Application to release *Stomphastis thraustica* (Peru population), an agent for the biological control of *Jatropha gossypiifolia* in Australia

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Executive summary

Jatropha gossypiifolia (Euphorbiaceae), commonly known as bellyache bush, is a serious weed of rangelands and riparian zones of northern Australia, and it has the potential to invade much of northern Australia. Biological control is an important component of the long-term management strategy for *J. gossypiifolia* in Australia. Biological control of bellyache bush was initiated in 1999. Since then, only one agent, the jewel bug, *Agonosoma trilineatum* has been released and there is no evidence of its establishment. A renewed biological control effort, involving exploration in South America identified a number of potential biological control agents, the most promising of which, a small leaf-mining moth *Stomphastis thraustica* (Lepidoptera: Gracillariidae), was imported from Peru into quarantine in 2014 for further research.

Biological studies conducted in quarantine demonstrated that *S. thraustica* (Peru population) has both a short generation time and high fecundity. Under quarantine conditions at 30^{o}C, a generation has been completed in as little as 13 days. This bodes well for its future as a biological control agent, allowing populations to build rapidly under favourable conditions. *Stomphastis thraustica* (Peru population) can complete development on all six biotypes of bellyache bush identified in Australia and shows no apparent preference. Gracillariidae are also known to be good dispersers. It is expected that *S. thraustica* (Peru population) will also be an adept disperser, a desirable characteristic given the expansive areas across which *J. gossypiifolia* occurs.

The host plant test list for the quarantine host testing of *S. thraustica* (Peru population) contains 50 species. The list focuses on phylogenetically related native species occurring in northern Australia where *J. gossypiifolia* is invasive. Several species reported as hosts of *S. thraustica* were also included. Host specificity testing was conducted as follows (Figure 1):

1. **No choice oviposition/larval development trials**: All test species were subject to no-choice trials. Indiscriminate egg lay under confined and/or no no-choice conditions is known to occur in many Lepidoptera. Eggs were laid on 35 species and egg hatch (utilising resources from the egg) occurred on 28 of these species. In all cases, except on *J. gossypiifolia* and *J. curcas*, the 1st instar larvae died, shortly after attempting to feed, demonstrating that these species are not suitable hosts for larval development and thus lifecycle completion. Exploratory feeding only affected the surface layers of leaves before the death of the larvae.
2. **Paired choice comparison trial**: This was conducted for species on which larval development occurred (*Jatropha curcas*). *Jatropha curcas* was found to be equally as acceptable as a host as *J. gossypiifolia* under quarantine conditions. *Jatropha curcas* has also invaded parts of northern Australia. It is a declared weed in some states and is an approved target for biological control. Utilisation of this species in the field would thus be beneficial.
3. **Choice oviposition trials**: These were conducted with 23 of the species on which eggs were laid during no-choice trials. When provided with a choice of the target and several non-target species, very few eggs were laid on the non-target species and in no cases did larvae develop beyond first instar on non-target species, including on two close relatives. These results further demonstrate that these species are not suitable hosts.
4. **Multiple-choice oviposition trial, without *J. gossypiifolia*:** This was conducted with six species on which high numbers of eggs were laid during no-choice trials. No eggs were laid on any of the plants in any of the replicates.

Results from quarantine host testing confirm that *S. thraustica* (Peru population) is highly host specific and suitable for release in Australia. In no cases was larval development possible past the first instar on any

species other than the target and *J. curcas.* The risk to species other than *J. curcas* is therefore predicted to be negligible.

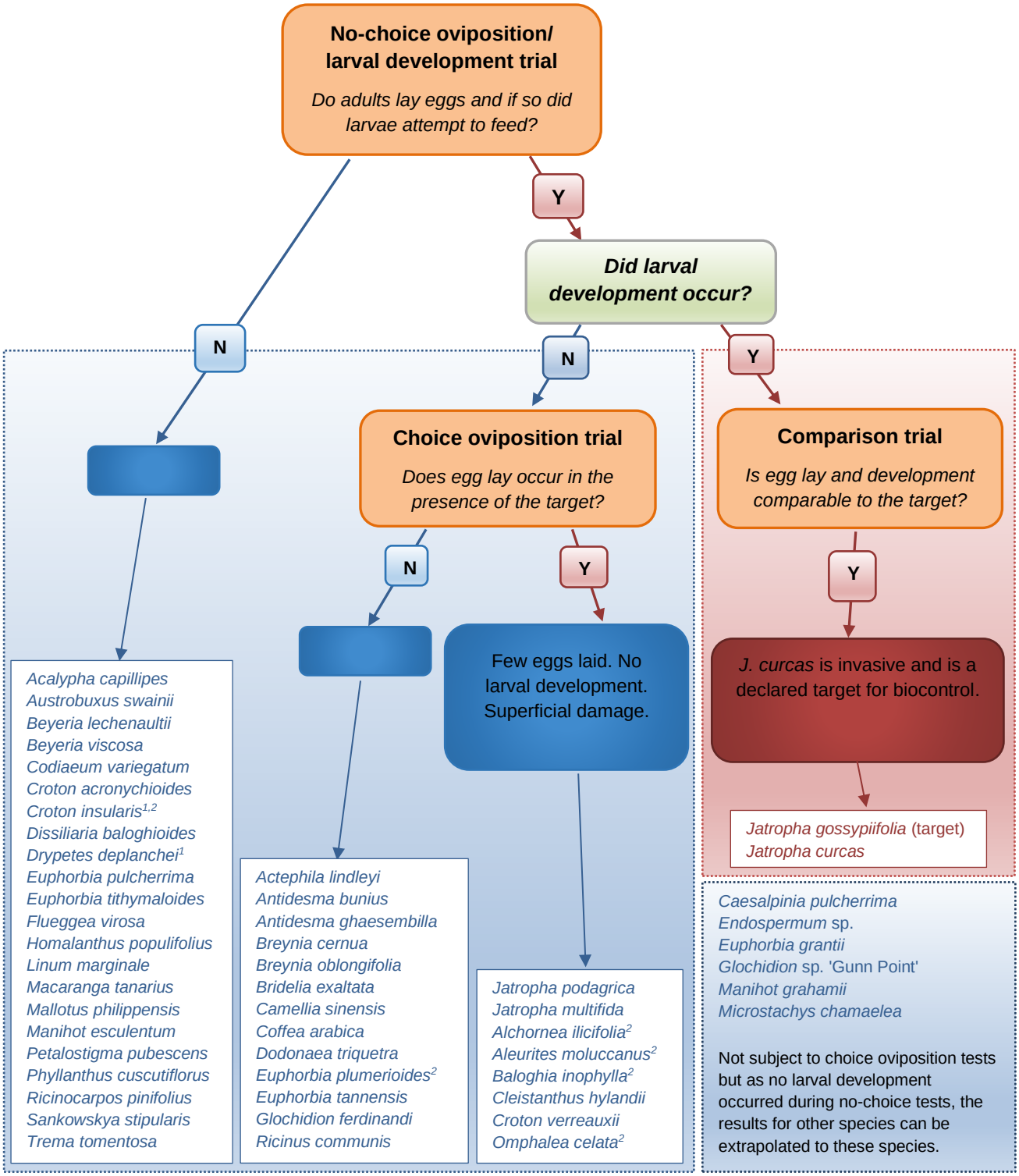

Figure 1 – Flow chart of host specificity testing conducted for* Stomphastis thraustica *(Peru population) [1]these species were included in a choice oviposition trial. [2]these species were also included in a multiple-choice oviposition trial without J. gossypiifolia.

1 Target

1.1 Classification

Order:	Malphigiales
Family:	Euphorbiaceae
Subfamily:	Crotonoideae
Tribe:	Jatropheae
Genus:	*Jatropha* L.
Species:	*gossypiifolia* L. 1753
Common name:	bellyache bush

1.2 Description

Jatropha gossypiifolia, commonly known as bellyache bush, is an erect, woody, deciduous, tropical or sub-tropical perennial shrub, commonly growing 2-3 m high (Figure 2; Bebawi *et al*. 2007). Stems are thick, semi-succulent, coarsely hairy, and exude a watery sap when injured. Leaves are alternate, petiolate, deeply divided into 3-5 lanceolate lobes with margins covered with sticky hairs. They range in colour from deep red-purple to bright green, depending on leaf age and plant biotype. Flowers are dioecious, small and red with yellow centres and occur in branched inflorescences in upper leaf axils. The fruit is a green three-lobed capsule, approximately 12 mm long and 10 mm wide. Seeds are dark coloured, carunculate and about 8 mm long. Roots are fleshy and tuberous (Parsons and Cuthbertson 1992; Bebawi *et al*. 2007). Reproduction can be by both seeds and vegetatively.

Jatropha gossypiifolia is a morphologically and genetically diverse species, and molecular genetic studies suggest that multiple introductions have occurred in Australia from throughout the native range (Prentis *et al*. 2009). This genetic diversity may contribute to the morphological, phenological and physiological diversity of *J. gossypiifolia* in Australia, where six biotypes have been identified. Queensland Bronze and Queensland Green grow in north Queensland, Queensland Purple in far north Queensland, Darwin Purple and Katherine Green occur in the Northern Territory, and Kununurra Green in Western Australia (Figure 3; Bebawi *et al.* 2007).

1.3 Native range and centre of origin

Jatropha gossypiifolia is found throughout Central and South America. It is believed to be native to the drier islands of the Caribbean and coastal Venezuela (Heard *et al.* 2002).

1.4 Australian and overseas distribution

Jatropha gossypiifolia has become widely naturalised in the tropics including Africa, Madagascar, India, Indonesia, Timor-Leste, Papua New Guinea, Sri Lanka, and Australia (Bebawi *et al*. 2007). It was introduced to Australia during the 1800s as a hardy ornamental and occurs as a weed in rangelands, particularly in

riparian zones (Figures 2 and 3; Csurhes 1999; Bebawi *et al*. 2007). *Jatropha gossypiifolia* is naturalised at many sites in Queensland (including Charters Towers, Mt. Isa, Cloncurry, Burdekin Catchment, Clermont, Springsure and Mornington Island) (Csurhes 1999), the Northern Territory (including Darwin, Middle Point, Tipperary, Katherine, Willeroo, Mataranka, Daly Waters and the Barry Caves) (Parsons and Cuthbertson 1992) and Western Australia. In Western Australia, widespread, uncontrolled populations occur in the east Kimberley, with the larger infestations at Lake Argyle and on the Bow River main road crossing. Small infestations occur in the west Kimberley region. According to Thorp and Lynch (2000), the potential distribution covers much of Queensland, Northern Territory and Western Australia (Figure 4). The potential distribution is limited by heat and dry stress in arid regions of central Australia and cold stress further south.

Figure 2 – Queensland Bronze and Queensland Green biotypes co-occurring near Charters Towers, Queensland.

1.5 Close relatives in Australia

The *Jatropha* genus contains approximately 170 species, most of which are native to tropical and subtropical America. There are no native *Jatropha* in Australia. *Jatropha curcas* is promoted in some countries as a biofuel, but not in Australia. In addition to *J. gossypiifolia, J. curcas*, *J. podagrica* Hook. and *J. multifida* L. are also known to be cultivated as ornamentals in Australia. Naturalised populations of *J. curcas* occur in northern Australia. It is a declared species in the Northern Territory and Western Australia and it has been approved as a target for weed biological control (Biosecurity Queensland 2016; Taylor *et al*. 2017).

Jatropha is part of the Euphorbiaceae family. There are around 30 genera and 195 species native to Australia in Euphorbiaceae. Until recently, the family was split into three subfamilies: Crotonoideae

Euphorboideae and Acalyphoideae. The genus *Jatropha* is a member of the Jatropheae tribe, within the subfamily Crotonoideae. There are no Australian native species within the tribe Jatropheae. The subfamily Crotonoideae contains about 13 genera and 95 species native to Australia, including Croton (12 spp.), *Beyeria* (15 spp.), *Bertya* (25 spp.), *Ricinocarpos* (15 spp.), and a number of cultivated species such as garden croton (*Codiaeum variegatum* (L.) A.Juss.) and cassava (*Manihot esculenta* Crantz). Subfamily Euphorboideae (43 Australian species in four genera) includes the genus *Euphorbia* (~35 native species). Many *Euphorbia* species are cultivated as ornamentals and numerous have become naturalized in Australia. The widespread weed castor oil plant (*Ricinus communis* L.) also belongs to this subfamily. Subfamily Acalyphoideae is represented in Australia by 57 species in 13 genera including *Monotaxis* (10 spp.) and *Mallotus* (12 spp.).

1.6 Pest status

Jatropha gossypiifolia can form monocultures in northern Australia and causes major negative economic and environmental impacts. Dense thickets crowd out useful pasture species from beneath its canopy, reducing pasture productivity (Bebawi *et al.* 2007). Stands also inhibit the movement of stock, including access to water bodies. All parts of *J. gossypiifolia* (and *J. curcas*) are highly toxic to stock and people. Death of animals have been attributed to the consumption of *J. gossypiifolia* during drought (Csurhes 1999). Environmentally, *J. gossypiifolia* infestations reduce biodiversity, alter fire regimes and increase soil erosion and destabilisation of creek and riverbanks due to its shallow root system.

Jatropha gossypiifolia has been declared a Weed of National Significance (http://www.environment.gov.au/biodiversity/invasive/weeds/weeds/lists/wons.html). It is a declared species in Western Australia, South Australia, Queensland, the Northern Territory and parts of New South Wales. In the Northern Territory *J. gossypiifolia* and *J. curcas* are proclaimed as noxious weeds for the whole of the Territory (*J. gossypiifolia*: class A/C and B/C; *J. curcas*: class A/C) under the *Weeds Management Act 2001* (https://nt.gov.au/__data/assets/pdf_file/0016/252133/declared-weeds-in-the-nt.pdf). In Western Australia, *J. gossypiifolia* is a declared pest (s22(2); *J. curcas* is a prohibited declared pest (s12) prohibited organism) under the *Biosecurity and Agriculture Management Act 2007* (https://www.agric.wa.gov.au/organisms). In South Australia *J. gossypiifolia* is declared under the Natural Resources Management Act 2004, with sale and movement prohibited (https://www.pir.sa.gov.au/__data/assets/pdf_file/0005/234536/bellyache_bush.pdf). In Queensland, *J. gossypiifolia* is a restricted invasive plant under the *Biosecurity Act 2014 (*https://www.daf.qld.gov.au/__data/assets/pdf_file/ 0004/383818/IPA-Restricted-plants-of-Qld.pdf*).* In New South Wales, *J. gossypiifolia* is declared under the *Biosecurity Act 2015* and is a Class 2 regionally prohibited weed in 19 local areas *(*http://weeds.dpi.nsw.gov.au/Weeds/ Details/266).

Figure 3* – Jatropha gossypiifolia *infestations; Charters Towers, Qld (top); Palmer River, Qld (middle); Hodgson River, N.T. (bottom).

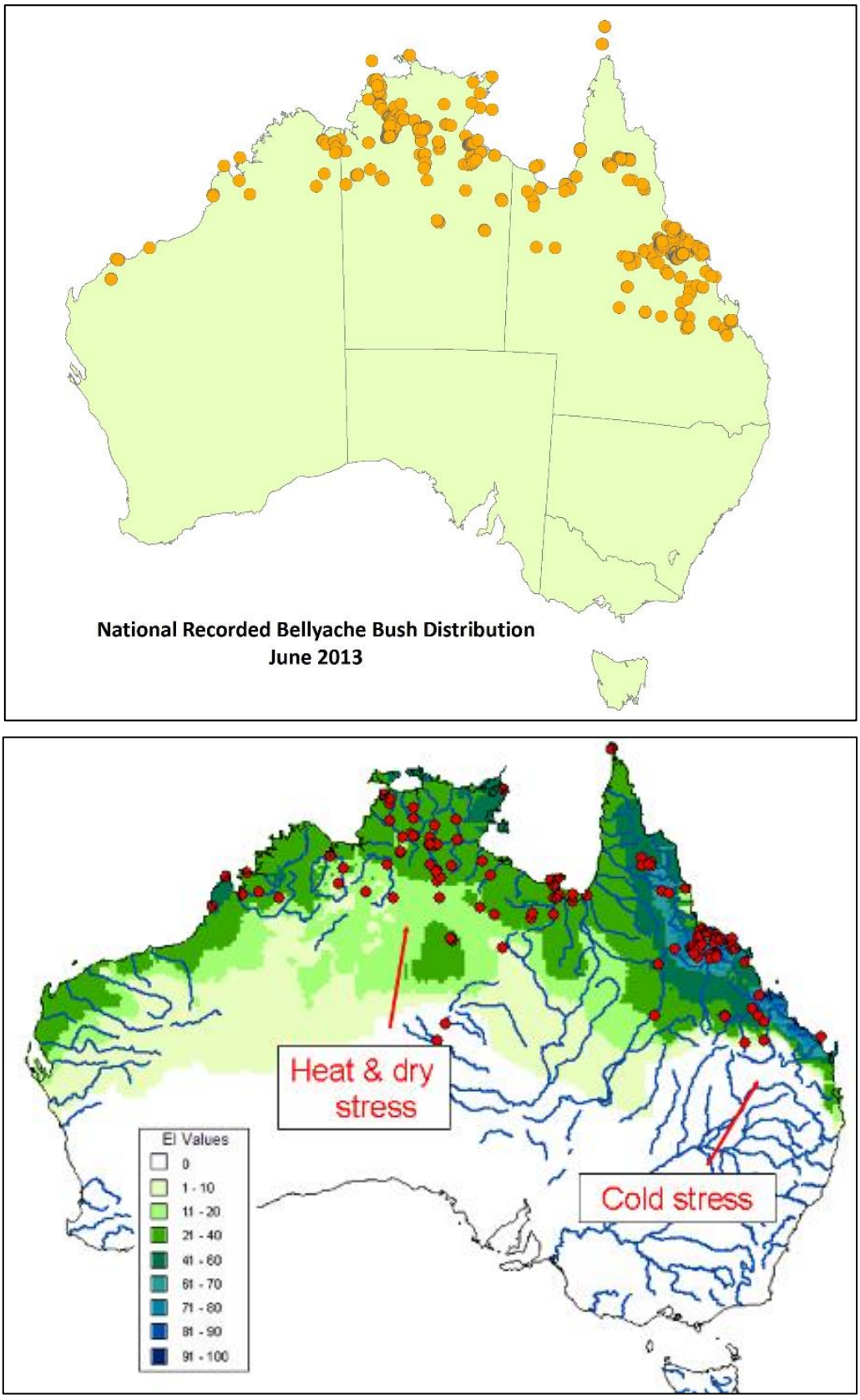

Figure 4* – Jatropha gossypiifolia *distribution as at June 2013 (top) and the predicted distribution based on climatic suitability (bottom).

1.7 Other control options

Chemical

Herbicides application is effective in controlling bellyache bush infestations (Vitelli and Madigan 2002), but huge recruitment from the soil seedbank follows chemical treatment resulting in the need for ongoing chemical treatments (Bebawi *et al*. 2007). Chemical control is therefore not an economically viable option in northern Australian grazing areas.

Mechanical

Mechanical control has potential where it is feasible to use machinery. Plants can be dug out and burned, provided most of the tuberous root system is removed to prevent regeneration from the roots (Parsons and Cuthbertson 1992). Cutting bellyache bush close to ground level is also effective in reducing the weed population (Bebawi and Campbell 2002a). However, both methods are suitable only in small areas, and not suitable for sensitive riparian areas, where bellyache bush is a serious problem.

Fire

Bellyache bush is also sensitive to fire (Bebawi and Campbell 2002b), but in areas with dense bellyache bush infestation fire may not be practical due to lack of adequate fuel load to carry the fire. Flaming using a hand-held burner is effective in killing bellyache bush plants (Vitelli and Madigan 2004). Fire has the potential to be a viable management tool in conjunction with grazing management (to exclude to stock). However, a large portion of the soil seed bank can survive fire, resulting in huge recruitment.

Biological control

In Australia a leaf-mining moth, *Stomphastis* sp. (formally identified as *Epicephala* sp.; Wilson 1997) and the castor oil looper, *Achaea janata* L. cause minor defoliation of bellyache bush (Bebawi et a. 2007; De Prins *et al.* in prep.). The seed-feeding jewel bug *Agonosoma trilineatum* F. (Scutelleridae) is the only biological control agent released against this weed in Australia. There is no sign of its field establishment in either Queensland or the Northern Territory (Bebawi *et al*. 2007).

1.8 Approved target for biological control

In 1999, the Standing Committee on Agriculture and Resource Management accepted a proposal, prepared by NTDPIF and CSIRO, to endorse *Jatropha gossypiifolia* and *J. curcas* as targets for biological control (http://weeds.ala.org.au/target.htm).

1.9 Stakeholders

Land managers across northern Australia, particularly cattle producers, have considerable interest in the control of *J. gossypiifolia*. Community and NRM groups (e.g. Mitchel River Catchment Group, Cape York indigenous groups, Desert Channels) are also concerned about the impact of the weed on land used for amenity, biodiversity conservation and agriculture. Meat and Livestock Australia and the Queensland State Government have provided substantial funding towards biological control research. The Northern Territory Government's Department of Environment and Natural Resources is also interested in the biological control of *J. gossypiifolia*.

2 Agent

2.1 Taxonomy

Order: Lepidoptera

Family: Gracillariidae

Subfamily: Ornixolinae

Genus: *Stomphastis* Meyrick

Species: *thraustica* (Meyrick, 1908)

Specimens collected from South America were initially identified by Ms V.M. Uys of the USDA; D. R. Davis of the Smithsonian Institution; and M. Kruger of Ditsong Museum, South Africa. *Jatropha gossypiifolia* leaves infested with larvae were imported into Australia from Peru on the 23rd of November 2014 (Table 1). A colony was established in quarantine from this material. All biological and host specificity studies refer to individuals descended from this initial importation. None of the individuals from the second importation from Bolivia were introduced into the colony or used for testing (Table 1).

Initially considered to be a new species by Gracillariidae expert Dr Jurate De Prins (Royal Belgian Institute of Natural Sciences), the species identification was confirmed to be *Stomphastis thraustica* (De Prins *et al*. in prep.; Appendix 1). A comparison of DNA with sequences available on GenBank found that the Peru population is less than 0.05% different from samples from Central Africa (Appendix 1). A full genome sequence of the Peru population has been completed and has been submitted to GenBank.

***Table 1 – Permits and importations made for* Stomphastis thraustica.**

PERMIT	DATE	SPECIES	LOCATION	IMPORTATIONS
IP3021062	09/12/13-09/12/15	*S. thraustica*	South Africa	None
IP3021066	09/12/13-09/12/15	*S. thraustica*	Bolivia	None
IP40008601	09/12/13-09/12/15	*S. thraustica*	Everywhere	23/11/14 – 550 individuals from Peru. Used for colony establishment and all work. 30/03/15 – 49 individuals from Bolivia. All destroyed.
IP15015683	23/10/15-23/10/17	*S. thraustica*	Everywhere	None
0001783549	15/12/17-15/12/19	*S.* sp. nov.	Peru	None

2.2 Biology

Stomphastis thraustica is a leaf miner. The biology and life cycle of *S. thraustica* have only been studied on the *J. curcas* (Xiao *et al.* 2009; Terren *et al.* 2012). Adult *S. thraustica* are nocturnal (Terren *et al.* 2012). They emerge from their pupal cases during the evening or early morning and mate during early morning (Xiao *et al.* 2009). Oviposition occurs during evening and early morning. Females lay eggs mostly on the underside of leaves near a vein. Larvae tunnel in the leaf blade resulting in brown patches and causing the leaves to dry out. They undergo five instars before pupating on the leaf surface. In China one generation takes 18-20 days. In the Panxhihua region of China the moth has over 10 generations per year, which overlap. The species endures the dry season as adults.

All of the following work with *S. thraustica* (Peru population) was conducted in a quarantine glasshouse maintained at 30°C and 65% relative humidity during the day and 20°C and 55% relative humidity during the evening (12 hour split). This temperature range approximates the average minimum and maximum temperatures experienced in northern Queensland, where bellyache bush is prevalent. Surveys in central South America were conducted in regions climatically similar to those conditions in Australia, broadly matched by the temperature and humidity range in the quarantine glasshouse. Results are presented as mean ± 1 standard error.

Adult *S. thraustica* (Peru population) are small (less than 1 cm long; Figure 5) and live for an average of 10 days in quarantine (15 days if provided with a sugar solution). They emerge during the evening/early morning and mate during the early morning. Females do not generally lay eggs on the morning that they emerge. They lay an average of 101 eggs (up to 172 eggs). Eggs are less than half a millimetre long (mean 390 µm ± 2 µm and are barely visible to the naked eye (Table 2). They are white in colour, translucent and oval in shape (Figure 5). Eggs are laid singularly on the leaf blade, usually next to a leaf vein on the underside of leaves, but sometimes on the upper leaf surface.

Egg hatch generally occurred three to four days after oviposition under quarantine conditions (mean 3.4 days ± 0.01 days), but up to six days after oviposition (Table 3). Newly emerged larvae mine directly into the leaf from the egg and remain in the leaf as they develop until pupation (Figure 7). Four larval instars were identified, based on head capsule width (Figure 6). This was verified by comparing discarded head capsules with new head capsules. The head capsule width of each successive instar increased by an average of 1.6 x (Table 2). Early instar larvae are transparent and devoid of colour, apart from a brown head capsule (Figure 5). Frass is visible inside the body. The final instar develops into a bright green colour (Figure 5). Larval development took an average of nine days (Table 3) before the larvae exited the leaf to pupate, at which point they were considered to be pre-pupae. The duration of the various instars is estimated to be 2-3 days for the 1st instar, 1-2 days for the second instar, 1-2 days for the third instar, and 2-3 days for the fourth instar (Figure 6). It is possible that an additional instar, that was not detected, occurs in the first two to three days of egg hatch as *S. thraustica* reportedly have five instars (e.g. Xiao *et al.* 2009; Ebadah *et al.* 2017).

Pre-pupae are highly mobile; most pupate on the leaves but some will also crawl to a suitable location or descend on a silk thread. They pupate within a small white cocoon which they spin on the selected surface. Pupation takes around seven days (Table 2). Pupal length averaged 4471 µm (± 37 µm; n=71). A generation from egg to adult takes 17-24 days under quarantine conditions (mean 19.6 ± 0.1 days; Table 2; Figure 7).

Under quarantine conditions, adults lived for an average of five days with no sustenance, eight days when supplied with water and 11 days when supplied with sugar solution (Table 2). Survival was significantly greater for adults supplied with water and significantly greater again when supplied with a sugar solution ($F_{2, 273} = 58.35$, $P < 0.001$).

Figure 5* – Stomphastis thraustica *(Peru population) (left to right): newly emerged larva, late instar larva, pupa, adult (top), egg (bottom).

Table 2. Mean egg length and larval head capsule widths of* Stomphastis thraustica *(Peru population) under quarantine conditions. n=sample size

Life stage	n	Mean width (µm) ± SE
Egg	48	389.7 ± 2.2
L I	199	142.2 ± 0.6
L II	70	225.0 ± 2.6
L III	70	357.3 ± 3.8
L IV	122	539.9 ± 3.2

Table 3 Adult survival and mean development time of* Stomphastis *thraustica* (Peru population) *under quarantine conditions. n=sample size; values with the same letter are not significantly different (Tukey's post hoc multiple comparison test).

		Duration (days)	
Life stage	**n**	**Mean ± SE**	**Range**
Adult survival (no sustenance)	90	4.8 ± 0.2[a]	1 – 10 days
Adult survival (water)	78	8.4 ± 0.4[b]	3 – 16 days
Adult survival (sugar solution)	108	11.1 ± 0.5[c]	2 – 28 days
Egg development	192	3.4 ± 0.1	3 – 6 days
Larval development	214	8.9 ± 0.1	7 – 12 days
Prepupa	175	1.0 ± 0.0	1 – 2 days
Pupa	159	6.6 ± 0.1	6 – 10 days
Total development time	204	19.6 ± 0.1	17 – 24 days

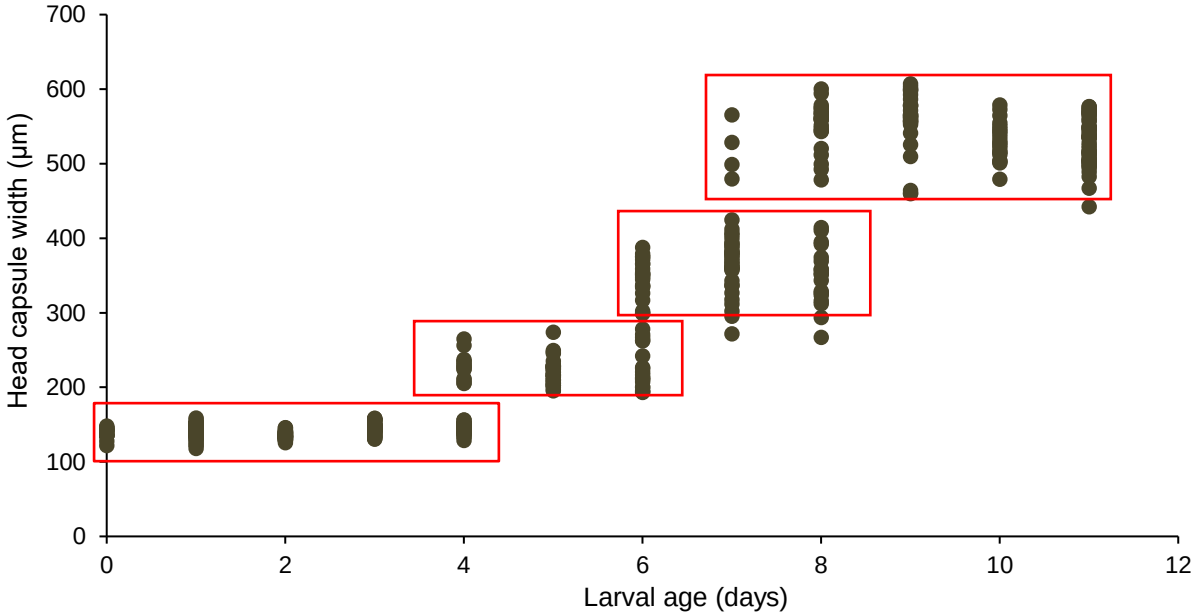

Figure 6. – Head capsule width of* Stomphastis thraustica *(Peru population) larvae from pre-eclosion (day 0) to pre-pupal stage (day 11).

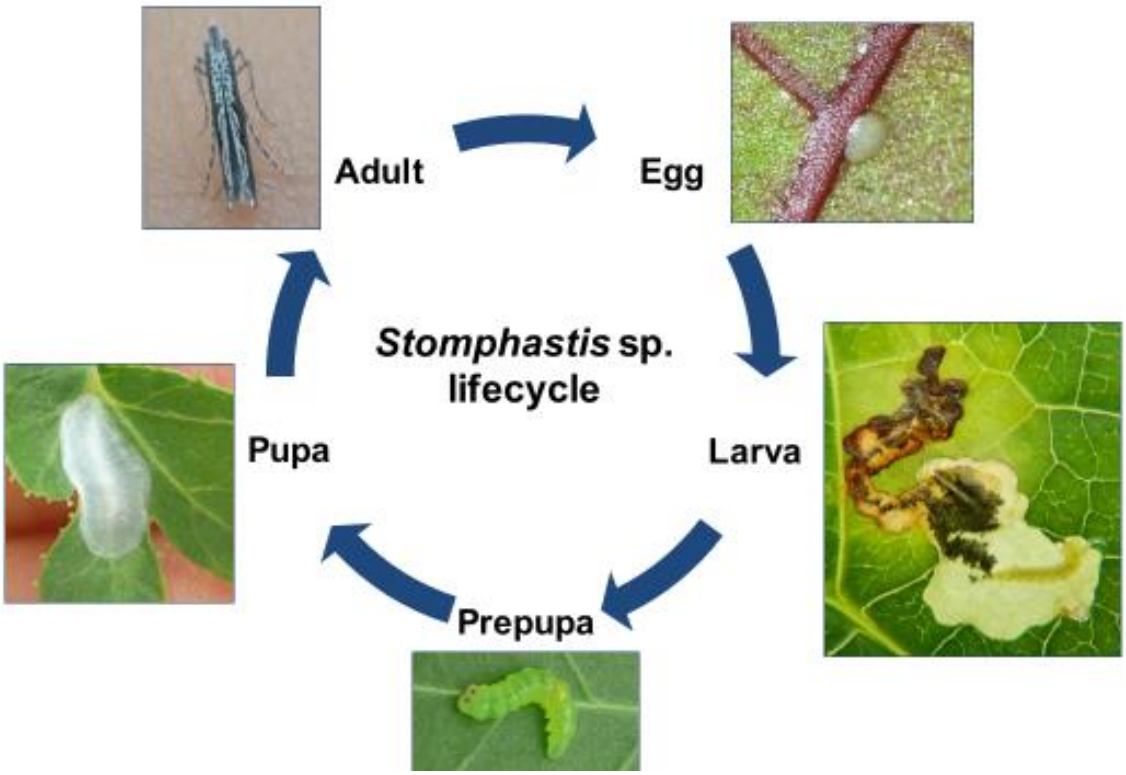

Figure 7* – Stomphastis thraustica *(Peru population) lifecycle.

2.3 Native range

Following extensive surveys for potential agents in countries north of the equator (Heard *et al.,* 20I2), survey effort was redirected to South America (Dhileepan *et al.* 2014). Prospective survey sites in various countries

in South America were identified based on herbarium records (Missouri Botanic Gardens, Kew Botanic Gardens, National Herbarium Nederland, New York Botanic Gardens, Rio de Janeiro Botanical Garden, and Harvard University Herbarium). In March-April 2012 , *J. gossypiifolia* was surveyed at 42 sites in Peru (San Martin, Tarapoto, Moyabamba, and Iquitos regions); all *J. gossypiifolia* sampled were in towns or in home gardens, except for the naturalized populations along the roadside between Picota and Bella vista, and on the river bank between Juan Guerra and Shapaja (Dhileepan *et al.* 2014).

Widespread and severe leaf damage was observed by *S. thraustica* (Peru population) on *J. gossypiifolia* in the San Martin province in northern Peru (18 out of 32 sites visited) and occasionally on *J. curcas* (1 of 10 sites where *J. gossypiifolia* and *J. curcas* co-occurred; Dhileepan *et al.* 2014; Figure 8). At sites where *J. gossypiifolia* co-occurred with the closely related plant *R. communis*, there was no evidence of the leaf miner on *R. communis* (Dhileepan *et al.* 2014). This is the first known record for a *Stomphastis* species from South America.

Stomphastis thraustica has been recorded from Africa (Benin, Botswana, Central African Republic of Congo, Ghana, Kenya, Madagascar, Mozambique, Namibia, Nigeria, Reunion Island, Senegal, South Africa, Uganda and Zimbabwe, and Asia (China, India, Indonesia and Malaysia (De Prins and De Prins 2021b).

Figure 8* – Stomphastis thraustica *damage to* J. gossypiifolia *in the field in Peru.

2.4 Related species

This is the first time that *Stomphastis* has been recorded in South America (Dhileepan *et al.* 2014). The genus *Stomphastis* contains 17 described species that are all from Africa and/or Asia. (De Prins and De Prins 2013; De Prins and De Prins 2021a,b; Table 4). *Stomphastis thraustica* is a well-known pest of *J. curcas* and *J. gossypiifolia* in Asia and Africa. It has also been recorded on *Microstachys chamaelea* (L.) Muell. Arg.in India. The Natural History Museum website (https://www.nhm.ac.uk/our-science/data/hostplants/) lists several other plants as hosts for *S. thraustica*, but they do not provide references for these reports. As such, the *Global Taxonomic Database of Gracillariidae*

(http://www.gracillariidae.net/) has omitted these species pending confirmation. It is likely that these reports of host plants (*Camellia sinensis* (L.) *Kuntze*, *Coffea* sp., *Hibiscus*, sp., *Leucas martinicensis* (Jacq.) W. T. Aiton, *R. communis* and *Triumfetta* sp.) are not accurate. Several species of *Stomphastis* utilise species of *Croton* as hosts; *S. dodonaeae* attacks *Dodonaea madagascariensis* and *D. viscosa* (Sapindaceae), *S. polygoni* attacks *Persicaria setosula* (Polygonaceae) and *S. tremina* attacks *Trema orientalis* (Ulmaceae). A similar looking *Stomphastis* species has also been collected from *J. gossypiifolia*, *J. curcas* and *J. clavuligera* Müll.Arg in Bolivia, but its species status is yet to be ascertained (Dhileepan *et al.* 2014). None of these individuals were introduced into our quarantine colony.

Searches of all available national and international library databases yielded no record of the presence of species in the genus *Stomphastis* in Australia. However, a leaf-mining moth, previously identified as *Epicephala* sp. (Gracillariidae) (Wilson 1997), has recently been identified as a *Stomphastis* species (De Prins *et al.* in prep.; Appendix 1). A comparison of DNA from specimens collected from the Northern Territory and Queensland with sequences lodged with GenBank suggest that the species in Australia is not *S. thraustica* (>5% difference). The Australian *Stomphastis* species is genetically most similar to a yet to be identified *Stomphastis* species from Madagascar. The genetics tree is presented in Appendix 1

Members of the Gracillariidae family have been released as weed biological control agents in Australia. *Neurostrota gunniella* has been released against *Mimosa pigra* (Wilson and Flanagan, 1990) and *Dialectica scalariella* has been released against *Echium plantagineum* (Johns and Hughes 2002).

Table 4* – Stomphastis *species and their recorded hosts (De Prins and De Prins 2021a,b).

Species	Distribution	Host species
S. adesa Triberti	Madagascar, Nigeria	*Monotes glaber* Sprague
S. aphrocyma Meyrick	South Africa, Zimbabwe	*Croton sylvaticus* Muell. Arg.
S. cardamitis Meyrick	South Africa, Namibia	*Croton gratissimus* Burch.
S. chalybacma Meyrick	India, SE Asia	*Caesalpinia pulcherrima* (L.)Sw., *C. decapetala* (Roth) Alston, *Samanea saman* (Jacq.) Merr.
S. conflua Meyrick	Africa, Asia	*Ricinus communis* L., *Juncus* sp., *Polygonum hydropiper* (L.) Delabre, *Pouzolzia hypoleuca* Wedd, *Pouzolzia mixta* Solms
S. crotoniphila Vari	South Africa	*Croton sylvaticus* Muell. Arg.
S. crotonis Vari	South Africa	*Croton menyhartii* Pax
S. dodonaeae Vari	South Africa, Madagascar	*Dodonaea viscosa* (L.) Jacq., *D. madagascariensis* Radlk.
S. eugrapta Vari	South Africa, Madagascar	Unknown
S. heringi Vari	Ethiopia	*Croton macrstachyus* Hochst. ex Delile
S. horrens Meyrick	Ethiopia	Unknown
S. labyrinthica Meyrick	India, Japan	*Guazuma tomentosa* Kunth, *Guazuma ulmifolia* Lam., *Trema orientalis* (L.) Blume
S. mixograpta Meyrick	South Africa	Unknown
S. polygoni Meyrick	Zimbabwe, China	*Persicaria setosula* (A. Rich.) K.L. Wilson
S. rorkei Vari	Southern Africa	*Croton gratissimus* Burch.
S. thraustica Meyrick	East Africa, South Africa, India, SE Asia	*Jatropha gossypiifolia* L., *J. curcas* L., *Microstachys chamaelea* (L.) Muell. Arg.
S. tremina Vari	South Africa	*Trema orientalis* (L.) Blume

2.5 Potential for control

Stomphastis thraustica is a known pest of *J. gossypiifolia* and *J. curcas* in Africa and Asia. *Jatropha curcas* is promoted in some countries as a biofuel and for this reason most studies looking at *S. thraustica* attack involve *J. curcas.* In Senegal, up to 98% of *J. curcas* plants were attacked, with an average of 32% of leaves having larval mines (Terren *et al.* 2012).

Biological studies conducted in quarantine demonstrated that *S. thraustica* (Peru population) has both a short generation time and high fecundity. Under quarantine conditions at 30°C, a generation has been completed in as little as 13 days (unpublished data). In two weeks, the Jatropha leaf-miner has the potential to destroy an entire leaf (Figure 9). This bodes well for its future as a biological control agent, allowing populations to build rapidly under favourable conditions. Indeed, numerous Gracillariidae species are known pests and their success is attributed in part to their short generation time (e.g. Guichard and Augustin 2002; Girardoz *et al.* 2007; De Prins *et al.* 2013). Gracillariidae are also known to be good dispersers (De Prins *et al.* 2013; De Prins and De Prins 2021a). The horse-chestnut leaf-mining moth, *Cameraria ohridella* Deschka & Dimic for example has spread across Europe and the UK at a rate of 40-65 km per year (Straw and Tilbury 2006; Tilbury *et al.* 2006). *Phyllonorycter platani* (Staudinger) is believed to have spread across Europe at a rate of 10 km per year (Sefrova 2001). It is expected that *S. thraustica* (Peru population) will also be an adept disperser, a desirable characteristic given the expansive areas across which *J. gossypiifolia* occurs.

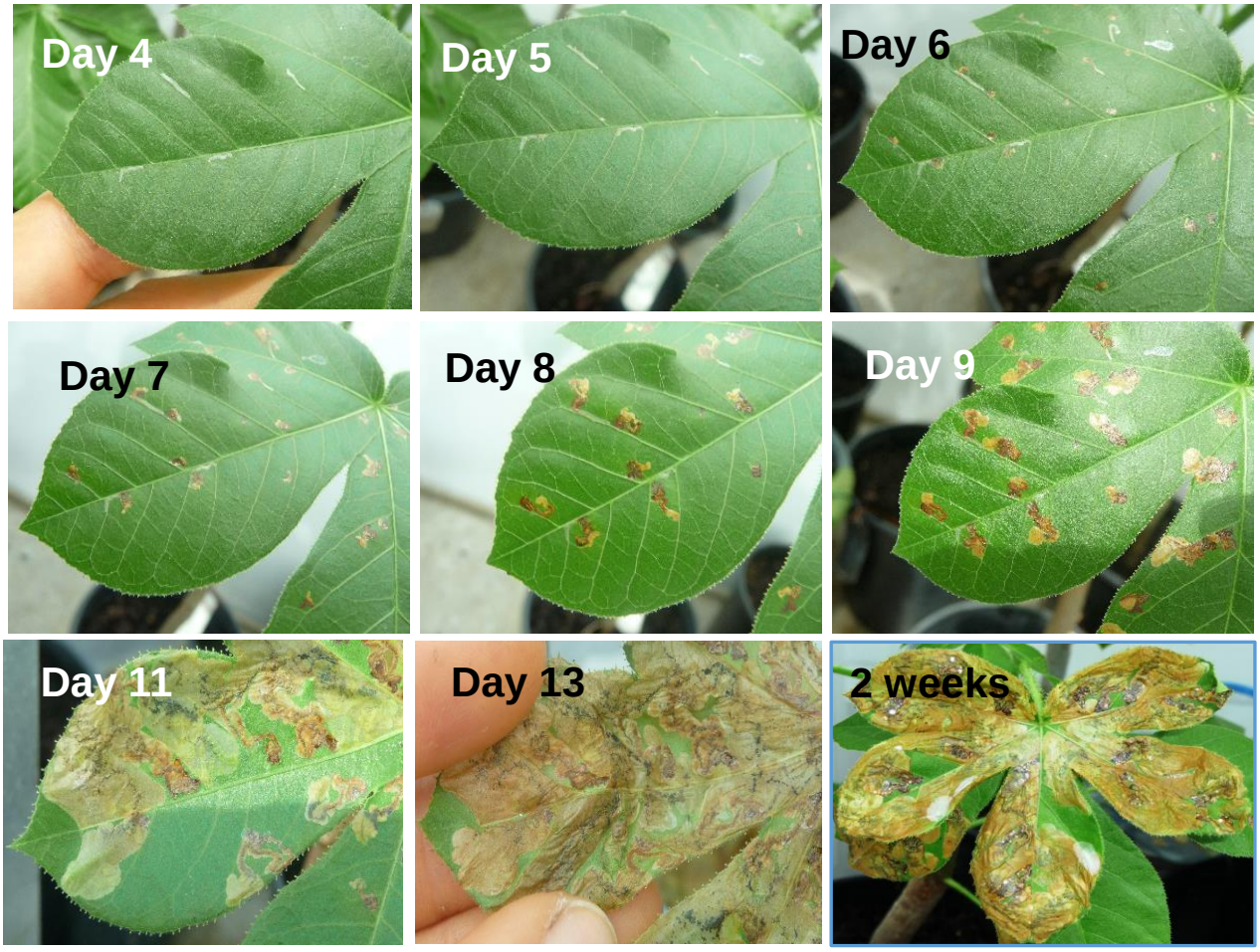

Figure 9* – Stomphastis thraustica *(Peru population) larval feeding damage over time in quarantine.

Stomphastis thraustica (Peru population) can complete development on all six biotypes identified in Australia. Under quarantine conditions *S. thraustica* (Peru population) females demonstrated no apparent

preference for a particular biotype ($F_{5,30}$ = 0.72, P = 0.611; Figure 10). The proportion of eggs on each biotype that developed to adults was also similar for all six biotypes ($F_{5,30}$ = 0.98, P = 0.444; Figure 11). The high variation for a given biotype across replicates can be attributed to its position within the cage. For each replicate biotypes were placed into a different arrangement (e.g. in a corner, in the middle), to minimise potential cage effects).

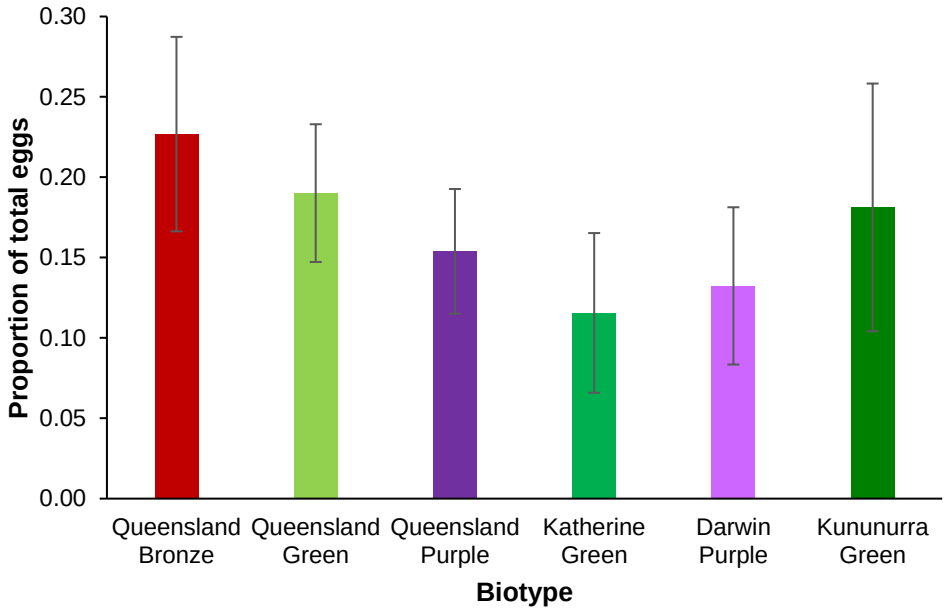

Figure 10 – Proportion of* Stomphastis thraustica *(Peru population) eggs laid on each of the six bellyache bush biotypes identified in Australia. Mean ± 1 standard error.

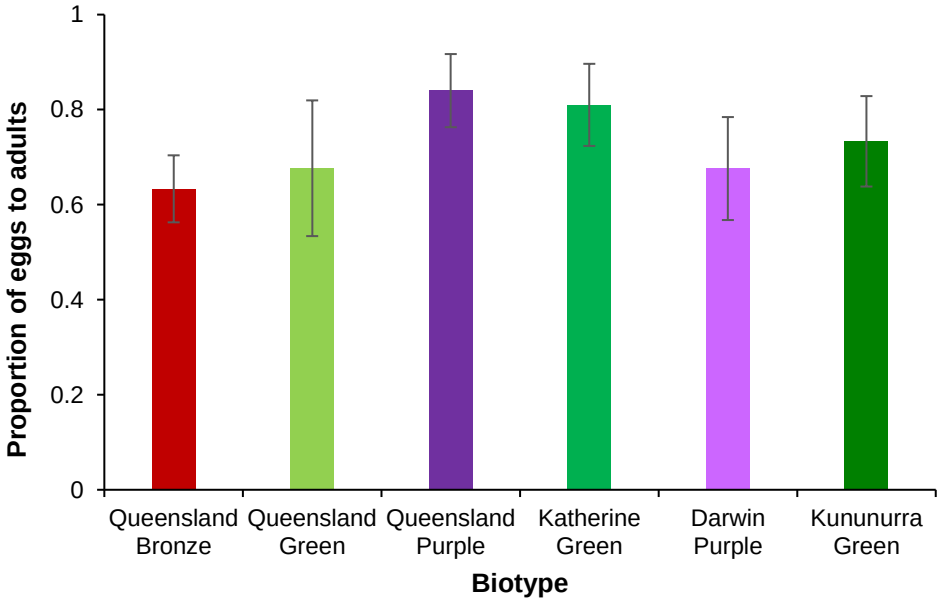

Figure 11 – Proportion of* Stomphastis thraustica *(Peru population) eggs laid on each bellyache bush biotype that develop into adults. Mean ± 1 standard error.

Stomphastis thraustica (Peru population) attack negatively impacted the growth of *J. gossypiifolia* plants under quarantine conditions. The average biomass of plants subjected to more than 50 larvae over three generations was half that of plants not attacked ($F_{2,13}$ = 4.96, P = 0.025; Figure 12). The average biomass of plants subjected to 20-40 larvae over three generation was not significantly different from the control plants.

2.6 Proposed source of the insect

The initial importation of *S. thraustica* from Peru was made in November 2014. A colony was established in our quarantine facility from this importation. We propose mass rearing and releasing individuals from this colony.

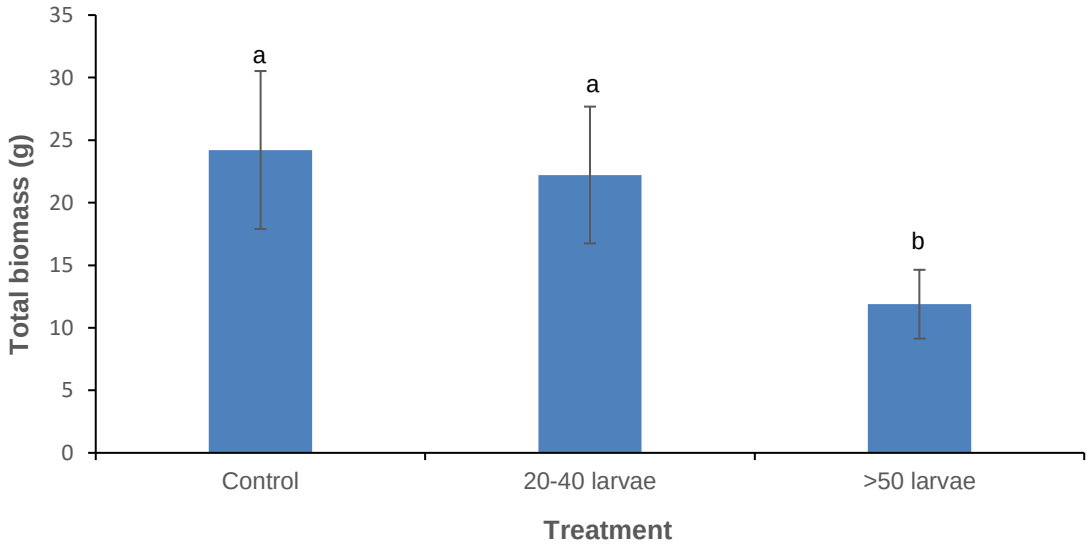

Figure 12 – Total biomass of* J. gossypiifolia *plants exposed to* Stomphastis thraustica *(Peru population) over three generations. Mean ± 1 standard error. Bars with the same letter are not significantly different (Tukey's post hoc multiple comparison test).

2.7 Proposed release strategy

A colony of *S. thraustica* (Peru population) is being maintained within quarantine facilities at the Ecosciences Precinct in Brisbane. Once approval for release has been granted, newly emerged adults will be collected within quarantine and examined under a microscope to ensure the absence of parasites. These adults will be removed from quarantine and placed into cages containing potted *J. gossypiifolia* plants, which will be located in one of the roof-top glasshouses of the Ecosciences Precinct.

The release effort will be focussed in areas with major bellyache bush infestations in Queensland, such as along the Burdekin River from Charters Towers to Home Hill, Hughenden, Gulf of Carpentaria, along the Gregory River, Normanton, and along the Palmer River in Cape York.

The Weed Management Branch of the Northern Territory Government's Department of Environment, Parks and Water Security has expressed interest in mass rearing and releasing at their Darwin research facility. We have also received interest from stakeholders in Western Australia. Given the ease with which the insect

can be reared, it will be valuable to involve organisations such as local councils and catchment groups with the mass rearing and release program. This will necessarily involve workshops with the relevant parties. The insect may be sent to some community groups/property managers via post. Pupae will be the best life stage to be transported via this method. Obviously, the extent of the release program involving community and NRM groups will depend on funding.

At this stage, we are unsure as to the best method for releases, but they will likely involve a combination of adults and pupae. Pupae will be the easiest method, particularly where multiple days of travel to release sites are required. Pupation takes 5-7 days. Pupae that are several days old can be removed from leaves or whole branches can be cut from an infested plant. The cut branches can be hung in bellyache bush plants in the field or a container with the pupae can strategically located. Conditions in quarantine are very different to the field so it is difficult to provide an optimal number of individuals for a successful release. This is something that we will ascertain early in the release program. A higher number of individuals means a greater level of genetic diversity, which is desired. Initial release numbers will range from 100-1000 individuals per release, until the optimal number for establishment is determined.

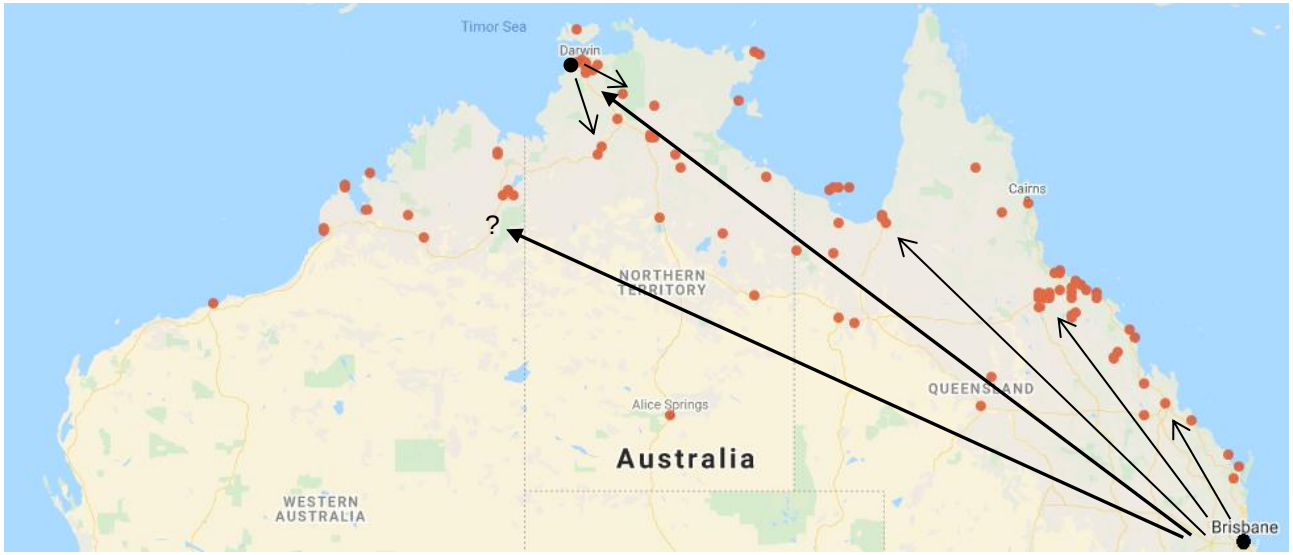

Figure 13 – Schematic representation of the proposed release strategy for* Stomphastis thraustica *(Peru population) on a map displaying bellyache bush herbarium records. Red dots are* J. gossypiifolia *herbarium records. Black dots represent potential mass rearing facilities from which releases will be made. Bold arrows represent movement from Brisbane to other mass rearing facilities and other arrows represent distribution (releases) from these facilities.

3 Host-specificity testing

3.1 Plant test list for *Stomphastis thraustica* (Peru population)

3.1.1 Background

For the last 40 years test lists have been developed following the centrifugal phylogenetic method (Wapshere 1974). The CPM emphasizes the testing of species most closely related to the target and then successively more distant taxa. Despite the name, test plant selection relied on hierarchical taxonomic groupings (and testing members from as many groups as possible) as well as the inclusion of unrelated 'safeguard' species. The CPM has been modernised, shifting the focus from taxonomic groupings to

phylogenetic relationships, taking into consideration ecological and biogeographic filters, and removing unrelated 'safeguard' species (Briese, 2003, 2005; Mehelis *et al*., 2015). The modernisation takes advantage of the huge advances made in plant phylogenetic relationships and host selection since the CPM was developed and shifts the focus to defining the host range rather than determining whether or not individual plant species were "safe" (Briese, 2005). Further, the inclusion of "safeguard" species is no longer considered to be beneficial: "They distract from the real purpose of host-specificity testing as they do not add information on host-range (see Briese and Walker, 2002), and would not be used in a modernised methodology for choosing test plant lists." (Briese 2005, but also see Briese and Walker 2002, Sheppard *et al.* 2005).

The host test list used for testing *S. thraustica* (Peru population) contains 50 species and was based on the list approved for the seed feeder *A. trilineatum*, (Heard *et al.* 2009; Table 5). The test list approved for *A. trilineatum* consisted of 49 species and was compiled following the CPM but focused on test species with adequate availability of fruit (as *A. trilineatum* is a seed feeder). The test list for *S. thraustica* (Peru population) was refined focusing on phylogenetically related native species occurring in northern Australia where *J. gossypiifolia* is invasive. It also includes several species recorded as hosts of *S. thraustica* (*J. curcas*, *R. communis* and *M. chamaelea;* Table 5).

3.1.2 Phylogenetic relationships and test list composition

Since the testing *of A. trilineatum*, the Euphorbiaceae has been split into seven families: Euphorbiaceae Jussieu sensu stricto (s.s.), Phyllanthaceae Martynov, Picrodendraceae Small, Putranjivaceae Meirner, Pandaceae Engl. & Gilg. Peraceae Klotzsch and Centroplacaceae Doweld & Reveal (Figure 14; Wurdack *et al.* 2005; Angiosperm Phylogeny Group 2016; Stevens 2018).

Until recently, Euphorbiaceae *s.s*. was separated into three subfamilies: Crotonoideae, Euphorboideae and Acalyphoideae.The current classification within Euphorbiaceae *s.s*. needs revision and relationships are still in a state of flux (Wurdack *et al.* 2005). Due to the uncertainty regarding relationships, the three recognised subfamilies (Crotonoideae, Euphorboideae and Acalyphoideae) are retained here. A schematic representation of relationships within Euphorbiaceae *s.s*. as per Wurdack *et al.* 2005 is presented in Appendix 2. Appendix 3 lists test plants from Euphorbiaceae *s.s*. as per the old tribal and subfamily classifications and using the groupings of Wurdack *et al.* 2005.

Euphorbiaceae *s.s.* is represented in Australia by approximately 195 native species from 30 genera (Zich *et al.* 2018). The genus *Jatropha* is a member of the Jatropheae tribe, within the subfamily Crotonoideae. There are no Australian native species within the tribe Jatropheae. Three *Jatropha* species other than the target are included in the test list (Table 5). The subfamily Crotonoideae (to which *J. gossypiifolia* belongs) contains about 13 genera and 95 species native to Australia and a number of cultivated species such as garden croton (*Codiaemum variegatum*) and cassava (*Manihot esculenta*). The test list includes representatives from all of the tribes present in Australia except Trigonostemoneae (which contains *Trigonostemon inopinatus*, a vulnerable species occurring in vine forests in central Queensland). The subfamily Euphorbioideae, represented in Australia by about 43 native species from 4 genera, includes the large genus *Euphorbia*. *Euphorbia* contains a number of cultivated and naturalized species including Poinsettia (*E. pulcherrima* Willd. ex Klotzsch) and petty spurge (*E. peplus* L.), as well as around 35 native species. Representatives from three of the four Euphorbioideae genera have been included in the list including several native and exotic Euphorbia species (Table 5). The subfamily Acalyphoideae is represented in Australia by about 57 native species in 13 genera, including the common weed castor oil plant (*Ricinus*

communis L.) and the native *Macaranga tanarius* (L.) Müll.Arg.). Representatives from seven genera have been included in the test list.

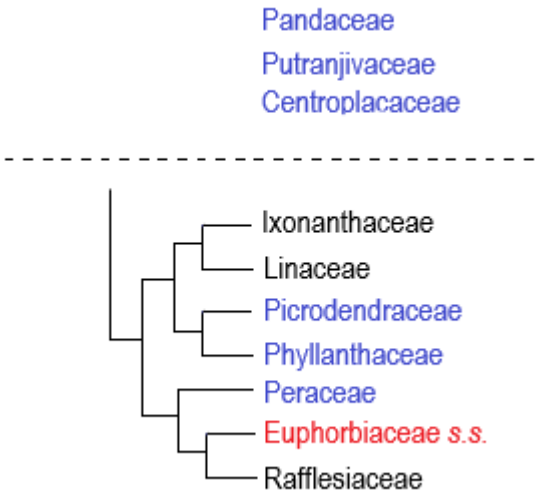

Figure 14 – Phylogenetic relationships of Euphorbiaceae* sensu stricta *and related families. Members of Euphorbiaceae* sensu lato *are in coloured text (Stevens 2018).

The Rafflesiaceae is a family of rare parasitic plants, closely related to Euphorbiaceae *s.s.* but not found in Australia. Peraceae is a small family of five genera, also not present in Australia. Neither are included in the test list. The Phyllanthaceae is represented in Australia by 12 genera (including *Phyllanthus* – 40 species and *Souropus* - 28 species) and the Picrodendraceae by around eight genera (Hunter 2005; ALA 2017). Representatives from both families are included. The Ixonanthaceae is not present in Australia and the Linaceae is a family of annual and perennial herbs; one species from Linaceae was included in the test list. The Putranjivaceae is no longer considered to be closely associated with the Euphorbiaceae family, but one representative of this family was included as an outlier.

We included five unrelated plants species in the list that are attacked by *S. thraustica* and other *Stomphastis* species (or native congeners of attacked species); *Camillea sinensis*, *Coffea arabica*, *Caesalpinia pulcherrima, Trema tomentosa,* and *Dodonaea triquetra*. Species included in the test list approved for *A. trilineatum* but omitted from the test list used here were either difficult to source or were replaced by species deemed more suitable. Species from unrelated families were also removed.

The sources of the various test plants are provided in Appendix 4. Plants were maintained in our shade house and/or heated glasshouse until required for testing. Plant pests were removed manually. Where this was not feasible plants were treated with host specific biological control (e.g. for spider mites) or treated with a contact spray such as white oil or soap spray. As a precaution, plants were not used for two weeks after use of a contact spray.

Table 5 – Host test list for* Stomphastis thraustica *(Peru population).

Test plants	Status[#]	Test plants	Status[#]
Malpighiales		*Euphorbia tithymaloides* L.	I
Euphorbiaceae		Hippomaneae	
Crotonoideae		*Homalanthus populifolius* Graham	N
Jatropheae		*Microstachys chamaelea* (L.) Hook.f.	N
Jatropha gossypiifolia L.	Target	Phyllanthaceae	
Jatropha curcas L.	I	Antidesmatoideae	
Jatropha podagrica Hook.	I	*Antidesma bunius* (L.) Spreng.	N
Jatropha multifida L.	I	*Antidesma ghaesembilla* Gaertn.	N
Adenoclineae		Phyllanthoideae	
Endopermum sp.	N	*Actephila lindleyi* (Steud.) Airy Shaw	N
Aleuritideae		*Breynia cernua* (Poir.) Mull.Arg.	N
Aleurites sp.	N	*Breynia oblongifolia* (Mull.Arg.) Mull.Arg	N
Codiaeae		*Bridelia exaltata* F. Muell.	N
Baloghia inophylla (G.Forst.) P.S.Green	N	*Cleistanthus hylandii* Airy Shaw	N
Codiaeum variegatum (L.) A.Juss.	NO	*Flueggea virosa* (Willd.) Voigt	N
Crotoneae		*Glochidion ferdinandi* (Muell.Arg.) F.M.Bailey	N
Croton acronychioides F.Muell.	N	*Glochidion* sp. 'Gunn Point'	N
Croton insularis Baill.	N	*Phyllanthus cuscutiflorus* S.Moore	N
Croton verreauxii Baill.	N	Picrodendraceae	
Manihoteae		*Austrobuxus swainii* (Beuzev. & C.T.White) Airy Shaw	N
Manihot esculentum Crantz	C	*Dissiliaria baloghioides* F.Muell. ex Baill.	N
Manihot grahamii Hook.	I	*Petalostigma pubescens* Domin	N
Ricinocarpeae		*Sankowskya stipularis* P.I.Forst.	N
Beyeria lechenaultii (DC.) Baill.	N	Putranjivaceae	
Beyeria viscosa (Labill.) Miq.	N	*Drypetes deplanchei* (Brongn. & Gris) Merr.	N
Ricinocarpos pinifolius Desf.	N	Linaceae	
Acalyphoideae		*Linum marginale* L.	N
Acalypheae		Fabales	
Acalypha capillipes Müll.Arg	N	Fabaceae	
Macaranga tanarius (L.) Müll.Arg.	N	*Caesalpinia pulcherrima* (L.)Sw.	IO
Mallotus philippensis (Lam.) Muell.Arg.	N	Rosales	
Ricinus communis L.	I	Ulmaceae	
Alchorneae		*Trema tomentosa var. aspera* (Brongn.) Hewson	N
Alchornea ilicifolia (J.Sm.) Muell.Arg.	N	Sapindales	
Omphaleae		Sapindaceae	
Omphalea celata P.I.Forst	N	*Dodonaea triquetra* J.C.Wendl.	N
Euphorbioideae		Ericales	
Euphorbieae		Theaceae	
Euphorbia grantii Oliv.	I	*Camellia sinensis* (L.) Kuntze	C
Euphorbia plumerioides Teijsm. ex Hassk.	N	Gentianales	
Euphorbia pulcherrima Willd. ex Klotzsch	IO	Rubiaceae	
Euphorbia tannensis Spreng.	N	*Coffea arabica* L.	C

[#]Status: I - invasive; O - ornamental; N - native; C – crop

3.2 Host specificity testing methods

3.2.1 Background information

The various methods used for testing weed biological control agents have been reviewed by a number of researchers (e.g. Heard and van Klinken, 1998; Sheppard, 1999; Heard 2000; Barton Browne and Withers, 2002; Briese 2005; Sheppard *et al*., 2005). An understanding of host selection behaviours, including the effects of motivation, prior learning, and experience provide the basis for selection, design, and interpretation of host specificity tests (Table 6). To mitigate the effects of these factors we used newly emerged individuals.

Host selection is not a single step, but rather a sequence of behavioural responses (Heard 2000). The sequence of steps in host selection includes habitat location, host location, host acceptance, and host use and may involve sensory cues including visual, olfactory, gustatory, and tactile stimuli. Different species express high specificity at different stages in the host selection process and test design can interfere with this selection process (Heard 2000). The confined conditions that are experienced during quarantine testing can influence agent behaviour (Heard 2000). For example, many arthropods, especially Lepidopterans lay eggs indiscriminately (egg dumping) in cage environments, particularly when they reach a high state deprivation (Marohasy, 1998; Heard 2000). In some cases insects will oviposit on cage walls.

There are three basic test designs: no-choice and choice (both conducted in quarantine), and field trials (usually choice trials conducted in the native range). It is widely accepted that no-choice testing provides the best initial screening of plants due to the conservative nature of the test. In no-choice trials, the agent is confined with a test species (usually) until death; the agent either attempts to feed (or reproduce) or dies. No choice tests identify the fundamental host range, which is the range of plants species that the agent is genetically capable of utilising (Figure 15). They do not consider behavioural or ecological factors that can affect host selection under natural conditions (realised host range).

The probability of an agent rejecting a species in a no-choice trial but accepting it in the field is negligible. However the probability of an agent accepting a species under the confined conditions of a no-choice trial but rejecting it in the field (i.e. false positive) is widely considered to be high. It is for this reason that test species identified under no-choice conditions to be potential hosts (i.e. supporting complete development of the agent) are then used in choice trials with the target or true host. Choice trials are useful for exploring host preference of species identified as potential hosts in no-choice tests. They allow assessment of how motivation, prior experience, and learning affect host preference with or without the target species (depending on test design; Sheppard *et al.* 2005). Like no-choice trials they can be affected by abnormal results due to confined conditions.

Host specificity testing of *S. thraustica* (Peru population) was conducted from December 2014 until December 2017 in a quarantine glasshouse maintained at 30°C and 65% relative humidity during the day and 20ºC and 55% relative humidity during the evening (12 hour split). Additional testing was conducted from December 2018 until September 2020. For a flow diagram of the testing conducted see Figure 1. In all cases individual plants were used for a single replicate only. Non-target plants were monitored until adults emerged on the *J. gossypiifolia* plant tested. If there was any evidence of live larvae on the non-target plants at this point, the test plants would be monitored until such time as no live *S. thraustica* (Peru population) were present. Similar sized plants were chosen for each replicate.

In many Lepidoptera, egg laying can be indiscriminate, especially in caged or no-choice conditions (e.g., Heard and van Klinken, 1998; Sheppard *et al.* 2005). Eclosion of larvae only requires resources within the egg. Subsequent larval development requires resources from a suitable host plant. In the context of using such insects as candidate biological control agents, larval feeding and development is the most appropriate

measure of risk to non-target plants. Hence, the host specificity tests were designed to characterize the risk of larval development on non-target plants.

Table 6 – Insect behavioural mechanisms and their consequences for the design and interpretation of host specificity tests (Heard 2000).

Behavior	Consequence
Host location stimuli	
Absence of early steps in host selection behaviour.	False positives in all cage tests.
Volatile chemicals from non-hosts mask those of hosts.	False negatives in most tests
Volatile chemicals from hosts are absorbed onto non-hosts.	False positives in cage choice tests
Experience and learning	
Associative learning	False positives in cage choice tests
Habituation to deterrents of non-hosts	False positives in cage choice and no-choice tests
Sensitization (including priming) to stimuli of hosts	False positives in choice tests and sequential no-choice tests
Central excitation	False positives in choice tests
Central inhibition	False negatives in cage and open field choice tests
Induced oviposition or adult feeding preferences	False negatives in all tests if adults experienced with test plant are used
Induced larval feeding preferences	False negatives in larval feeding and development trials
Time-dependent effects	
Insects increase their response to lower ranked hosts as they approach a deprived state	False negatives in cage and open field choice tests
	Choice trials run for short times may not be appropriate
Age: females become less discriminating as they age.	False negatives in all tests if old insects are not used
Other behaviors	
Inhibitory cage environment / escape responses	False positives in all cage tests

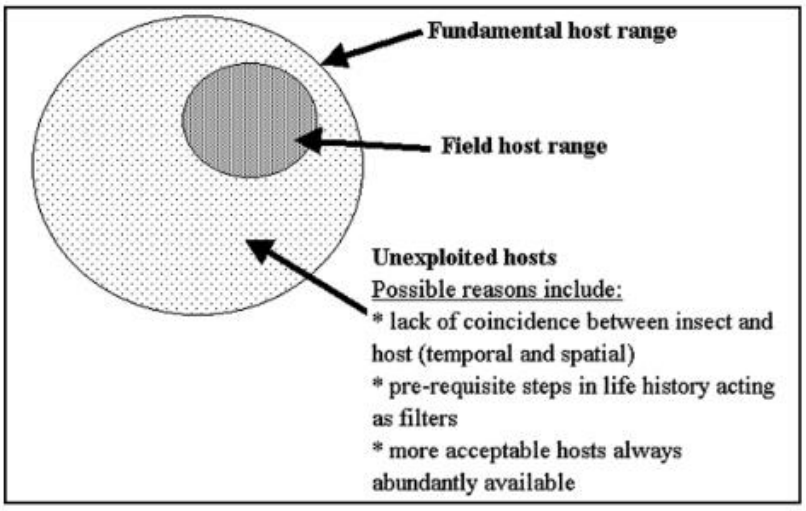

Figure 15 – Possible reasons for the fundamental host range not being fully realised in the field (van Klinken 2000).

3.2.2 No-choice tests

All test plant species were subject to no choice oviposition/larval development tests (Figure 1). Since sexing the highly mobile adults is very time consuming, unsexed adults were used. Twenty newly-emerged (i.e. unexperienced) unsexed *S. thraustica* (Peru population) adults were released into a 45 x 45 x 90 cm gauze covered cage, containing a single potted test plant and a small sealed container of a sugar solution with a dental wick protruding from the lid (to provide the moths with sustenance; Figure 16). The probability of a replicate having zero females out of the 20 individuals is 0.000001 (based on a 1:1 sex ratio, which was determined by sexing all adults emerging from 12 different *J. gossypiifolia* plants and calculating the mean

for the 12 plants). With each round of testing, at least one *J. gossypiifolia* plant was also included as a control, however only ten newly emerged adults were released into this cage (so as not to over-burden plants). The probability of a replicate having zero females out of the 10 individuals is 0.001 but this did not occur (had it occurred (i.e. zero eggs laid), the round would have been repeated). Plants were checked periodically for eggs and larval mines (which were counted) and again when all adults had died. Most test species were subjected to a minimum of five replications. *Euphorbia plumerioides* Teijsm. ex Hassk. and *Ricinocarpos pinifolius* Desf. were subject to three replicates due to the difficultly procuring them.

For the final two species tested (*Camellia sinensis* (L.) Kuntze and *Coffea arabica* L.), ten newly emerged adults were contained with each plant. The probability of zero females being present in a replicate is 0.001 (based on a 1:1 sex ratio).

Figure 16 – Examples of no-choice test arenas for* Stomphastis thraustica *(Peru population).

3.2.3 Paired-choice comparison tests

Any test plant species on which larval development occurred was subjected to a comparison trial with the target. *Jatropha curcas* was the only species other than the target that supported larval development. Ten newly emerged unsexed *S. thraustica* (Peru population) adults were released into a 100 x 45 x 90 cm gauze covered cage containing a single potted *J. curcas* plant and one *J. gossypiifolia* plant (arranged so that no plants were touching). Adults were removed from the cage after one day and the number of eggs on each plant counted. Plants were monitored and the number of larvae, pupae and adults and the duration of each life stage were recorded. Similar-sized plants were chosen for each replicate and eight replicates were completed.

3.2.4 Choice oviposition tests

Many Lepidoptera lay eggs indiscriminately in confined and or no-choice conditions (Withers and Barton Browne 1998; van Klinken and Heard 2000). Female *S. thraustica* (Peru population) also laid eggs on many non-target species under no-choice conditions. To examine oviposition behavior in the presence of the target plant, multiple-choice oviposition trials were conducted, focusing on species on which eggs were most often laid. For the preliminary trial (which was conducted in 2017 and 2018) 10 newly emerged unsexed *S.*

thraustica (Peru population) adults were released into a 100 x 45 x 90 cm gauze covered cage containing a single potted plant each of *Croton verreauxii* Baill., *Baloghia inophylla* (G.Forst.) P.S.Green, and *Aleurites moluccanus* (L.) Willd.) and a single *J. gossypiifolia* plant (arranged so that no plants were touching; Table 7). Plants were checked weekly and the number of eggs and mines were counted**.** Five replicates were completed with plants placed in a different arrangement for each replicate. The same sized cage was used to test *Camellia sinensis* and *Coffea arabica* (Figure 17; Table 7). One plant each of these plus one *J. gossypiifolia* plant were arranged so that no plants were touching. Plants were checked weekly, and the number of eggs and mines were counted**.** Five replicates were completed with plants placed in a different arrangement for each replicate.

For the remaining choice oviposition trials, which were conducted in 2019 and 2020, three newly emerged sexed *S. thraustica* (Peru population) adult male-female pairs were used. These trials followed the same methods used for the initial choice trial but used a larger cage size (Figure 18; Table 7). The larger cage size provided an opportunity for the adults to fly around to facilitate choice for egg laying. For trials conducted in the 215 x 140 x 210 cm cage, plants were moved to a 100 x 45 x 90 cm gauze covered cage once all adults had died, and then monitored as above.

3.2.5 Multiple-choice oviposition test, in the absence of *J. gossypiifolia*

We conducted a multiple-choice test without the target, with six non-target species: *Alchornea ilicifolia, Antidesma bunis, Baloghia inophylla, Bridelia exaltata, Croton insularis*, and *Omphalea celata*. The species were chosen due to the high number of eggs laid on them during no-choice trials and their availability. Three newly-emerged sexed *S. thraustica* (Peru population) adult male-female pairs were released into a 215 x 140 x 210 cm cage containing a single potted plant of each test plant species. Plants were checked for eggs once all adults had died (generally one week after their release into the cage) and then again several days later. If any eggs were found, plants were monitored until such time as no live *S. thraustica* (Peru population) were present. Five replicates were completed with plants placed in a different arrangement for each replicate.

Figure 17 – An example of a choice oviposition trial with* J. gossypifolia*,* Coffea arabica *and* Camellia sinensis*.

Figure 18 – Examples of choice oviposition trials conducted in the 215 x 140 x 210 cm cage.

Table 7. Choice oviposition tests conducted with* Stomphastis thraustica *(Peru population) and the target* (Jatropha gossypiifolia*)

Trial	Non-target plant species	Stomphastis adults (newly emerged)	Cage size (cm)	Replicates
1	*Aleurites moluccanus* *Baloghia inophylla* *Croton verreauxii*	10 unsexed adults	100 x 45 x 90	5
2	*Camellia sinensis* *Coffea arabica*	3 sexed ♂♀ pairs	100 x 45 x 90	5
3	*Actephila lindleyi* *Breynia oblongifolia* *Bridelia exaltata* *Cleistanthus hylandii*	3 sexed ♂♀ pairs	215 x 140 x 210 cm	5
4	*Alchornea ilicifolia* *Antidesma ghaesembilla* *Breynia cernua*	3 sexed ♂♀ pairs	215 x 140 x 210 cm	3
5	*Antidesma bunius* *Euphorbia plumerioides* *Omphalea celata,*	3 sexed ♂♀ pairs	215 x 140 x 210 cm	5
6	*Dodonaea triquetra* *Euphorbia tannensis* *Glochidion ferdinandi*	3 sexed ♂♀ pairs	215 x 140 x 210 cm	3
7	*Jatropha multifida* *Jatropha podagrica* *Ricinus communis*	3 sexed ♂♀ pairs	215 x 140 x 210 cm	5
8	*Aleurites moluccanus* *Baloghia inophylla* *Croton insularis* *Drypetes deplanchei*	3 sexed ♂♀ pairs	215 x 140 x 210 cm	5

3.2.6 Statistical analysis

Proportion data from the paired-choice comparison trial and the choice oviposition trials (where eggs were laid on non-target plants) were arcsine transformed using the modified Freeman and Tukey (1950) formula (Zar 2010). The transformed data was then subject to Analysis of Variance tests. Where a significance difference was found, Tukey's Post Hoc Test was applied.

3.3 Host specificity testing results

3.3.1 No-choice tests

Under no-choice conditions *S. thraustica* (Peru population) females laid eggs on 35 of the 50 test species, predominantly on leaves at the top of the plants (Table 8). Egg hatch occurred on 28 of these species. Hatching of larvae from eggs occurred on both target and non-target plants, as the embryos in the eggs utilized the resources within the eggs to complete egg development. Subsequent development of hatched larvae requires resources from a suitable host plant. As a result, in all test plant species, except on *J. gossypiifolia* and *J. curcas*, the first instar larvae attempted to feed and then died, shortly after emerging, as they are not suitable hosts; this includes on congeners *J. multifida* and *J. podagrica.* The test plants were then kept, with the dead larvae for an additional 2-3 weeks while development proceeded on *J. gossypiifolia* (and *J. curcas,* where tested). The exploratory larval mines on these non-target species ranged in size from 1 mm to 30 mm in length and 0.5mm wide per mine (Figure 19). These mines affected only the surface layers of the leaves. In no cases did the exploratory mines cause leaf death, leaf drop or a change in leaf colour. Larval development was only observed on *J. gossypiifolia* and *J. curcas* plants. Figure 20 shows an example of a larval mine on *J. gossypiifolia,* for comparison with Figure 18. The number of adults that emerged from *J. gossypiifolia* plants during no-choice trials ranged from 21 to 308 (mean 100 ± 10) (Table 8).

Table 8 – Results from no-choice host specificity testing with* Stomphastis *thraustica (Peru population). Results presented as mean (standard error).

Test plants	Status	Reps	Eggs*	1st instar larvae	Larval devel.#	Adults
Malpighiales						
Euphorbiaceae						
Crotonoideae						
Jatropheae						
Jatropha gossypiifolia L.	Target	46	161 (13)	158 (12)	Y	100 (10)
Jatropha curcas L.	I	5	70 (25)	69 (24)	Y	51 (29)
Jatropha podagrica Hook.	I	5	91 (33)	82 (33)	N	N
Jatropha multifida L.	I	5	129 (28)	96 (17)	N	N
Adenoclineae						
Endopermum sp.	N	5	-	10 (7)	N	N
Aleuritideae						
Aleurites sp.	N	5	46 (10)	41 (9)	N	N
Codiaeae						
Baloghia inophylla (G.Forst.) P.S.Green	N	6	36 (17)	19 (17)	N	N

Codiaeum variegatum (L.) A.Juss.	NO	5	0	0	N	N
Crotoneae						
Croton acronychioides F.Muell.	N	6	6 (3)	0	N	N
Croton insularis Baill.	N	5	1 (1)	0	N	N
Croton verreauxii Baill.	N	5	140 (61)	133 (57)	N	N
Manihoteae						
Manihot esculentum Crantz	C	6	0	0	N	N
Manihot grahamii Hook.	I	6	15 (7)	9 (6)	N	N
Ricinocarpeae						
Beyeria lechenaultii (DC.) Baill.	N	6	3 (3)	0	N	N
Beyeria viscosa (Labill.) Miq.	N	5	0	0	N	N
Ricinocarpos pinifolius Desf.	N	3	0	0	N	N
Acalyphoideae						
Acalypheae						
Acalypha capillipes Müll.Arg	N	5	0	0	N	N
Macaranga tanarius (L.) Müll.Arg.	N	5	0	0	N	N
Mallotus philippensis (Lam.) Muell.Arg.	N	5	0	0	N	N
Ricinus communis L.	I	5	6 (5)	1 (1)	N	N
Alchorneae						
Alchornea ilicifolia (J.Sm.) Muell.Arg.	N	5	60 (39)	4 (3)	N	N
Omphaleae						
Omphalea celata P.I.Forst	N	6	17 (7)	16 (6)	N	N
Euphorbioideae						
Euphorbieae						
Euphorbia grantii Oliv.	I	5	2 (2)	2 (2)	N	N
Euphorbia plumerioides Teijsm. ex Hassk.	N	3	29 (11)	4 (3)	N	N
Euphorbia pulcherrima Willd. ex Klotzsch	IO	5	0	0	N	N
Euphorbia tannensis Spreng.	N	5	81 (32)	62 (26)	N	N
Euphorbia tithymaloides L.	I	5	0	0	N	N
Hippomaneae						
Homalanthus populifolius Graham	N	5	0	0	N	N
Microstachys chamaelea (L.) Hook.f.	N	5	29 (28)	26 (25)	N	N
Phyllanthaceae						
Antidesmatoideae						
Antidesma bunius (L.) Spreng.	N	5	10 (9)	8 (7)	N	N
Antidesma ghaesembilla Gaertn.	N	5	43 (13)	35 (12)	N	N
Phyllanthoideae						
Actephila lindleyi (Steud.) Airy Shaw	N	5	2 (1)	1 (1)	N	N
Breynia cernua (Poir.) Mull.Arg.	N	5	32 (16)	32 (16)	N	N
Breynia oblongifolia (Mull.Arg.) Mull.Arg	N	5	1 (1)	1 (1)	N	N
Bridelia exaltata F. Muell.	N	6	30 (24)	14 (8)	N	N
Cleistanthus hylandii Airy Shaw	N	6	1 (1)	1 (1)	N	N
Flueggea virosa (Willd.) Voigt	N	5	4 (4)	0	N	N
Glochidion ferdinandi (Muell.Arg.) F.M.Bailey	N	6	1 (1)	1(1)	N	N
Glochidion sp. 'Gunn Point'	N	5	72 (11)	11(3)	N	N
Phyllanthus cuscutiflorus S.Moore	N	5	0	0	N	N
Picrodendraceae						
Austrobuxus swainii (Beuzev. & C.T.White) Airy Shaw	N	**5**	**0**	0	N	N

Dissiliaria baloghioides F.Muell. ex Baill.	N	**5**	**0**	0	N	N
Petalostigma pubescens Domin	N	5	0	0	N	N
Sankowskya stipularis P.I.Forst.	N	5	2 (2)	0	N	N
Putranjivaceae						
Drypetes deplanchei (Brongn. & Gris) Merr.	N	5	1 (1)	1(1)	N	N
Linaceae						
Linum marginale L.	N	5	0	0	N	N
Fabales						
Fabaceae						
Caesalpinia pulcherrima (L.)Sw.	IO	5	7 (3)	5 (3)	N	N
Rosales						
Ulmaceae						
Trema tomentosa var. aspera (Brongn.) Hewson	N	5	<1 (1)	0	N	N
Sapindales						
Sapindaceae						
Dodonaea triquetra J.C.Wendl.	N	5	8 (3)	8 (2)	N	N
Ericales						
Theaceae						
Camellia sinensis (L.) Kuntze	C	5	9 (5)	2 (1)	N	N
Gentianales						
Rubiaceae						
Coffea arabica L.	C	5	38 (10)	38 (10)	N	N

Status[@]: I – invasive, C – crop, N – native, O – ornamental; Eggs*: eggs are very difficult to see and could not be seen on *Endospermum* leaves due their pubescence; Larval development[#] (beyond 1st instar): Y – yes, N - no

3.3.2 Paired-choice comparison tests

In paired-choice trials with *J. curcas* and *J. gossypiifolia*, 51% (0.51 ± 0.05) of eggs were laid on *J. curcas* and 49% (0.49 ± 0.05) were laid on *J. gossypiifolia,* which was not significantly different ($F_{1,16}$ = 0.02, P = 0.885; Figure 21). Development of these eggs through to adult were similar for both species: *J. gossypiifolia*: 0.74 ± 0.05, *J. curcas* 0.73 ± 0.47; $F_{1,14}$ = 0.09, P = 0.771.

3.3.3 Choice oviposition tests

In choice oviposition tests, very few eggs were laid on non-target plants. In no cases did larval development past first instar occur on any species other than the target, *J. gossypiifolia*. Eggs were laid on the target plants in all replicates for all trials. When adult females were offered a choice of *J. gossypiifolia*, *C. verreauxii, B. inophylla* and *A. moluccanus* in the preliminary choice trial, an average of 89.9 ± 2.0 % of eggs were laid on bellyache bush; significantly greater than the percentage laid on the non-target species (*C. verreauxii*: 2.3 ± 0.8 %; *B. inophylla*: 2.7 ± 1.1 %; *A. moluccanus*: 5.2 ± 2.3%; $F_{3,16}$ = 171.60, P<0.001; Table 9). No larval development occurred on any species other than bellyache bush. The test plants were then kept, with the dead larvae for an additional 2-3 weeks while development proceeded on *J. gossypiifolia.* In no cases did these exploratory mines cause leaf death, leaf drop or leaf colour change. Figure 22 demonstrates that the exploratory mines affected only the surface layers of the leaves. There is no evidence of mines on the other side of the leaves.

Figure 19 – Adaxial and abaxial surface of the same* Croton verreauxii *leaf (above) and* Coffea arabica *leaf (below), demonstrating that the exploratory mines by 1st instar* Stomphastis thraustica *(Peru population) larvae were restricted to the surface of the leaf. There is no evidence of the mines on the other side of the leaf.

Figure 20 – Adaxial and abaxial surface of a* Jatropha gossypiifolia *leaf with single* Stomphastis thraustica *(Peru population) larval mine.

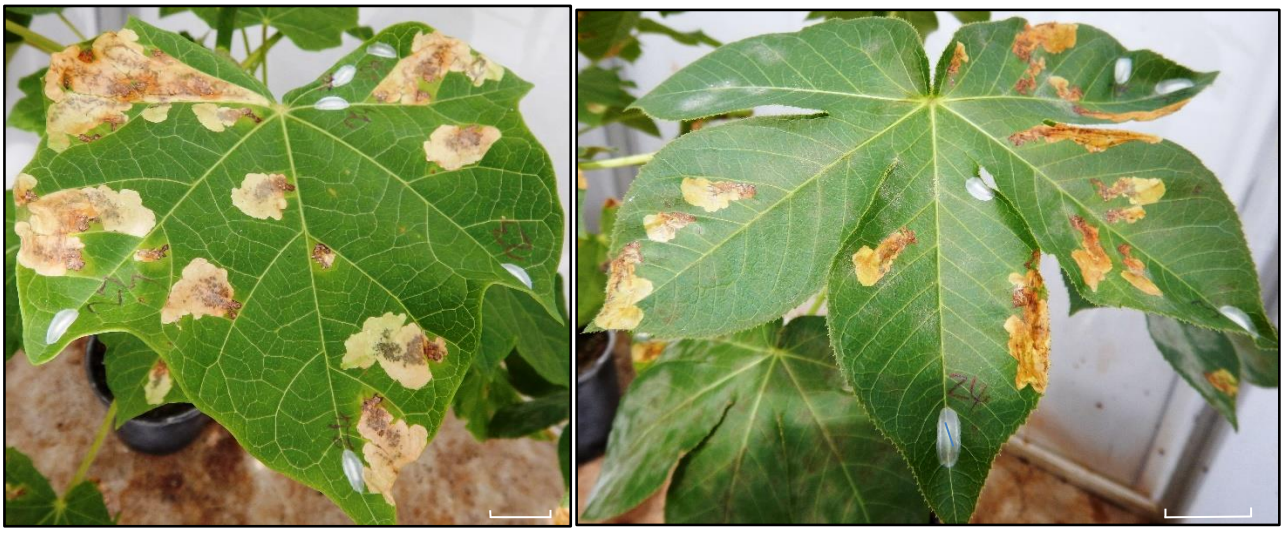

Figure 21 – Damage by* Stomphastis thraustica *(Peru population) on* Jatropha curcas *leaf (left) and* J. gossypiifolia *leaf (right).

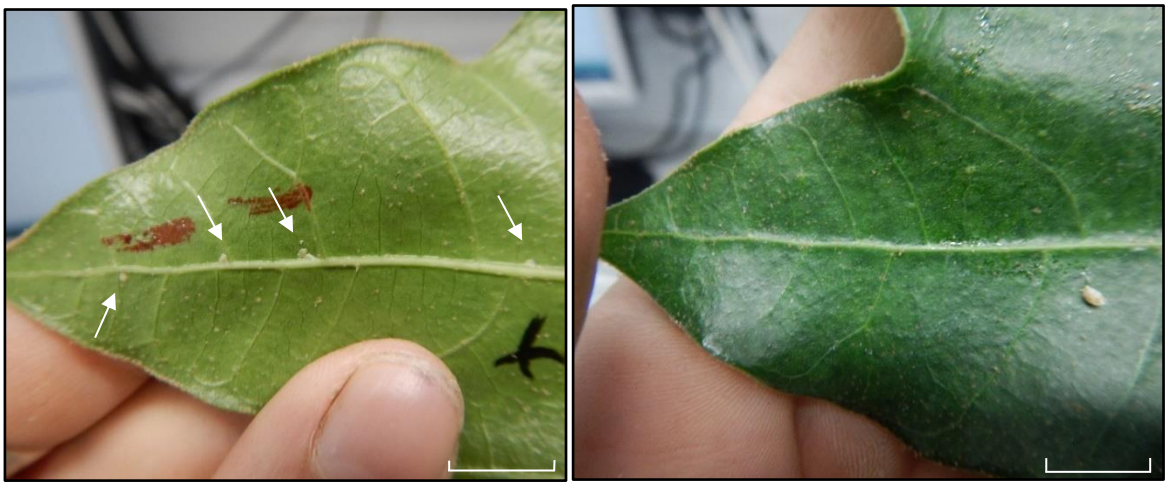

Figure 22 – Adaxial and abaxial surface of the same* Aleurites moluccanus *leaf with exploratory mines by first instar* Stomphastis thraustica *(Peru population) larvae.

When adult females were offered a choice of *J. gossypiifolia*, *Actephila lindleyi, Bridelia exaltata, Breynia oblongifolia* and *Cleistanthus hylandii* plants, 98.6 ± 0.9% of eggs were laid on the target plant. This was significantly more than the 1.4 ± 0.9% (six eggs in total) that were laid on the non-target plants *($F_{4,20}$* = 681.56; *P<0.001*; Table 9). No feeding by first instar larvae was detected on non-target plants in this trial. When adult females were offered a choice of *J. gossypiifolia, A. ilicifolia, Antidesma ghaesembilla* and *Breynia cernua* plants, one egg was laid on one *A. ilicifolia* plant in one replicate. Significantly more eggs were laid on *J. gossypiifolia* than the non-target plants ($F_{3,8}$ = 838.78, *P< 0.001*; Table 9*).* No feeding by first instar larvae was detected on non-target plants in this trial. When adult females were offered a choice of *J. gossypiifolia*, *J. multifida, J. podagrica* and *R. communis*, an average of 86% of eggs were laid on the target plant; significantly greater than the percentage laid on the non-target species ($F_{3,16}$ = 107.70, *P*< 0.001; Table 9). No larval development occurred on any species other than bellyache bush. In no cases did these exploratory mines cause leaf death, or leaf drop. In the other choice oviposition trials, no eggs were laid on any of the non-target plants (Table 9).

3.3.4 Multiple-choice oviposition test, without *J. gossypiifolia*

When adult females were offered a choice of the non-target species *Alchornea ilicifolia, Antidesma bunis, Baloghia inophylla, Bridelia exaltata, Croton insularis*, and *Omphalea celata* in a large walk-in cage, no eggs were laid on any of the plants in any of the five replicates. It is possible that eggs were laid on the cage walls but due to the small size of the eggs cage walls were not inspected.

Table 9. Results from choice oviposition tests conducted with* Stomphastis thraustica *(Peru population) and the target* J. gossypiifolia*.

Trial	Species	Mean proportion of eggs laid[#]	Mean number of eggs laid	Larval development
1	*Jatropha gossypiifolia*	0.90 ± 0.02[a]	246 ± 31	Y
	Aleurites moluccanus	0.05 ± 0.02[b]	16 ± 7	N
	Baloghia inophylla	0.03 ± 0.01[b]	6 ± 2	N
	Croton verreauxii	0.02 ± 0.01[b]	7 ± 2	N
2	*Jatropha gossypiifolia*	1.00 ± 0.00	79 ± 20	Y
	Camellia sinensis	0.00 ± 0.00	0 ± 0	N
	Coffea arabica	0.00 ± 0.00	0 ± 0	N
3	*Jatropha gossypiifolia*	0.99 ± 0.00[a]	83 ± 14	Y
	Actephila lindleyi	0.00 ± 0.00[b]	0 ± 0	N
	Breynia oblongifolia	0.00 ± 0.00[b]	0 ± 0	N
	Bridelia exaltata	0.01 ± 0.00[b]	1 ± 1	N
	Cleistanthus hylandii	0.00 ± 0.00[b]	0 ± 0*	N
4	*Jatropha gossypiifolia*	0.99 ± 0.01[a]	41 ± 4	Y
	Alchornea ilicifolia	0.01 ± 0.01[b]	0 ± 0*	N
	Antidesma ghaesembilla	0.00 ± 0.00[b]	0 ± 0	N
	Breynia cernua	0.00 ± 0.00[b]	0 ± 0	N
5	*Jatropha gossypiifolia*	1.00 ± 0.00	67 ± 12	Y
	Antidesma bunius	0.00 ± 0.00	0 ± 0	N
	Euphorbia plumerioides	0.00 ± 0.00	0 ± 0	N
	Omphalea celata,	0.00 ± 0.00	0 ± 0	N
6	*Jatropha gossypiifolia*	1.00 ± 0.00	67 ± 11	Y
	Dodonaea triquetra	0.00 ± 0.00	0 ± 0	N
	Euphorbia tannensis	0.00 ± 0.00	0 ± 0	N
	Glochidion ferdinandi	0.00 ± 0.00	0 ± 0	N
7	*Jatropha gossypiifolia*	0.86 ± 0.02[a]	41 ± 8	Y
	Jatropha multifida	0.11 ± 0.03[b]	6 ± 2	N
	Jatropha podagrica	0.03 ± 0.03[bc]	1 ± 1	N
	Ricinus communis	0.00 ± 0.00[c]	0 ± 0	N
8	*Jatropha gossypiifolia*	1.00 ± 0.00	55 ± 6	Y
	Aleurites moluccanus	0.00 ± 0.00	0 ± 0	N
	Baloghia inophylla	0.00 ± 0.00	0 ± 0	N
	Croton insularis	0.00 ± 0.00	0 ± 0	N
	Drypetes deplanchei	0.00 ± 0.00	0 ± 0	N

[#]Different letters within a trial represent values that are statistically significantly different at $P < 0.05$; *One egg was laid on each of these species in one replicate.

3.4 Discussion

Host specificity testing has confirmed that *Stomphastis thraustica* (Peru population) is highly host specific and suitable for release in Australia. Across all of the host specificity testing trials, larval development was only possible on *J. gossypiifolia* and *J. curcas.* None of the other species tested were suitable hosts, as demonstrated by test results. In Peru, *Stomphastis thraustica* was observed on *J. gossypiifolia* and *J. curcas*, so complete development on *J. curcas* was not unexpected. It is not a hindrance to the moth being released for the biological control of *J. gossypiifolia* in Australia. *Jatropha curcas* has invaded parts of northern Australia. It is a declared weed in some states and is an approved target for biological control (Taylor et al. 2017; Biosecurity Queensland 2016). Utilisation of this species in the field by *S. thraustica* (Peru population) would thus be beneficial. *Jatropha curcas* was the only congener of three tested to support any development of the insect past the first instar.

Stomphastis thraustica (Peru population) laid eggs on many of the test plants during no-choice trials. This is neither unexpected nor concerning. Insects often oviposit on non-target test plants under confined, no-choice conditions. Lepidopterans in particular are known to display indiscriminate oviposition when placed into confined conditions (e.g. Heard 2000). The eggs laid on the test species by *S. thraustica* (Peru population) females in the no-choice trials is therefore an artefact of the laboratory conditions (i.e. a false positive result). In a no-choice situation, where females have no access to a suitable host, they have no choice but to lay eggs on the test plant. When larger cages were used, virtually no eggs were laid on native plant species. The likelihood of *S. thraustica* (Peru population) laying eggs on a non-target plant in the field, where they are able to express their full host selection behaviour, is very small. Indeed, in choice trials very few eggs were laid on non-target plants. It is possible that a moth may accidentally lay the occasional egg on a non-target species growing amongst *J. gossypiifolia* in the field, but nowhere near the numbers encountered in the no-choice trials.

Hatching of a larva from an egg only requires resources within egg. Subsequent larval development requires resources obtained from a suitable host plant. In this context, larval feeding and development, rather than eggs laid, is the most appropriate measure of risk to non-target plants. On some of the species on which eggs were laid in the no-choice trials, newly emerged *S. thraustica* (Peru population) larvae attempted to feed, but in all cases (except *J. curcas),* the test plants were not suitable as hosts and the larvae died without developing further. Of the hundreds of larvae that attempted to feed on the non-target plants, none could utilise these species as a host, including congeners *J. podagrica* and *J. multifida*. Newly emerged larvae produce tiny surface mines as they feed; 0.5 mm across. Figures 18, 20 and 21 show that these mines affect only the surface layers of the leaves – there is no evidence of the mines on the other side of the leaves. Even with an artificially high number of exploratory mines (for reasons explained above), in no cases did leaf drop and or senescence occur, and test plants remained healthy. Irrespective of the number of eggs laid during no-choice trials, the act of oviposition is not damaging in itself, so egg lay should not be concerning unless it results in significant feeding damage and development of hatching immatures (Hill 1999), and in the case of *S. thraustica* (Peru population), it did not. Rapid death of newly emerged larvae without further development is further evidence that species other than *J. curcas* are not at risk of attack by *S. thraustica* (Peru population). In a comparable example, a water hyacinth (*Eichhornia crassipes*) weevil *Neochetina bruchi* fed to some extent on 50 species and laid eggs on 22 species during host specificity testing, and a second weevil (*N. eichhorniae)* attempted to feed on 25 plant species and oviposited on seven species. However, both only completed development only on water hyacinth (Julien *et al*. 1999). Both weevils have been released around the world and no non-target attack has been recorded under natural field conditions (Julien *et al.* 1999).

In the no-choice trials there was a high level of variability in the number of eggs laid between replicates for a given species, both for the test species and the target in the no-choice trials. This can be attributed to the

fact that unsexed adults were used. For the test species, there may have been anywhere from zero to 20 females in each replicate. The probability of zero females in a replicate is extremely small (0.000001 where 20 individuals were released and 0.001 where 10 individuals were released). Over five replicates 100 adults were released on to most of the test species. Even on the target, which only had 10 adults released onto each plant, egg numbers ranged from eight to 380.

During the host testing of *S. thraustica* (Peru population) no larval development was possible on species other than the target and *J. curcas*. Even if an agent completes development on a non-target species during host testing (which are conducted under ideal conditions), the ability of the agent to successfully colonise the plant in the field may still be limited. A Gracillariidae moth approved for release in Australia, *Neurostrota gunniella* (Busck), laid eggs on many of the test species during quarantine host testing (Davis *et al.* 1991). It completed development on five closely related non-target species. On the remaining species "larvae died in the first instar after mining in a few pinnules (leaflets)", which was considered an artefact of testing (Davis *et al.* 1991). Only the species that supported complete development were considered to be potential hosts in the field, but due to high mortality these species were considered to be suboptimal hosts that would only be attacked when occurring in close proximity to the target. Indeed, in the field the non-target attack of the native *Neptunia major* was restricted to populations occurring within the vicinity of the target weed (Taylor *et al.* 2007).

As mentioned above, *S. thraustica* (Peru population) is not the first Gracillariidae moth to be utilised for weed biological control in Australia. Released against *Mimosa pigra* L. in the Northern Territory, *Neurostrota gunniella* dispersed rapidly following its release and is now present wherever *M. pigra* occurs in the Northern Territory (Wilson and Flanagan 1990; Wilson and Forno 1995). Gracillariidae are known to be good dispersers. It is expected that *Stomphastis thraustica* (Peru population) will also be an adept disperser, a desirable characteristic given the expansive areas across which bellyache bush occurs.

These test results provide strong evidence that *S. thraustica* (Peru population) is highly host specific and is suitable for release in Australia. The high level of host specificity, short generation time and projected rapid dispersal bodes well for its future as a biological control agent.

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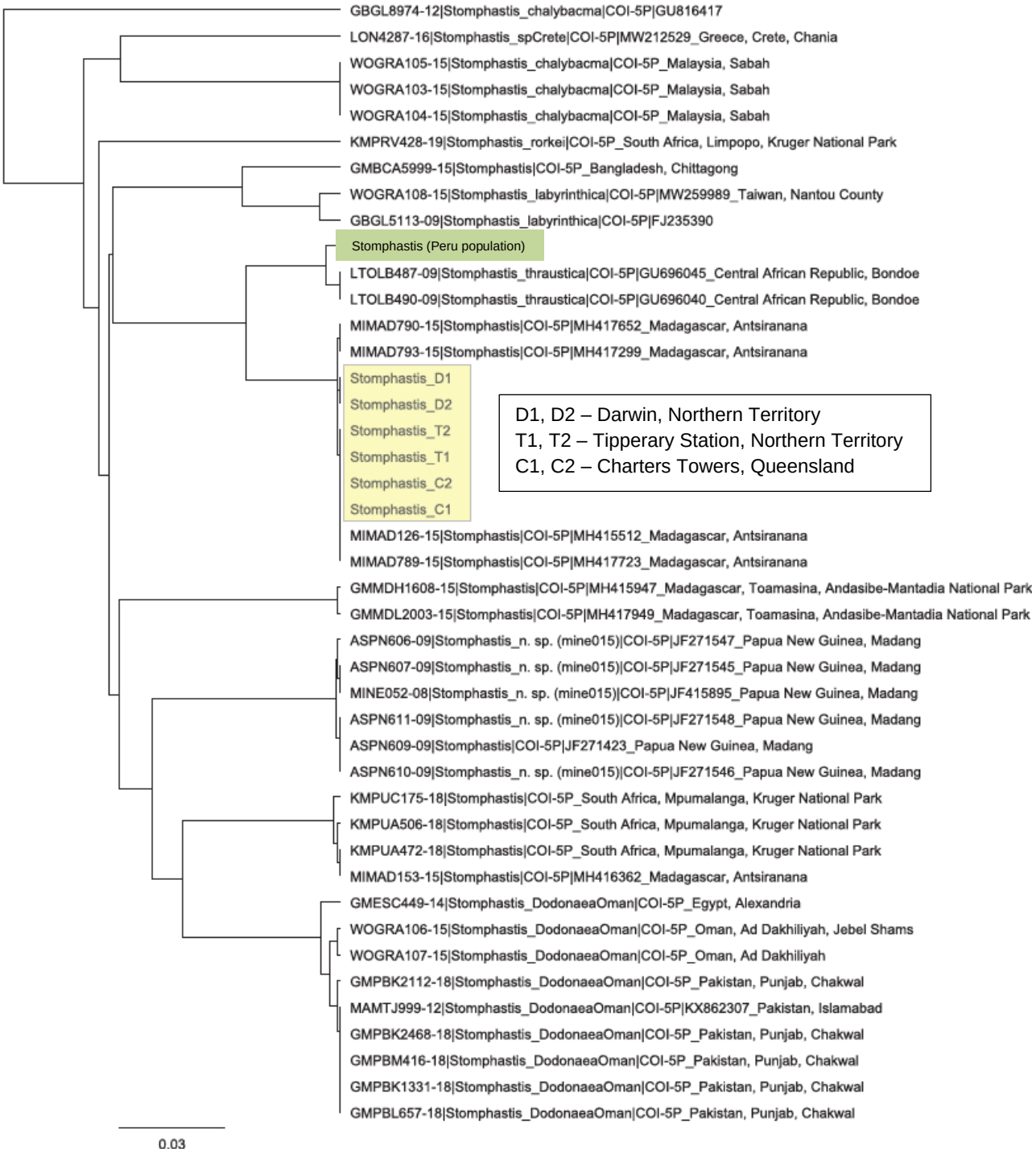
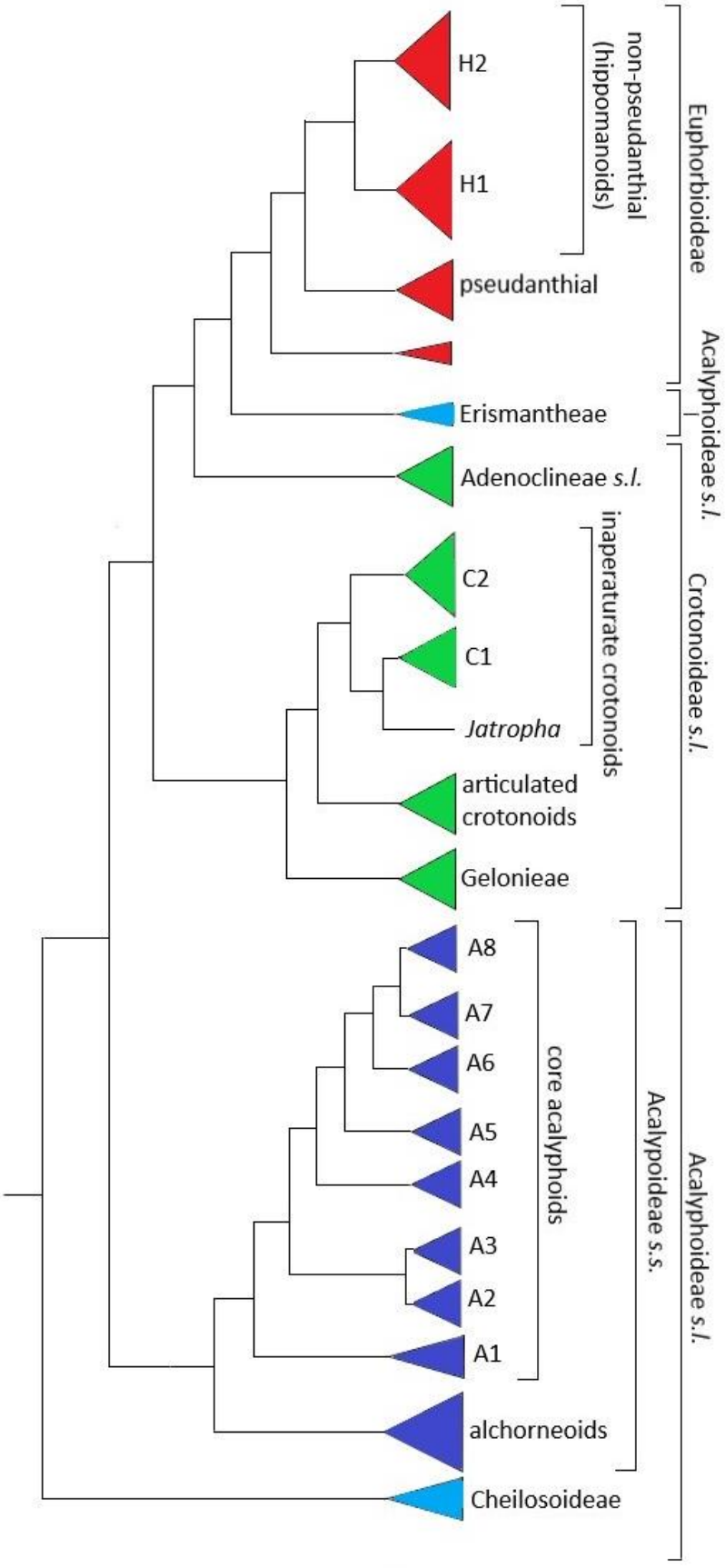
Appendix 1. *Stomphastis* genetics tree showing the quarantine population from Peru (green) and samples from Australia (yellow) compared with samples lodged with GenBank.



Appendix 2. Schematic representation of relationships within Euphorbiaceae s.s. based on Wurdak *et al.* 2005



Appendix 3. Test plants from Euphorbiaceae *s.s.* grouped following old tribal and subfamily classification and following the relationships presented in Wurdack *et al.* 2005.

Old tribal and subfamily classification	Wurdack et al. 2005
Family	Family
Subfamily	Subfamily
Tribe	Clade
	Subclade
Euphorbiaceae	Euphorbiaceae
Crotonoideae	Crotonoideae
Jatropheae	Inaperturate crotonoids
***Jatropha gossypiifolia* L.**	C1
Jatropha curcas L.	***Jatropha gossypiifolia* L.**
Jatropha podagrica Hook.	*Jatropha curcas* L.
Jatropha multifida L.	*Jatropha podagrica* Hook.
Adenoclineae	*Jatropha multifida* L.
Endopermum sp.	*Croton acronychioides* F.Muell.
Aleuritideae	*Croton insularis* Baill.
Aleurites sp.	*Croton verreauxii* Baill.
Codiaeae	C2
Baloghia inophylla (G.Forst.) P.S.Green	*Aleurites* sp.
Codiaeum variegatum (L.) A.Juss.	*Baloghia inophylla* (G.Forst.) P.S.Green
Crotoneae	*Codiaeum variegatum* (L.) A.Juss.
Croton acronychioides F.Muell.	*Beyeria lechenaultii* (DC.) Baill.
Croton insularis Baill.	*Beyeria viscosa* (Labill.) Miq.
Croton verreauxii Baill.	*Ricinocarpos pinifolius* Desf.
Manihoteae	Articulated crotonoids
Manihot esculentum Crantz	*Manihot esculentum* Crantz
Manihot grahamii Hook.	*Manihot grahamii* Hook.
Ricinocarpeae	Adenoclineae s.l.
Beyeria lechenaultii (DC.) Baill.	*Endopermum sp.*
Beyeria viscosa (Labill.) Miq.	*Omphalea celata* P.I.Forst
Ricinocarpos pinifolius Desf.	Euphorbioideae
Acalyphoideae	Nonpseudanthial Euphorbioideae
Acalypheae	H1
Acalypha capillipes Müll.Arg	*Homalanthus populifolius* Graham
Macaranga tanarius (L.) Müll.Arg.	*Microstachys chamaelea* (L.) Hook.f.
Mallotus philippensis (Lam.) Muell.Arg.	Pseudanthial Euphorbioideae
Ricinus communis L.	*Euphorbia grantii* Oliv.
Alchorneae	*Euphorbia plumerioides* Teijsm. ex Hassk.
Alchornea ilicifolia (J.Sm.) Muell.Arg.	*Euphorbia pulcherrima* Willd. ex Klotzsch
Omphaleae	*Euphorbia tannensis* Spreng.
Omphalea celata P.I.Forst	*Euphorbia tithymaloides* L.
Euphorbioideae	Acalyphoideae
Euphorbieae	Acalyphoideae s.s.
Euphorbia grantii Oliv.	Core acalyphoid A4
Euphorbia plumerioides Teijsm. ex Hassk.	*Ricinus communis* L.
Euphorbia pulcherrima Willd. ex Klotzsch	Core acalyphoid A3
Euphorbia tannensis Spreng.	*Acalypha capillipes* Müll.Arg
Euphorbia tithymaloides L.	Core acalyphoid A1
Hippomaneae	*Macaranga tanarius* (L.) Müll.Arg.
Homalanthus populifolius Graham	*Mallotus philippensis* (Lam.) Muell.Arg.
Microstachys chamaelea (L.) Hook.f.	alchorneoids
	Alchornea ilicifolia (J.Sm.) Muell.Arg.

*There are no representative from Erismantheae or Cheilosoideae in Australia

Appendix 4. Source of plant species used in host specificity testing

Test plants	Status	Source
Malpighiales		
Euphorbiaceae		
Crotonoideae		
Jatropheae		
Jatropha gossypiifolia L.	Target	Field collected seed and cuttings; various locations (NQ, FNQ)
Jatropha curcas L.	I	Field collected seed and cuttings; various locations (NQ)
Jatropha podagrica Hook.	I	Various retail outlets
Jatropha multifida L.	I	Various retail outlets
Aleuritideae		
Aleurites sp.	N	Various retail outlets; Mt Coot-tha Botanic Gardens
Codiaeae		
Baloghia inophylla (G.Forst.) P.S.Green	N	Burringbar Rainforest Nursery (seed grown)/native plant sale
Codiaeum variegatum (L.) A.Juss.	NO	Various retail outlets
Crotonoideae		
Croton acronychioides F.Muell.	N	Burringbar Rainforest Nursery (seed grown)
Croton insularis Baill.	N	Yuruga Native Plant Nursery
Croton verreauxii Baill.	N	Burringbar Rainforest Nursery (seed grown)
Manihoteae		
Manihot esculentum Crantz	C	Various retail outlets
Manihot grahamii Hook.	I	Field collected – SEQ
Ricinocarpeae		
Beyeria lechenaultii (DC.) Baill.	N	Goldfields Revegetation Nursery
Beyeria viscosa (Labill.) Miq.	N	Plants of Tasmania Nursery and field collected (Queensland).
Ricinocarpos pinifolius Desf.	N	Goldfields Revegetation Nursery
Acalyphoideae		
Acalypheae		
Acalypha capillipes Müll.Arg	N	Toowoomba grower
Macaranga tanarius (L.) Müll.Arg.	N	Paten Park Native Nursery; Burringbar Rainforest Nursery (seed grown)
Mallotus philippensis (Lam.) Muell.Arg.	N	Various retail outlets
Ricinus communis L.	I	Field collected – SEQ
Alchorneae		
Alchornea ilicifolia (J.Sm.) Muell.Arg.	N	Paten Park Native Nursery; Burringbar Rainforest Nursery (seed grown)
Adenoclineae		
Endopermum sp.	N	Field collected
Omphaleae		
Omphalea celata P.I.Forst	N	Sourced from Mackay Regional Botanic Gardens in 2007
Euphorbioideae		
Euphorbieae		
Euphorbia grantii Oliv.	I	Field collected – SEQ
Euphorbia plumerioides Teijsm. ex Hassk.	N	Cuttings sourced from a collector
Euphorbia pulcherrima Willd. ex Klotzsch	IO	Various retail outlets
Euphorbia tannensis Spreng.	N	Seed sourced from a collector
Euphorbia tithymaloides L.	I	Sourced from a collector
Hippomaneae		
Homalanthus populifolius Graham	N	Burringbar Rainforest Nursery (seed grown)

Microstachys chamaelea (L.) Hook.f.	N	Field collected – CQ
Phyllanthaceae		
Antidesmatoideae		
Antidesma bunius (L.) Spreng.	N	Burringbar Rainforest Nursery
Antidesma ghaesembilla Gaertn.	N	Field collected - Top End, N.T.
Phyllanthoideae		
Actephila lindleyi (Steud.) Airy Shaw	N	Burringbar Rainforest Nursery
Breynia cernua (Poir.) Mull.Arg.	N	Greening Australia Nursery - Darwin
Breynia oblongifolia (Mull.Arg.) Mull.Arg	N	Burringbar Rainforest Nursery (seed grown)
Bridelia exaltata F. Muell.	N	Various retail outlets
Cleistanthus hylandii Airy Shaw	N	Go Green Native Nursery
Flueggea virosa (Willd.) Voigt	N	Various retail outlets
Glochidion ferdinandi (Muell.Arg.) F.M.Bailey	N	Various retail outlets
Glochidion sp. 'Gunn Point'	N	Field collected - Top End, N.T.
Phyllanthus cuscutiflorus S.Moore	N	Go Green Native Nursery and Burringbar Rainforest Nursery
Picrodendraceae		
Austrobuxus swainii (Beuzev. & C.T.White) Airy Shaw	N	Burringbar Rainforest Nursery (seed grown)
Dissiliaria baloghioides F.Muell. ex Baill.	N	Barung Landcare Nursery
Petalostigma pubescens Domin	N	Various retail outlets
Sankowskya stipularis P.I.Forst.	N	Yuruga Native Plant Nursery
Putranjivaceae		
Drypetes deplanchei (Brongn. & Gris) Merr.	N	Burringbar Rainforest Nursery (seed grown)
Linaceae		
Linum marginale A.Cunn. ex Planch.	N	Plants of Tasmania Nursery
Fabales		
Fabaceae		
Caesalpinia pulcherrima (L.)Sw.	IO	Various retail outlets
Rosales		
Ulmaceae		
Trema tomentosa var. aspera (Brongn.) Hewson	N	Burringbar Rainforest Nursery (seed grown)
Sapindales		
Sapindaceae		
Dodonaea triquetra J.C.Wendl.	N	Burringbar Rainforest Nursery (seed grown)
Ericales		
Theaceae		
Camillea sinensis (L.) Kuntze	C	Multiple sources
Gentianales		
Rubiaceae		
Coffea arabica L.	C	Daley's Fruit Tree Nursery (multiple varieties)

Status: I – introduced; W – weed; O – ornamental; C – crop; N - native
CQ – Central Queensland; FNQ – Far North Queensland; NQ – North Queensland; SEQ – South-east Queensland; NT – Northern Territory

Appendix 5. Statistical test results

Aleurites muluccanus, Baloghia inophylla, Croton verreauxii* and *Jatropha gossypiifolia

Analysis of variance

Variate: C32

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
C31	3	14485.09	4828.36	171.60	<.001
Residual	16	450.20	28.14		
Total	19	14935.29			

Information summary

All terms orthogonal, none aliased.

Tables of means

Variate: C32

Grand mean 25.3

C31	Am	BB	Bi	Cv
	12.1	71.8	8.8	8.4

Standard errors of differences of means

Table	C31
rep.	5
d.f.	16
s.e.d.	3.35

Tukeys test

C31

	Mean	
Cv	8.39	a
Bi	8.76	a
Am	12.07	a
BB	71.80	b

Actephila lindleyi*, *Breynia oblongifolia, Cleistanthus hylandii, Bridelia exaltata* and *Jatropha gossypiifolia

Analysis of variance

Variate: C3

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
C1	4	25337.122	6334.280	681.56	<.001
Residual	20	185.877	9.294		
Total	24	25522.999			

Information summary

All terms orthogonal, none aliased.

Message: the following units have large residuals.

units 13	7.13	s.e.	2.73
units 23	-7.19	s.e.	2.73

Tables of means

Variate: C3

Grand mean 20.13

C1	Actephila	BELLYACHE	Breynia	Bridelia	Cleistanthus
	3.24	83.77	3.24	6.16	4.25

Standard errors of differences of means

Table	C1
rep.	5
d.f.	20
s.e.d.	1.928

Tukeys test

	Mean	
Actephila	3.24	a
Breynia	3.24	a
Cleistanthus	4.25	a
Bridelia	6.16	a
BELLYACHE	83.77	b

Antidesma ghaesembilla, Breynia cernua, Alchornea ilicifolia* and *Jatropha gossypiifolia

Analysis of variance

Variate: C13

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
C11	3	14034.254	4678.085	838.78	<.001
Residual	8	44.618	5.577		
Total	11	14078.872			

Information summary

All terms orthogonal, none aliased.

Message: the following units have large residuals.

units 11 -3.92 s.e. 1.93

Tables of means

Variate: C13

Grand mean 24.87

C11	Alchornea	Antidesma	BELLYACHE	Breynia
	6.39	4.49	84.08	4.49

Standard errors of differences of means

Table	C11
rep.	3
d.f.	8
s.e.d.	1.928

Tukeys test

C11

	Mean	
Antidesma	4.49	a
Breynia	4.49	a
Alchornea	6.39	a
BELLYACHE	84.08	b

Jatropha multifia, Jatropha podagrica, Ricinus communis* and *Jatropha gossypiifolia

Analysis of variance

Variate: C22

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
C21	3	16459.69	5486.56	134.87	<.001
Residual	20	813.60	40.68		
Total	23	17273.28			

Information summary

All terms orthogonal, none aliased.

Message: the following units have large residuals.

units 10	-12.7	s.e.	5.8
units 16	15.1	s.e.	5.8

Tables of means

Variate: C22

Grand mean 24.8

C21	Jatriopha multifida	Jatropha gossypiifolia	Jatropha podagrica
	17.2	69.4	8.3

C21	Ricinus communis
	4.2

Standard errors of differences of means

Table	C21
rep.	6
d.f.	20
s.e.d.	3.68

Tukeys test

Ricinus communis	4.25	a
Jatropha podagrica	8.31	ab
Jatriopha multifida	17.18	b
Jatropha gossypiifolia	69.42	c