warm temperate New World and have evolved large inflated spherical seed capsules (balloons) as an elaborated seed defence system against the piercing sucking mouthparts of soapberry bugs native there. This mechanical defence system is absent from all native Australian Sapindaceae species and so represents a novel challenge to feeding by native Australian soapberry bugs.

Cardiospermum halicacabum was likely introduced into the northern parts of Australia's Northern Territory (NT) between 1600 and 1800 (Bean 2007, Le Roux et al. 2025) and was colonised by both *L. tagalicus* subspecies (Andres et al. 2013). This led to the establishment of a highly differentiated hybrid *L. tagalicus* biotype (hereafter referred to as "halicacabum" insects). While these hybrid insects remained small-bodied, their proboscides increased by as much as 38% (Andres et al. 2013). This rapid evolutionary response allowed them to more efficiently reach seeds inside the inflated fruit of *C. halicacabum* (Andres et al. 2013).

Cardiospermum grandiflorum was first recorded in Australia in 1923 as a botanical specimen of a horticultural escape (Carroll et al. 2005b). Particularly since the 1970s, it has invaded large areas along the country's east coast in New South Wales (NSW) and Queensland (Qld) (Carroll et al. 2005b, Le Roux et al. 2025). This balloon vine was colonised by *L. tagalicus* subsp. *tagalicus*. *Cardiospermum grandiflorum* has larger fruits than *C. halicacabum* and by 2004 field measurements showed that the proboscides of insects on *C. grandiflorum* were around 10% longer than those of insects on the neighbouring native host plant, *Alectryon tomentosus* (Carroll et al. 2005a). Insects found in association with these two host species are hereafter referred to as "grandiflorum" and

"tomentosus" insects, respectively. The independent colonisation of two invasive balloon vine species by *L. tagalicus* in Australia, and their similar evolutionary responses under different demographic scenarios and temporal scales, provide valuable opportunities to study the evolutionary impacts of balloon vines on the insect.

The evolutionary trajectories and geographic isolation of "halicacabum" and "grandiflorum" insects in Australia may ultimately lead to reproductive isolation between populations found on different host plants. Reproductive isolation will initially manifest as limited gene flow between populations and, eventually, as limited capacity for interbreeding (e.g., Schwarz et al., 2005). We aim to determine whether "grandiflorum", "halicacabum" and "tomentosus" biotypes differ genetically from one another by inferring the genetic structure and comparing the fitness of different crosses between them. We hypothesise that, given the differences in residence time of the two *Cardiospermum* host plant species, together with their geographic isolation in Australia, "halicacabum" insects will be more genetically differentiated from "grandiflorum" and "tomentosus" insects than the latter two will be from each other. We also hypothesised that the greater genetic differentiation between "grandiflorum" and "halicacabum" insects will correspond to significant differences in the fitness of their hybrids compared to the that of both parental biotypes, but that experimental hybrids between "grandiflorum" and "tomentosus" insects will have similar fitness to parental biotypes due to their recent divergence and largely overlapping distributions.

Material and Methods

Field sampling

We collected *L. tagalicus* subsp. *tagalicus* from *A. tomentosus* (i.e., "tomentosus" insects) from two sites: Sherwood Arboretum (SA; Qld) and Bahr Scrub Road (BSR; Qld). Putative hybrids between *L. tagalicus* subsp. *tagalicus* and subsp.

vulgaris from *C. halicacabum* (i.e. "halicacabum" insects) were collected from four sites: Daly River Crossing (DRC; NT), East Alligator River boat ramp (EAR; NT), Mango Farm Tourist Park (MFTP; NT), and Mamukala Wetland (MKW; NT). *Leptocoris tagalicus* subsp. *tagalicus* was collected from *C. grandiflorum* (i.e. "grandiflorum" insects) from five sites: Davy Gooding Bridge (DGB, Qld), Corner of Keevers Drive and Valery Road (KD:VR; NSW), Wyrallah Road and Ingram Road Junction (WR:IR; NSW), Long pocket boat ramp (LPBR; Qld), and Sherlock Road (SR; Qld) (Fig. 1, see Table S1 for more details on collection sites). These collections were made under the permits issued by the NSW Department of Planning, Industry and Environment (licence number: SL102654), the Director of National Parks, Australian Government (permit number RK737) and the Parks and Wildlife Commission of the Northern Territory (permit number 71037). *Alectryon tomentosus* is one of the main hosts of the *L. tagalicus* subsp. *tagalicus* insects that have colonised both balloon vine species (Carroll et al. 2005a; Andres et al. 2013). At each site we collected up to 30 insects (adults and/or nymphs) of which 15 were euthanised and preserved in 80% ethanol for genetic analyses and kept at -80°C until further use. The remaining insects were used to start laboratory breeding lines for the hybridisation experiment (see below).

For each host plant species, we collected five fruits from five plants at each location (i.e. n=25/site). When fewer than five individual plants were present, 25 fruits were collected from across all individuals found at the site. We measured the size of fruits of all three host plant species using digital callipers (± 0.001 mm). The measurements mode differed between the host genera given their different fruit morphologies. For *A. tomentosus* we measured the fruit size as the width (diameter) and calculated fruit radius, and for the two *Cardiospermum* species we measured the distance from the balloon outer capsule to the seed and the seed diameter. These measurements were used to calculate the distance to the

centre of the seed for all host plant species, which provides a comparable metric of seed "reachability".

DArT library preparation and sequencing

The head, thorax, and legs of 166 *Leptocoris tagalicus* insects collected in the field were transferred to 96-well plates and sent to Diversity Arrays Technology (DArT) for DNA extraction and next generation sequencing. DNA was extracted from these samples using the NucleoMag kit (MACHEREY-NAGEL). *PstI* and *HpaII* compatible adaptors with two different restriction enzyme overhangs (Sansaloni et al. 2011) were designed to include the Illumina flowcell attachment sequence, sequencing primer sequence and "staggered", varying length barcode region (also see Elshire et al. 2011). The reverse adaptor contained the flowcell attachment region and *HpaII* compatible overhang sequence. Only mixed *PstI* - *HpaII* restriction fragments were PCR amplified using the following conditions: initial denaturation at 94 °C for 1 min followed by 30 cycles of 94 °C for 20 s, 58 °C for 30 s, 72 °C for 45 s and a final extension at 72 °C for 7 min.

After PCR amplification, equimolar amounts of amplified product were bulked and subjected to 100 cycles of sequencing (single reads) on the Illumina NovaSeq, NovaSeq X+ sequencer. Sequences generated from each lane were processed using proprietary DArT analytical pipelines. In the initial pipeline, poor quality sequences were removed, with more stringent filtering parameters applied to the barcode region compared to the rest of the sequence, ensuring the reliable assignment of the sequences to specific samples (based on the "barcode split"). Technical replicates were created for ~20% of samples and were carried through the entire sequencing and library preparation process to generate a 'reproducibility' score for each locus.

Single Nucleotide Polymorphism (SNP) filtering

Single Nucleotide Polymorphisms (SNPs) were filtered using the *DartR* R package (Gruber et al. 2018; R Core Team 2023). First, all monomorphic loci were removed, followed by a reproducibility filter of 0.9 and individual call-rate of 0.7. Individual locus call-rates were filtered to 0.85 and the depth of coverage was bounded between 8 and 200. Minor allele frequency was restricted to 0.02 after which monomorphic loci were again filtered out. Secondaries (i.e., where more than one SNP per tag was present) were filtered at random. The resulting genotype dataset was then filtered for Hardy-Weinberg Equilibrium at each site using a false discovery rate of 0.5 and was corrected for multiple comparisons (all sites had more individuals than the minimum of five used to exclude sites from the analysis, and as such, no insects were excluded). This dataset was then filtered for linkage disequilibrium (LD) using the indep-pairwise function in PLINK V1.9 (Purcell et al. 2007), for which we used comparisons between all loci and a window size of 1000 kb, with an R^2 of 0.5.

Population genetic diversity and structure

We used the filtered genomic dataset to calculate site-level genetic diversity metrics (observed (*H*o) and unbiased expected heterozygosities (*uHe*) and inbreeding coefficients (F_{IS}) for bugs at each collection site) and pairwise F_{ST} values between all collection sites using dartR. We inferred the number of genetic clusters using the sparse non-negative matrix factorisation algorithm of the "snmf" function in the *LEA* R package (Frichot & François 2015), which identifies the optimal number of clusters (*K*) through a minimum cross-entropy analysis. This analysis was run for *K* values ranging between one and 11, using 10000 iterations.

Hybridisation experiment

Live insects collected from each site were kept in a temperature-controlled glasshouse, covered internally and externally by shade cloth, at Macquarie

University's Plant Growth Facility (6:00-10:30 at 26°C, 10:30-16:00 at 28°C, 16:00-18:00 at 26°C, and 18:00-06:00 at 20°C). For our "tomentosus" × "grandiflorum" crosses (breeding experiment 1) we created separate breeding lines from all insects (i.e. from two sites) collected on *A. tomentosus.* The "grandiflorum" breeding line for this cross originated from the DGB site. For our "halicacabum" × "grandiflorum" crosses (breeding experiment 2) we created a "halicacabum" breeding line from insects collected on *C. halicacabum* from all sites. The "grandiflorum" breeding line for this cross included insects collected from sites KD:VR, WR:IR, LPBR and SR. All these initial breeding lines were maintained for six months (i.e., 5-6 generations) before setting up the breeding experiments. Throughout both experiments insects were fed *C. grandiflorum* seed only, as this host plant fruits all year round. Note that the two breeding experiments form part of separate research projects that differed slightly in some details, as outlined below.

Breeding experiment 1

We started isolating fifth instars (i.e., last instar stage) of "tomentosus" and "grandiflorum" bugs in February 2023, until we obtained at least 20 male and 20 female virgins from each of the two breeding lines. Insects were kept in 90 mm petri dishes lined at the bottom with filter paper. Water was provided in 1.5 mL Eppendorf tubes with a cottonwool plug and changed every 3-4 days. On the 6th of March 2023, isolated adults were randomly paired into four parental (P1) breeding lines: control tomentosus (i.e. ♀ "tomentosus" × ♂ "tomentosus"), two reciprocal hybrid crosses (i.e., ♀ "tomentosus" × ♂ "grandiflorum" and ♀ "grandiflorum" × ♂ "tomentosus"), and control "grandiflorum" (i.e. ♀ "grandiflorum" × ♂ "grandiflorum"). Each P1 breeding line consisted of 10 insect pairs. All paired insects were arranged in a randomised block design consisting of 10 blocks, with each block containing four pairs. Paired insects were placed in

350mL plastic circular tubs, lined at the bottom with a 90mm filter paper, with single carboard cell from an egg carton for shelter, and with a mesh window in the lid for ventilation. Pairs of insects were fed two halved *C. grandiflorum* seeds every 3-4 days; old food was only removed if observed to be mouldy. Insect pairs were also provided with a 5mL vial of water (plugged with cottonwool) which was changed weekly. The position of petri dishes was randomised in each block using RANDOM.ORG (Haahr 2023). The position of blocks in the glasshouse was randomised every seven days.

Eggs laid during the first two days following pairing were discarded as *L. tagalicus* females can lay unfertilised eggs. From day three onwards, we counted the daily number of eggs laid by each pair for 15 days and transferred these using forceps to a new 'egg container' (350mL plastic circular tubs, lined at the bottom with a 90mm filter paper, with a bent carboard rectangle from an egg carton's lid for shelter for subsequent hatchlings, and with a mesh window in the lid for ventilation; these containers were provided a with 5mL water vial but not food). In pairs where the male died before the female, the female was kept alive and continued laying fertile eggs. At the end of 15 days, we measured proboscis and body length (distance between the to the distal tip of the head and anterior tip of the abdomen) for all P1 adults using digital callipers (± 0.001 mm).

One of the first hatchlings to emerge from each P1 pair was moved into a corresponding nymph tracking container (350mL plastic circular tubs, lined at the bottom with a 90mm filter paper, with a single carboard cell from an egg carton for shelter, and with a mesh window in the lid for ventilation; nymphs were fed and watered identically to their parents), while all subsequent hatchlings were moved to a corresponding nymph box (500mL plastic rectangular tubs, lined at the bottom with paper towel cut to size, with four carboard cells from an egg carton for shelter, and with a mesh window added in the lid for ventilation; these containers received two 5mL water vials and half of a *C. grandiflorum* seed for every three nymphs, both of which were changed on the same schedule as parental

containers). Nymph boxes contained up to 20 nymphs before a new container was made to prevent overcrowding and competition for resources. The nymph tracking containers were checked daily and the dates that individuals moulted into different instar stages (5 instar stages in total) and adulthood were recorded. Tracked nymphs that died prior to reaching their 5th instar moult were replaced with a freshly hatched nymph on the same day of death where possible or were replaced with a nymph from the associated nymph box at first or second instar stage. Any individuals that did not make it to adulthood within 40 days were excluded from the experiment. Once nymphs reached adulthood, they were given two days to sclerotise before being measured and sexed. The tracked nymphs were kept alive and used for the next generation where possible.

The number of nymphs in each egg container was counted daily from when the first hatchling emerged until less than five hatchings occurred across all insect pairs in a two-day span (after 18 days). Fifth instar nymphs from P1 parents were removed and isolated in 90 mm Petri dishes to be used as virgin parents in the next generation (G1) pairings. One hundred and fifty bugs from across all 40 P1 pairs were isolated to ensure enough insects to create 48 G1 pairs. These insects were paired on the 22nd May 2023 using the same design as for the P1 insects. Data collection of all G1 pairs was the same as for P1 pairs.

<u>Breeding experiment 2</u>

We started isolating 5th instars of “grandiflorum” and “halicacabum” insects in March 2023 until we had obtained at least 30 male and 30 female virgins from each breeding line. Husbandry of isolated insects followed that for experiment 1. Isolated insects were paired on the 12th June 2023 into four parental (P1) breeding lines: control “halicacabum” (i.e. ♀ “halicacabum” × ♂ “halicacabum”), two reciprocal hybrid crosses (i.e., ♀ “halicacabum” × ♂ “grandiflorum” and ♀ “grandiflorum” × ♂ “halicacabum”), and control “grandiflorum” (i.e. ♀

316 “grandiflorum” × ♂ “grandiflorum”). Each P1 breeding line consisted of 15 insect
317 pairs. All paired insects were arranged in a randomised block design consisting
318 of 15 blocks, with each block containing up to eight pairs. The position of petri
319 dishes was randomised in each block using RANDOM.ORG (Haahr 2023). The
320 position of blocks in the glasshouse was randomised every seven days.
321 The number of eggs laid by each pair was counted on the third day after
322 pairing (with eggs laid in the first two days being discarded before the first count)
323 and then every third and fourth day (alternating) up until the 15th day following
324 pairing. As in experiment 1, in pairs where the male died before the female, the
325 female was kept alive and continued laying fertile eggs. At the end of 15 days,
326 we measured traits of all P1 adults as in experiment 1.
327 The tracking of nymph development and the isolation of 5th instars for G1
328 pairings followed the methods described for experiment 1. Up to ten 5th instar
329 nymphs from each P1 pair were isolated and raised to adulthood. These insects
330 were paired on the 11th August 2023. Methods for the “grandiflorum” and
331 “halicacabum” lines were the same as P1. For our hybrid lines we paired eight
332 female offspring of the ♀ “halicacabum” × ♂ “grandiflorum” P1 crosses with
333 eight male offspring of the ♀ “grandiflorum” × ♂ “halicacabum” P1 crosses and
334 eight female offspring of the ♀ “grandiflorum” × ♂ “halicacabum” P1 crosses
335 with eight male offspring of the ♀ “halicacabum” × ♂ “grandiflorum” P1 crosses.
336 Data collection of all G1 pairs was the same as for P1 pairs.
337
338 *Insect and host plant trait analyses*
339 Differences in the distance to seed centre for the three different host plant
340 species were assessed using a one-way ANOVA and a Tukey’s post hoc analysis.
341 Insect proboscis length and body size were analysed using two-way ANOVAs
342 with sex and host plant, and their interaction, as explanatory variables. Significant

means were separated using Tukey HSD post hoc analyses. These analyses were done in the R statistical environment (R Core Team, 2023).

Variance-to-mean ratios were computed for fixed effects to assess their dispersion. When modelling egg production, relative fecundity, and development time using generalized linear mixed-effects models (GLMMs), the random effect of "experiment" had minimal variance (σ^2: 3.46×10^{-12} to 4.55×10^{-10}; results not shown). We therefore analysed these data using generalized linear models (GLMs).

We compared egg production (data overdispersed, see results) between cross types by fitting a GLM with a quasipoisson error distribution using the *gml* function in the base R statistical environment. Relative fecundity (data overdispersed) was compared between cross types fitting a GLM that included an offset (log number of nymphs of best performing pair) for estimating the model coefficients, with a quasipoisson error distribution using the base R statistical environment. We compared development time (data underdispersed) using a GLM with a Conway-Maxwell-Poisson error distribution using the *glmmTMB* R package (Brooks et al. 2017). Post hoc pairwise comparisons for all GLMs were performed to identify significant differences between the different cross types, using estimated marginal means (EMMs) implemented in the *emmeans* R package (Lenth 2022).

We used separate linear regressions to estimate the correlation between mid-parent values and those of their offspring for proboscis length and body length. We then estimated the heritability of these traits with animal models using the *MCMCglmm* R package (Hadfield, 2010). We used a Gaussian model with 100,000 iterations, 100,000 burn-in, and 10 thinning. We included offspring sex as a random effect in the models. We checked all variables for autocorrelation and convergence and used the posterior distribution of variance components to infer mean narrow sense-heritability and a 95% confidence interval for all traits that returned significant pMCMC values (< 0.05).

Results

Single Nucleotide Polymorphism (SNP) filtering

Initial quality filtering of the SNP data resulted in 165 individuals and 11, 846 SNPs that were retained for further downstream filtering. At site-level, none of the SNPs were identified as violating assumptions of the Hardy-Weinberg Equilibrium. Filtering for linkage disequilibrium resulted in the removal of 7,841 loci. We therefore retained a final genetic data set consisting of 165 individuals and 4,005 SNPs.

Population genetic diversity and structure

At site level, observed heterozygosity ranged between 0.195 and 0.225 (average 0.21 ±0.011), uH_E between 0.272 and 0.291 (average 0.282 ±0.008) and F_{IS} between and 0.282 (average 0.257 ±0.020) (Table 1). Pairwise F_{ST} values ranged between 0.226 and 0.0024 (i.e. between insects collected from BSR and SR) and 0.05 (i.e. between insects collected from KD:VR and MFTP). The average pairwise F_{ST} across all populations were 0.019 (±0.016) (Table 2).

Our admixture analysis identified the most likely number of genetic clusters as two (Fig. 1). These two clusters appear to be strongly influenced by geography. That is, genomic assignment of "halicacabum" insects collected in the NT corresponded mostly to one genetic cluster while the assignment of "grandiflorum" and "tomentosus" insects from NSW and Qld largely corresponded to the other genetic cluster (Fig. 1).

Table 1 Summary of genetic diversity metrics of *Leptocoris tagalicus* biotypes collected from different sites (average per sites and standard deviation).

L. tagalicus biotype	Population ID	*Ho*	*Ho* SD	uH_E	uH_E SD	F_{IS}
"halicacabum" insects	**EAR**	0.198	0.161	0.274	0.179	0.278
	MWK	0.198	0.163	0.272	0.179	0.271
	MFTP	0.195	0.160	0.272	0.180	0.282
	DRC	0.198	0.162	0.273	0.180	0.277
"grandiflorum" insects	**LPBR**	0.214	0.161	0.283	0.173	0.245
	SR	0.215	0.160	0.286	0.173	0.250
	DGB	0.209	0.157	0.285	0.174	0.268
	WR:IR	0.214	0.159	0.291	0.171	0.263
	KD:VR	0.218	0.167	0.287	0.175	0.241
"tomentosus" insects	**SA**	0.225	0.161	0.291	0.167	0.226
	BSR	0.223	0.165	0.289	0.171	0.228
	Overall	**0.210**		**0.282**		**0.257**

Table 2 Pairwise F_{ST} values between collection sites of different *Leptocoris tagalicus* biotypes.

		"halicacabum" insects				"grandiflorum" insects					"tomentosus" insects	
		EAR	**MWK**	**MFTP**	**DRC**	**LPBR**	**SR**	**DGB**	**WR:IR**	**KD:VR**	**SA**	**BSR**
"halicacabum" insects	**EAR**	0										
	MWK	-0.0011										
	MFTP	0.0009	0.0013									
	DRC	0.0015	0.0018	0.0013								
"grandiflorum" insects	**LPBR**	0.0331	0.0316	0.0355	0.0344							
	SR	0.0271	0.0254	0.0299	0.0280	-0.0016						
	DGB	0.0322	0.0313	0.0332	0.0336	0.0068	0.0001					
	WR:IR	0.0289	0.0273	0.0297	0.0293	0.0035	0.0006	0.0031				
	KD:VR	0.0472	0.0473	0.0499	0.0501	0.0215	0.0159	0.0208	0.0147			
"tomentosus" insects	**SA**	0.0279	0.0277	0.0307	0.0302	0.0019	-0.0022	0.0032	0.0012	0.0158		
	BSR	0.0274	0.0262	0.0288	0.0295	0.0028	-0.0024	0.0035	-0.0022	0.0151	-0.0020	0

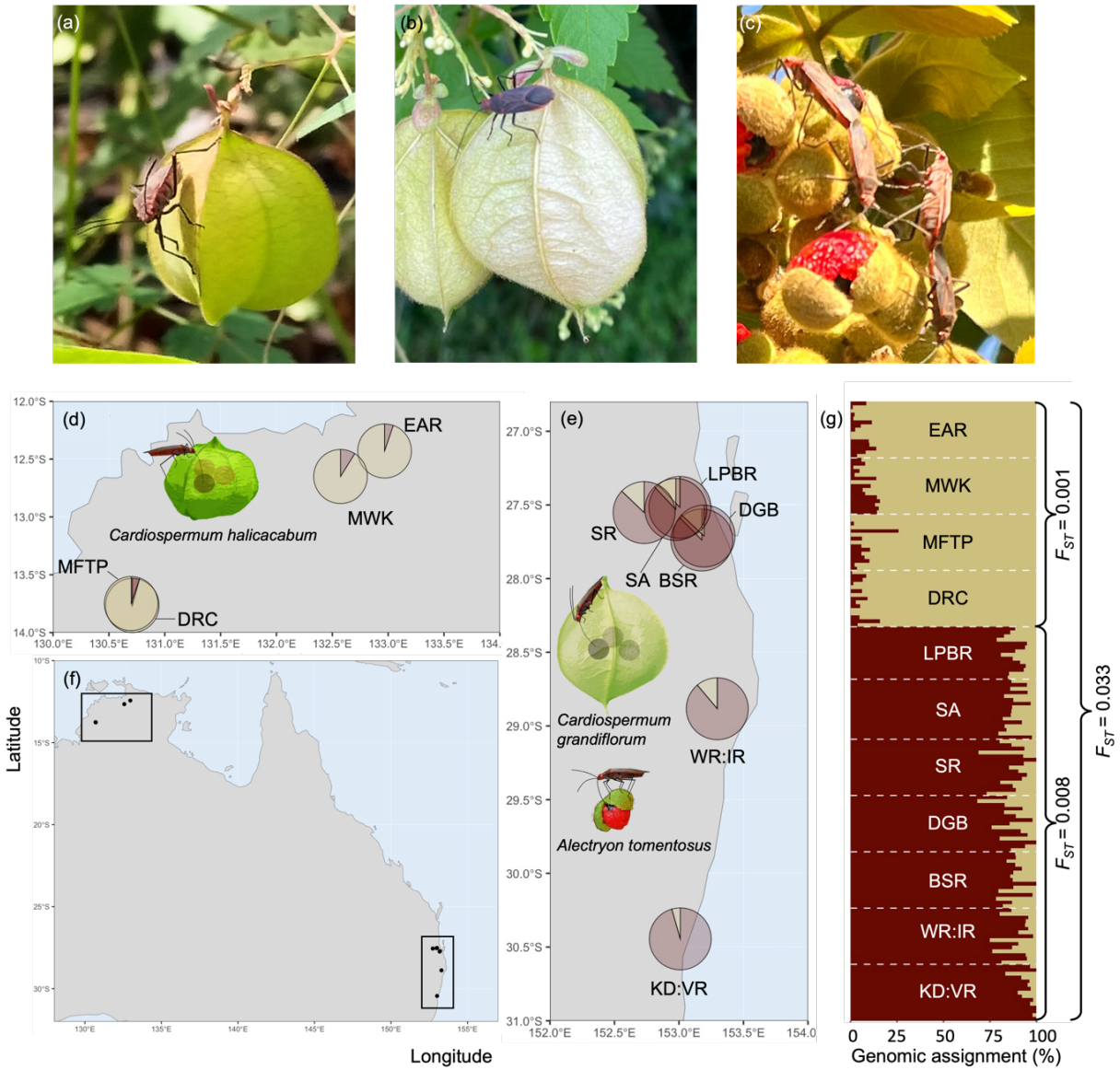

(a)
(b)
(c)
(d)
EAR
MWK
Cardiospermum halicacabum
MFTP
DRC
(e)
LPBR
DGB
SR
SA
BSR
Cardiospermum grandiflorum
WR:IR
Alectryon tomentosus
KD:VR
(f)
Latitude
Longitude
(g)
EAR
MWK
MFTP
DRC
LPBR
SA
SR
DGB
BSR
WR:IR
KD:VR
F_{ST} = 0.001
F_{ST} = 0.008
F_{ST} = 0.033
Genomic assignment (%)

Figure 1 *Leptocoris tagalicus* pictured on two of its invasive host plants (a) *Cardiospermum halicacabum* (b) and *C. grandiflorum* and one of its native host plants *Alectryon tomentosus* (c). Included are maps illustrating the collection sites in NT of *Leptocoris tagalicus* on *Cardiospermum halicacabum* in the Northern Territory (d) and *C. grandiflorum* and *Alectryon tomentosus* in the States of New South Wales and Queensland (e). These insert maps correspond to the rectangles on the larger map (f). Pie charts in insert maps show the average genomic assignment values of insects at each site to one of two population genetic clusters. (g) Genetic structure of *L. tagalicus* inferred from ancestral admixture analysis. The vertical bar plot shows the proportion of each individual insect's genomic assignment (y-axis) to each cluster. Average site pairwise F_{ST} values within and between the two main population genetic clusters are shown. *Leptocoris tagalicus* individuals collected on *C. halicacabum* in the Northern Territory had genomic assignment values corresponding mostly to one cluster (tan colour) while insects collected on *C. grandiflorum* and *A. tomentosus* in New South Wales and Queensland were primarily assigned to a second cluster (maroon colour). Photo credits: Levi Brown (pane a), Johannes Le Roux (pane b) and Dylan Geraghty (pane c).

Insect and host plant traits in the field

The reachability of seeds of the different host plant species, measured as the distance from the outside of the fruit to the centre of the seed, was significantly different ($F_{2,350}$ = 679.4, $p < 0.001$; Fig. 2a). The distance to the seed centre was shortest in *A. tomentosus* (4.59 ± 0.06 mm), followed by *C. halicacabum* (6.1± 0.13 mm) and *C. grandiflorum* (11.76 ±0.09 mm).

A two-way ANOVA to evaluate the effects of *L. tagalicus* sex and host plant species on insect proboscis length found significant main effects for both sex ($F_{1, 379}$ = 595.51, ($p < 0.001$) and host species ($F_{2, 379}$ = 53.24, $p < 0.001$), as well as for the interaction between these factors ($F_{2, 379}$ = 5.49, $p = 0.0044$; Fig 2b). Tukey's post hoc analyses showed that, irrespective of host plant, female insects always had longer proboscides than male insects. Further, female insects collected from *C. halicacabum* had longer proboscides than those collected from the two other host plants, while male insects collected from the two *Cardiospermum* species had longer proboscides than those collected from *A. tomentosus*.

A two-way ANOVA to evaluate the effects of *Leptocoris tagalicus* sex and host plant species on insect body size found significant main effects for sex ($F_{1, 379}$ = 1125.76, ($p < 0.001$) and host species ($F_{2, 379}$ = 59.81, $p < 0.001$), as well as for the interaction between these two factors ($F_{2, 379}$ = 6.79, $p = 0.0012$; Fig 2c). Tukey's post hoc analyses showed that female insects had larger bodies than male insects. For both sexes, body size varied between host plants, with "tomentosus" insects being the largest, "grandiflorum" insects being intermediate, and "halicacabum" insects being the smallest (Fig. 2c).

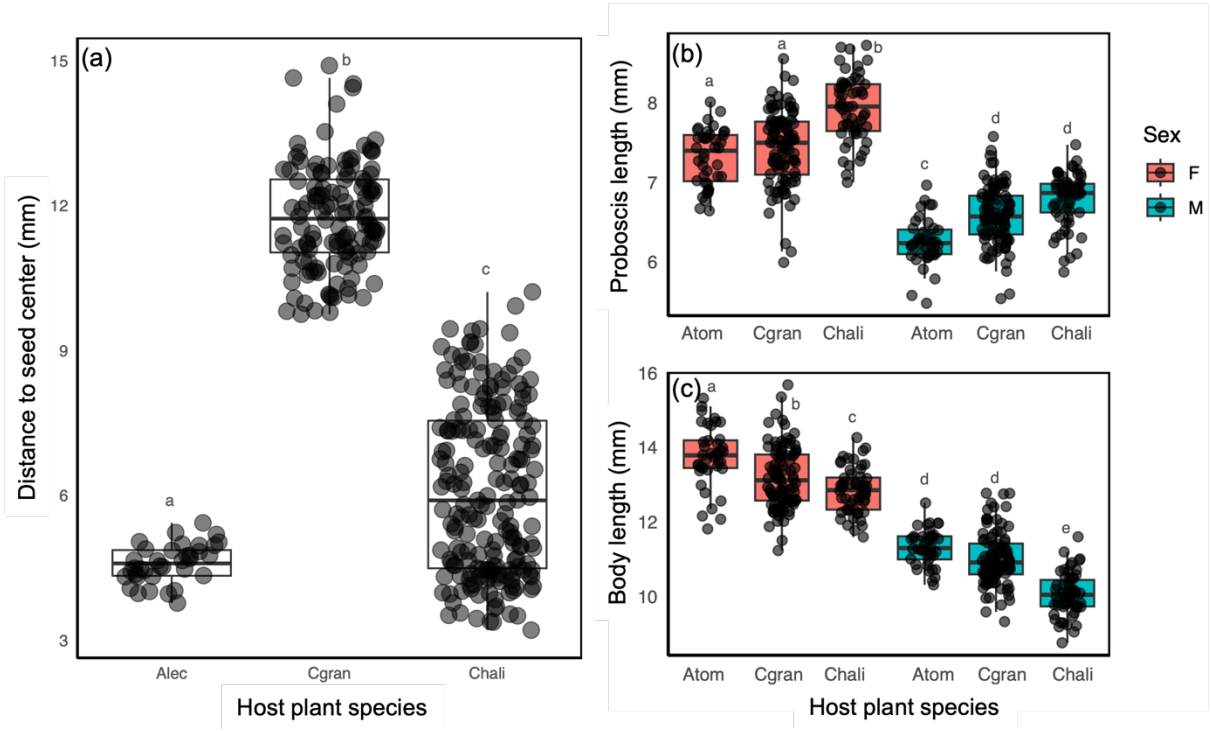


Figure 2 (a) The reachability of seeds in the three study host plant species; *Alectryon tomentosus*, *Cardiospermum grandiflorum* and *C. halicacabum*. Reachability was calculated as the distance to seed centre for each species. Significant differences between different host plants are indicated by different letters ($p<0.05$). (b) The proboscis length of male and female *L. tagalicus* insects collected from three different host plant species, with significant differences between different host plant x insect sex combinations indicated by different letters above boxplots ($p<0.05$). (c) The body length of male and female *L. tagalicus* insects collected from three different host plant species, with significant differences between different

host plant x insect sex combinations indicated by different letters ($p<0.05$). Alec = *Alectryon tomentosus*, Cgran = *Cardiospermum grandiflorum*, Chali = *C. halicacabum*.

Hybridisation experiment

For egg production, the variance-to-mean ratio was 37.03, indicating significant overdispersion in the data. The fitted GLM revealed a significant intercept (estimate = 5.16, SE = ±0.09, t = 54.64, $p < 0.001$; Table S2). The "tomentosus" × "grandiflorum" F1 cross pairs produced significantly more eggs compared to pairs in the "tomentosus" cross (0.33 ±0.12, t = 2.80, p = 0.005), while the "grandiflorum" × "halicacabum" pairs showed a significant decrease in egg production (−0.36 ±0.13, t = -2.71, p = 0.0075). The "tomentosus" × "grandiflorum" and "grandiflorum" × halicacabum" crosses showed marginal decreases in egg production compared to the "tomentosus" control cross (p = 0.0525 and 0.0722, respectively). The remaining crosses did not show significant differences in egg production. The EMMs post hoc analysis showed that pairs in the "tomentosus" × "grandiflorum" F1 cross had the highest egg production, significantly different from all other crosses except the "grandiflorum" × "halicacabum" F1 and "tomentosus" control crosses. The remaining crosses produced a similar number of eggs compared to one or more of the other crosses, indicating varying degrees of differences or similarities in their fecundity (Fig. 3).

The variance-to-mean ratio was 36.8 for the number of hatchlings, indicating significant overdispersion in the data. The fitted GLM for relative fecundity had a significantly negative intercept (-0.881 ±0.132, t = -6.695, $p < 0.0001$; Table S3). Both F1 crosses had significantly higher relative fecundity than the "tomentosus" control cross ("grandiflorum" × "halicacabum" F1 cross: 0.477 ±0.181, t = 2.640, p = 0.009 and "tomentosus" × "grandiflorum" F1 cross: 0.326 ±0.166, t =

1.958, p = 0.052). In contrast, the “tomentosus” × “grandiflorum” had marginally significant lower relative fecundity compared with the “tomentosus” control cross (-0.369 ±0.209, t = -1.766, p = 0.079). The remainder of the crosses had similar relative fecundity to the “tomentosus” control cross. The EMMs post hoc analysis indicated that the two F1 crosses had significantly higher relative fecundity than the “grandiflorum” × “halicacabum” and “tomentosus” × “grandiflorum” crosses. The relative fecundity of control crosses was also found to be similar to most crosses except for the “grandiflorum” control cross that had lower relative fecundity than the “grandiflorum” × “halicacabum” F1 cross (Fig. 3).

For development time, the variance-to-mean ratio was 0.19, indicating significant underdispersion in the data. The GLM for development time showed a significant effect of cross type (AIC = 670.6, log-likelihood = -327.3, dispersion parameter = 0.132, Table S4). Compared to the “tomentosus” control cross, the “grandiflorum” × “halicacabum” (0.087 ±0.022, z = 3.95, p < 0.001), “grandiflorum” × “halicacabum” F1 (0.053 ±0.025, z = 2.07, p = 0.038), “grandiflorum” control (0.052 ±0.020, z = 2.56, p = 0.010) and “halicacabum” control (0.115 ±0.021, z = 5.37, p < 0.001) crosses had significantly longer development times. Other cross types did not show significant differences. The EMMs showed that the “halicacabum” control cross had the longest average development time which was statistically similar to development time of the “grandiflorum” × “halicacabum” and “grandiflorum” × “halicacabum” F1 crosses, but significantly longer than all other cross types. The remaining crosses had development times similar to the “grandiflorum” × “halicacabum” F1 cross.

Mid-parent and offspring values for proboscis length and body size were significantly correlated (Fig. 4) and our animal models predicted narrow sense heritability as being 46% (30-55%; 95% CI) and 32% (23-43%; 95% CI) for these two traits, respectively.

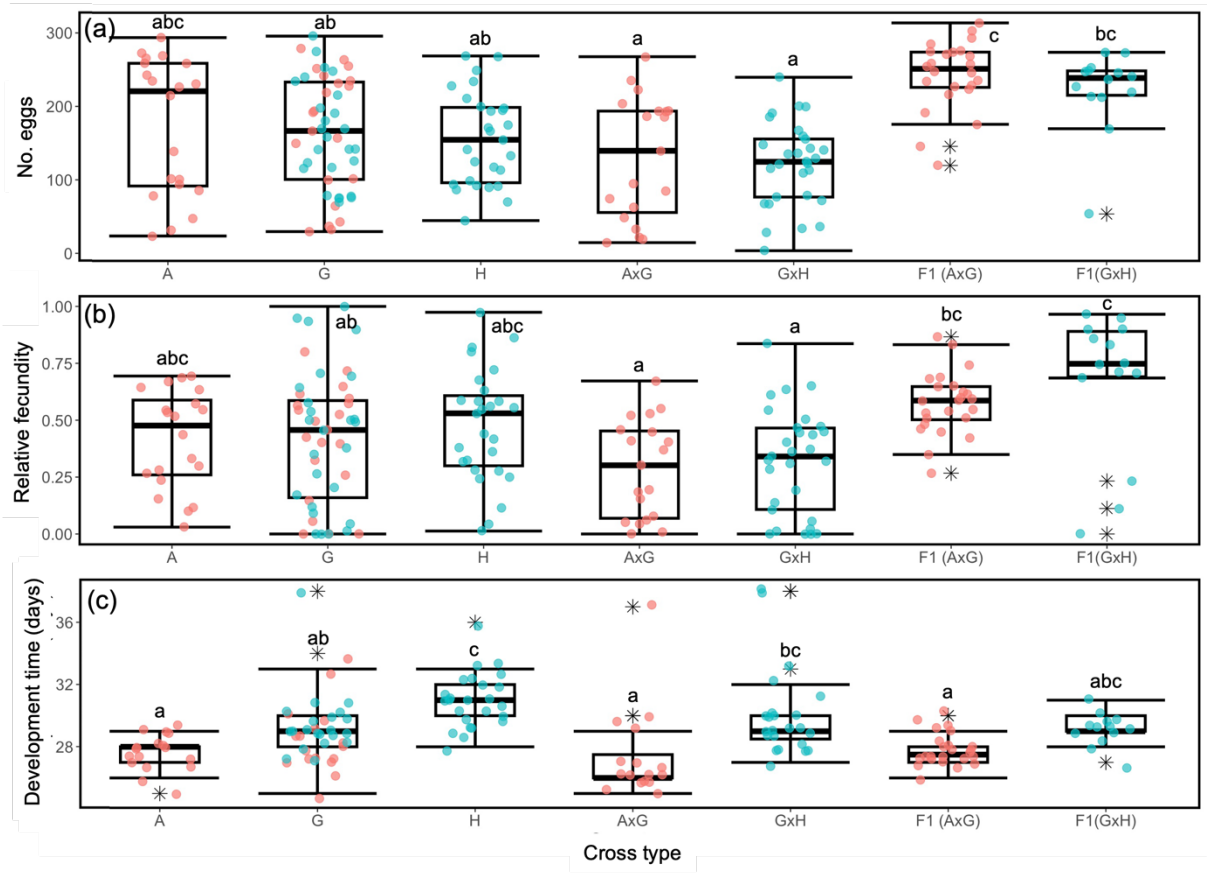


Figure 3 Boxplots of developmental and fitness metrics of *Leptocoris tagalicus* for different cross types: (**a**) number of eggs laid in 15 days, (**b**) relative fecundity, (**c**) number of days taken for tracked nymphs to reach adulthood after hatching. For relative fecundity, each datapoint represents the number of nymphs produced by an insect pair divided by the highest number of nymphs produced by a pair in the hybridisation experiment. Boxes show the interquartile range, the thick bar inside the

box is the median, the whiskers represent the highest and lowest non-outlier data points. Stars show outliers (points outside of 1.5* the interquartile range). Symbol colours represent the breeding experiment: experiment 1 = red, experiment 2 = blue. X-axis labels refer to crosses: A = control “tomentosus”, G = control “grandiflorum”, H = control “halicacabum”, AxG = “tomentosus” × “grandiflorum”, GxH = “grandiflorum” × “halicacabum”, F1(AxG) = “tomentosus” × “grandiflorum” F1, and F1(GxH) = “grandiflorum” × “halicacabum” F1. Significant differences between cross types ($p<0.05$) are indicated by lower case letters in all figures.

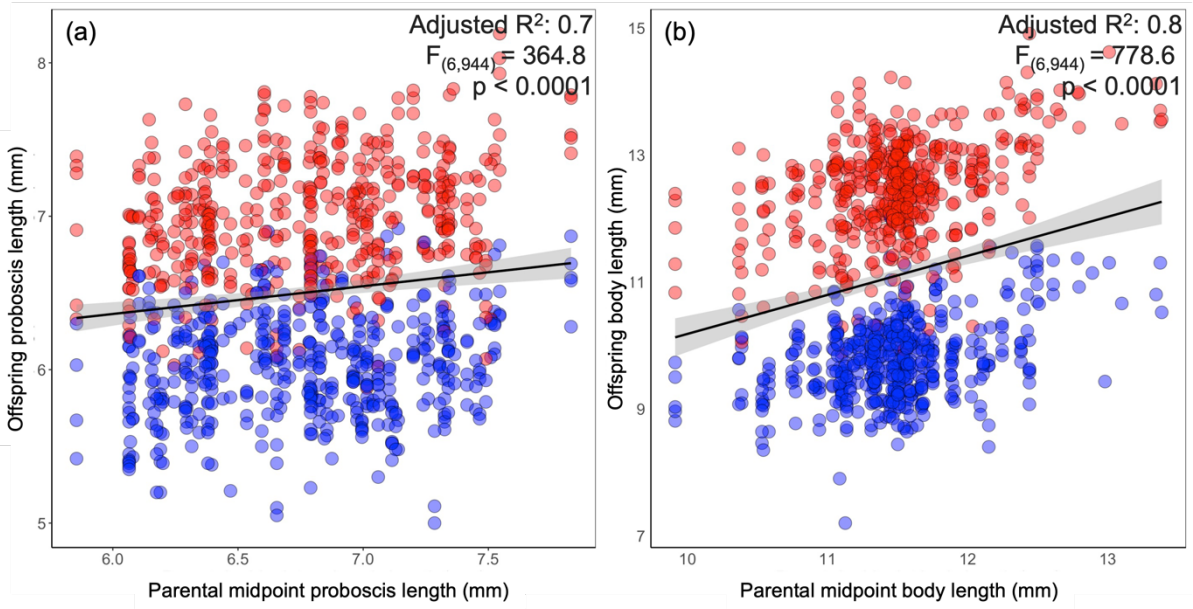


Figure 4 Scatter plots representing mid-parent offspring linear regressions for (**a**) proboscis length and (**b**) body length. Red symbols represent female offspring, and blue symbols represent male offspring. The grey shaded areas around the best fit line represent 95% CIs.

Discussion

We found support for our first hypothesis that insects associated with the two invasive *Cardiospermum* species would be genetically isolated. "Halicacabum" insects from the NT were genetically differentiated from "tomentosus" and "grandiflorum" insects in NSW and Qld (Fig. 1). In contrast, the latter two biotypes, which were often collected from proximate locations (within a few kilometres), showed relatively low genetic differentiation. Our data do not allow us to determine whether the genetic divergence between NT insects and those from NSW and Qld is due to geographic isolation, high host plant fidelity, or both.

The time needed for populations to evolve reproductive isolation remains an important question in evolutionary biology, with some studies suggesting that this can happen quickly (e.g. Schwarz et al. 2005, 2007). We tested whether reproductive isolation is evolving in *L. tagalicus* following its colonisation of, and adaptation to, invasive balloon vines. The timing, geography and evolutionary underpinnings of these colonisation events by *L. tagalicus* is markedly different. *Cardiospermum halicacabum* was introduced to Australia 200-400 years ago (Le Roux et al. 2025) and now serves as the host for a hybrid biotype of the two *L. tagalicus* subspecies (Andres et al. 2013). On the other hand, *C. grandiflorum* was introduced to the east coast of Australia around a century ago and subsequently colonised by *L. tagalicus* subsp. *tagalicus* (Carroll et al. 2005a).

Given the likely influence of geographic isolation on genetic differentiation in our study, our analyses of reproductive isolation only partially assessed whether the performance (i.e. number of eggs produced, relative fecundity, development time) of "grandiflorum", "halicacabum", "tomentosus" insects, and their hybrids reflects their residence time on different host plants. Not only did we find differences in the performance genetically differentiated and spatially isolated "grandiflorum" and "halicacabum" insects, but we also found differences between the genetically and spatially proximate "grandiflorum" and "tomentosus" insects. In both instances, inter-biotype crosses (i.e., "tomentosus" × "grandiflorum" and "grandiflorum" × "halicacabum" crosses) performed similarly to parental control crosses (Fig. 3). F1 crosses, however, had higher performance levels (i.e. eggs production and relative fecundity) compared to inter-biotype crosses. It is worth noting the slower development of "halicacabum" control insects and the nymphs produced by their crosses involving other biotypes, which could be due to these bugs being fed *C. grandiflorum* seed, a host that these bugs do not encounter in the NT. We also note that our fecundity data (number of eggs laid and hatchling emergence) were overdispersed, suggesting additional sources of variation in these data that were not accounted for in our models, such as developmental plasticity or genetic attributes (e.g. inbreeding over successive generations in the laboratory).

Experimental hybridisation is a powerful approach to investigate the magnitude and genetic architecture of population differentiation (Galloway and Etterson 2005). For instance, genetic drift can lead to the fixation of alternate alleles across populations. When these populations hybridise, hybrid vigour (heterosis) may arise if loci exhibit overdominance or if fixed alleles in different populations are mildly deleterious and recessive. This increase in performance is typically greatest in F1 hybrids (Lynch and Walsh, 1998), as was the case in our study.

The persistence of host-specific insect trait differences under common garden conditions across multiple generations (Fig. 2), along with our estimates of trait heritability and performance differences between inter-biotype and F1 crosses, provides strong evidence for genetic differentiation among all studied *L. tagalicus* biotypes. Notably, these genetic differences were observed in both geographically and genetically more isolated (i.e. "halicacabum" and "grandiflorum" insects; F_{ST}=0.033) than proximate ("tomentosus" and "grandiflorum" insects; F_{ST}=0.008) biotypes. While ongoing gene flow between "grandiflorum" and "tomentosus" insects may slow the evolutionary adaptation of "grandiflorum" insects to their new host plant, it is not preventing adaptation altogether (Carroll et al. 2005a; Fig. 2). Although we did not have the opportunity to sample *L. tagalicus* insects on nearby small-fruited native *Atalaya hemiglauca* host plants in the NT, it is likely that limited ongoing gene flow also occurs between them and "halicacabum" insects (Andrés et al. 2013). Previous studies have shown that trait differences can persist between populations despite high levels of gene flow (e.g., Comeault et al. 2015; Yadav et al. 2019; Kardum Hjort et al. 2023). In *L. tagalicus*, individuals with short proboscides may experience lower fitness on *Cardiospermum* host plants compared to those with long proboscides. Conversely, while the performance of insects with long proboscides on smaller fruited native hosts will likely be suboptimal (Carroll et al. 1997, Kunte 2007), they will not be physically excluded from feeding. This asymmetry could enable long-proboscis individuals to mediate gene flow between host plant species, even as trait divergence continues. Our findings contribute to a growing body of evidence showing that invasive species not only impact native biodiversity ecologically, such as by outcompeting native species, but also drive rapid evolutionary responses in native populations.

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Data accessibility statement

Genotype (insect SNP) as well as plant and insect morphological data are available in the Zenodo online repository (https://zenodo.org/records/15054308).

Competing interests statement

The authors have no competing interests to declare.

Author Contributions

J.J.L.R. conceived and designed the study with input from L.B. and D.M.G. G.M.D. led the genetic analyses with input from J.A.O. The hybridisation experiments were led by L.B. and D.M.G. with substantial input from J.M.H., N.M.D., N.B., S.I., R.M.C. and S.K. All insect and host plant data were analysed by J.J.L.R. while heritability of insect traits was analysed by D.M.G.. J.J.L.R. wrote the first draft of the manuscript and all co-authors provided feedback.

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Supplementary Materials

Table S1. Details of localities from where *Leptocoris tagalicus* individuals were collected for this study.

Host plant	Site	State/territory	Lat	Long	Experiment
A. tomentosus	BSR	Queensland	-27.74	153.18	1
A. tomentosus	SA	Queensland	-27.53	152.98	1
C. grandiflorum	DGB	Queensland	-27.71	153.19	1
C. grandiflorum	WR:IR	New South Wales	-28.88	153.30	2
C. grandiflorum	KD:VR	New South Wales	-30.44	153.01	2
C. grandiflorum	LPBR	Queensland	-27.52	153.01	2
C. halicacabum	DLC	Northern Territory	-13.77	130.71	2
C. halicacabum	MWK	Northern Territory	-12.65	132.57	2
C. halicacabum	EAR	Northern Territory	-12.43	132.97	2
C. halicacabum	MFTP	Northern Territory	-13.75	130.70	2
C. grandiflorum	SR	Queensland	-27.55	152.73	2

Table S2. Summary of GLM for *Leptocoris tagalicus* egg production.

	Estimate	SE	t value	Pr(>|t|)
(Intercept)	5.157905	0.094349	54.66829	4.4E-111
crossTypegrandiflorum_control	-0.05791	0.11403	-0.50782	0.612229
crossTypehalicacabum_control	-0.11402	0.126602	-0.90061	0.369046
crossTypeAG_inter	-0.28552	0.14618	-1.95324	0.052404
crossTypeCH_inter	-0.36041	0.133059	-2.70862	0.007435
crossTypeAG_F1	0.334299	0.119214	2.804191	0.005621
crossTypeCH_F1	0.248305	0.137195	1.809873	0.072051

Table S3. Summary of GLM for *Leptocoris tagalicus* relative fecundity.

	Estimate	SE	t value	Pr(>|t|)
Intercept	-0.88083	0.131564	-6.69506	2.89E-10
crossTypeAG_inter	-0.3691	0.208957	-1.76641	0.079091
crossTypeAG_F1	0.326005	0.166495	1.958051	0.051832
crossTypeCH_inter	-0.2572	0.180963	-1.42126	0.157041
crossTypeCH_F1	0.477055	0.180707	2.639938	0.00905
crossTypegrandiflorum_control	0.004625	0.156973	0.029465	0.976528
crossTypehalicacabum_control	0.141282	0.168645	0.837748	0.403328

Table S4. Summary of GLM for *Leptocoris tagalicus* development time.

	Estimate	SE	t value	Pr(>|t|)
Intercept	5.157905	0.094349	54.66829	4.4E-111
crossTypeAG_F1	-0.05791	0.11403	-0.50782	0.612229
crossTypealectryon_control	-0.11402	0.126602	-0.90061	0.369046
crossTypeCH_inter	-0.28552	0.14618	-1.95324	0.052404
crossTypeCH_F1	-0.36041	0.133059	-2.70862	0.007435
crossTypegrandiflorum_control	0.334299	0.119214	2.804191	0.005621
crossTypehalicacabum_control	0.248305	0.137195	1.809873	0.072051