Core funded aligned research on insects and fungi on species other than radiata pine 2017/2018

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Introduction

Plantation species other than *Pinus radiata* (radiata pine), specifically Douglas-fir and eucalypts, form an important part of a diversified forest estate. Douglas-fir is planted over approx. 105,000 ha. and is the most widely planted tree species after radiata pine. *Eucalyptus nitens* is planted over approximately 14,000 ha and contributes ~$40 million pa in hardwood chip exports. Throughout New Zealand the biggest threat to *E. nitens* plantations is from the eucalyptus tortoise beetle, *Paropsis charybdis*. In addition, the recent arrival of myrtle rust may pose further challenges to the *Eucalyptus* forestry industry, although no evidence of damage in planted forests has been found in Australia to date.

Eucalyptus and *Paropsis*

To manage pest populations of *Paropsis charybdis*, chemical control with aerial spraying of insecticides occurs in up to a quarter of large plantations annually. However, the costs associated with aerial spraying are prohibitive for many growers and a major barrier to increasing eucalypt plantations. Other undesirable outcomes, such as environmental and ecological harm and risking FSC certification could also result from long-term use of chemical insecticides. An alternative approach to insecticides is using classical biological control.

In the case of *Paropsis charybdis*, four potential agents have been introduced already in previous decades, but only two of these have been helpful, so a more effective control is needed. A promising new agent is the Australian parasitoid *Eadya daenerys* (Hymenoptera: Braconidae). Host-testing of non-target species against this parasitoid in a quarantine facility at Scion has been completed and we are now ready to present these findings and apply to the EPA for the release of *Eadya* in New Zealand.

Douglas-fir and Swiss needle cast (SNC)

Generous genetic resources of Douglas-fir exist in New Zealand and breeding objectives through Diverse Forests have targeted improved growth, form, resistance to SNC, and wood stiffness. However, SNC remains a problem as this disease is the major limiting factor for growing Douglas-fir more widely in the Central North Island.

Since the late 2000s, Scion and Oregon State University have been undertaking collaborative research into factors affecting SNC on Douglas-fir in New Zealand. As part of this work a large number of isolates of *Nothophaeocryptopus gaeumannii* (the fungus that causes SNC) were collected by Prof. Stone, OSU and Scion. We are now in a position to publish new knowledge of the pathogen populations compared regionally within NZ and overseas.

Funding and outcomes

Core funding (now known as Strategic Science Investment Fund or SSIF) of $150K pa to the Forest Protection team is used to undertake research on insects or fungi on *Eucalyptus* and Douglas-fir, and is included as the Scion contribution to the SWP research partnership. This milestone report will summarise the research undertaken in 2017-2018.

The research summarised here is broken down into three objectives, the outputs of which are supplied herein to SWP. The first objective relates to the biocontrol of *Paropsis*, the second to SNC and the third to myrtle rust incursion.

Progress results and discussion

Objective One

Undertaking a project team meeting to approve EPA application content, completing host specificity testing on non-target beetles, completing mass rearing of the current generation of *Eadya* in quarantine, engaging with the public and Māori, reporting the results to industry, and lodging an application (if appropriate) with the EPA to release *Eadya*.

The NZ Farm Forestry Association led project "Improved Biological Control of *Paropsis* charybdis" made great progress this financial year. The parasitoid wasp *Eadya daenerys* remains a strong candidate for being considered as safe to release as a biological control agent for eucalyptus tortoise beetle in New Zealand. Host range testing has been completed for nine non-target chrysomelid beetles, including a native species. Host-specificity testing is arguably the most important aspect of assessing the risk of a potential biological control agent to the New Zealand environment. A technical note summarising the results has been produced and is attached below. Most significantly, the physiological host range of *Eadya daenerys* in New Zealand is limited to *Paropsis charybdis* and *Trachymela sloanei*, both pest paropsine beetles, but cocoons produced from *T. sloanei* were very small, indicating that this species may not be a viable host in the field. In addition, observations of adult female *Eadya* behaviour revealed the parasitoids sometimes attempted to attack other non-target beetle larvae. This has been observed only within the artificial confines of a petri dish, and does not mean the parasitoid will do this in the wild. Four other non-target Chrysomelinae species were incomplete physiological hosts (internal parasitism) but Eadya was unable to emerge. No *Eadya* offspring were produced from any of the beneficial beetles (the weed biocontrol agents) exposed to *Eadya*, even though some of them are relatively closely related to tortoise beetles, especially *Chrysolina*, the Tutsan leaf beetle, and *Gonioctena*, the broom leaf beetle. The native veronica leaf beetle, *Allocharis* nr. *tarsalis* was also infrequently attacked, and the impact of being stung prevented these sub-alpine beetles from proceeding to pupation. Climatic modelling may help to establish whether there is any likelihood that *E. daenerys* will ever reach sub-alpine zones to cause any impact on these natives beetles. It is most likely that attack on non-target species will be limited to eucalyptus-feeding paropsine beetles.

In the last year a number of publications have been produced that are directly related to the objective outlined here. First, a manuscript describing and revising *Eadya daenerys* species has been accepted for publication (see abstract and citation below). This has resulted in a name change for our focal *Eadya* species, from *E. paropsidis* to *E. daenerys*. We have checked all of the *Eadya* we have retained from host testing, and as expected all of these were found to be *E. daenerys*. A second manuscript on the molecular phylogeny of *Eadya* in relation to host identity has also been accepted for publication in Plos One (see abstract and citation below). This confirms the field host range of *Eadya daenerys* in Tasmania as *Paropsisterna agricola, Paropsisterna nobilitata, Paropsisterna bimaculata*, and *Paropsis charybdis*.

An unexpected outcome of the work relates to another fairly recent incursion into New Zealand of the Eucalyptus variegated beetle (EVB), *Paropsisterna variicollis*. We can now confirm *Eadya annleckiae* Ridenbaugh 2018 should be examined in future research for its potential to control *Paropsisterna variicollis* in New Zealand and *Paropsisterna selmani* in Ireland, as its field host range appears to encompass these two beetle species.

Challenges

The milestone to complete an application to the EPA for the release of *Eadya* is currently 95% complete, but the expected completion date of 29 June 2018 may be missed. The current projection is that this application will be completed in July.

The Paropsis biocontrol project continues to struggle with rearing *Eadya* in quarantine and we have adapted to this by changing our proposed release strategy for these parasitoids. We propose to release parasitized *Paropsis* larvae directly into *Eucalyptus* plantations in New Zealand so they can spin their own cocoons in the soil. This should negate the problems captive *Eadya* had in spinning adequate cocoons in artificial quarantine conditions, and may also reduce the male-bias we had with first-generation captive *Eadya*.

Objective Two

Complete data analysis on 1250 genotyped isolates to obtain the geographic molecular diversity of *N. gaeumannii*, and to produce a publication presenting the results of this work in an internationally recognised journal.

All analyses on this objective (SNC) have been completed by Ian Hood and collaborators. A manuscript from this work has been written and is currently under consideration for publication at the international journal Phytopathology (see citation and abstract below). The main findings relate to the genetic differentiation of *N. gaeumannii* isolates in the North and South Island, which were weakly differentiated. This suggests that gene flow has occurred between the North and South Island's *N. gaeumannii* populations, and between the local *N. gaeumannii* populations within each island. Furthermore eighteen isolates of *N. gaeumannii* Lineage 2, which has previously been reported only from western Oregon, were recovered from two sites in the North Island and four sites in the South Island. The most likely explanation for the current distribution of *N. gaeumannii* in New Zealand is that it was introduced on infected live seedlings through the forestry or ornamental nursery trade.

Objective Three (extra)

The incursion of myrtle rust to New Zealand poses a risk to New Zealand's *Eucalyptus* industry. Scion has used SSIF funding to undertake an extra third objective, research into the susceptibility and risk of myrtle rust to New Zealand *Eucalyptus* by undertaking the following:
Identification of the species of *Eucalyptus* that myrtle rust was found on in Kerikeri, New Zealand – *Eucalyptus globoidea*. This identification was difficult due to the age of the seedling and the close phylogenetic relationship to other *Eucalyptus* species in New Zealand. It involved substantial molecular testing beyond morphological identification and the Myrtaceae barcode database to identify ($10K SSIF project). This finding may have repercussions for the NZ DFI as *Eucalyptus globoidea* is one of the top species of interest for producing a future ground-durable hardwood pole industry. Furthermore, E. *globoidea* is one of the few species of interest to the NZ DFI that shows complete resistance to the Eucalyptus variegated beetle, EVB. The next stage of research will tell us more about the risks myrtle rust poses to this industry. Observations in Australia are encourage, with no reported damage to planted eucalypt forests.

Preliminary stages of testing *Eucalyptus* species against the myrtle rust strain present in New Zealand, as well the *Eucalyptus* strain in South America have been initiated. This has involved Scion pathologist Dr Beccy Ganley travelling to Uruguay to meet with Uruguayan and Brazilian researchers to develop collaborations for testing industry relevant strains of *Eucalyptus* against the strain of myrtle rust causing issues in *Eucalyptus* in both countries. This strain is different from the ones present in New Zealand, Australia and other countries worldwide, which is known as the 'pandemic strain'.

Next financial year will involve obtaining and germinating seed from industry relevant *Eucalyptus* species for testing. Material will be tested against the eucalyptus strain and the pandemic strain.

We will also be continuing collaborations with South African researchers to gain insight into the risk the strain of myrtle rust present in South Africa poses to their *Eucalyptus* spp. industry. So far this strain has not been found on *Eucalyptus* and only on *Metrosideros.*

Outputs

Ridenbaugh RD, Barbeau E, Sharanowski BJ (2018) Description of four new species of *Eadya* (Hymenoptera, Braconidae), parasitoids of the *Eucalyptus* Tortoise Beetle (*Paropsis charybdis*) and other *Eucalyptus* defoliating leaf beetles. Journal of Hymenoptera Research. https://doi.org/10.3897/jhr.@@.24282

Abstract
Eucalyptus L'Heritier, 1789 (Mrytales: Myrtaceae) plantations are a global economic resource with a wide array of uses. As this forestry crop grows in popularity around the world, the exotic introduction of pests such as the leaf beetles belonging to the genera *Paropsis* Oliver, 1807 and *Paropsisterna* Motschulsky, 1860 increases in frequency. These pest introductions have spurred a need to understand the natural enemies of these pests for use in classical biological control programs. One such enemy, *Eadya paropsidis* Huddleston & Short, 1978 (Hymenoptera: Braconidae), has shown potential as a biological control agent against *Paropsis charybdis*, an exotic pest of New Zealand *Eucalyptus* plantations. However, observations made by biocontrol researchers have raised concerns that *E. paropsidis* is a complex of cryptic species. A comprehensive large-scale phylogenetic study utilizing both host and molecular data (Peixoto et al. 2018), as well as a morphological multivariate ratio analysis, was utilized (Peixoto et al. 2018) to ensure accurate delimitation of the species of *Eadya*. Here we formally describe the three new species (*Eadya annleckieae* Ridenbaugh, 2018, **sp. n.**, *Eadya daenerys* Ridenbaugh, 2018, **sp. n.**, *Eadya spitzer* Ridenbaugh, 2018, **sp. n.**), and one additional new species discovered in the Australian National Insect Collection (*Eadya duncan* Ridenbaugh, 2018, **sp. n.**). All distributions and host associations for *Eadya* are listed as well as a redescription of the originally described *E. paropsidis* and *E. falcata*. An illustrated key to all known species is included to assist biological control researchers. The value of citizen science observations is discussed, along with the need for a further understanding of mainland *Eadya* populations given the recent spread of paropsine pests. Finally, we discuss the subfamilial placement of *Eadya*, and suggest it belongs within Euphorinae based on morphological characters.

Peixoto L, Allen GR, Ridenbaugh RD, Quarrell S, Withers TM, Sharanowski BJ (2018) When taxonomy and biological control researchers unite: species delimitation of *Eadya* parasitoids (Braconidae) and consequences for classical biological control of invasive paropsine pests of *Eucalyptus*. Plos One in press.

Abstract
The invasive Eucalyptus Tortoise Beetle, *Paropsis charybdis*, defoliates plantations of *Eucalyptus nitens* in New Zealand. Recent efforts to identify host specific biological control agents (parasitoids) from Tasmania, Australia, have focused on the larval parasitoid wasp, *Eadya paropsidis* (Braconidae), first described in 1978. In Tasmania, *Eadya* has been reared from *Paropsisterna agricola* (genus abbreviated *Pst*.), a smaller paropsine that feeds as a larva on juvenile rather than adult foliage of *Eucalyptus nitens*. To determine which of the many paropsine beetle hosts native to Tasmania are utilized by *E. paropsidis*, and to rule out the presence of cryptic species, a molecular phylogenetic approach was combined with host data from rearing experiments from multiple locations across six years. Sampling included 188 wasps and 94 beetles for molecular data alone. Two mitochondrial genes (COI and Cytb) and one nuclear gene (28S) were analyzed to assess the species limits in the parasitoid wasps. The mitochondrial genes were congruent in delimiting four separate phylogenetic species, all supported by morphological examinations of *Eadya* specimens collected throughout Tasmania. *Eadya paropsidis* was true to the type description, and was almost exclusively associated with *Pst. tasmanica*. A new cryptic species similar to *E. paropsidis*, *Eadya* sp. 3, was readily reared from *Pst. agricola* and *P. charybdis* from all sites and all years. *Eadya* sp. 3 represents the best candidate for biological control of *P. charybdis* and was determined as the species undergoing host range testing in New Zealand for its potential as a biological control agent. Another new species, *Eadya* sp. 1, was morphologically distinctive and attacked multiple hosts. The most common host was *Pst. variicollis*, but was also reared from *Pst. Nobilitata* and *Pst. selmani*. *Eadya* sp. 1 may have potential for control against *Pst. variicollis*, a new incursion in New Zealand, and possibly *P. selmani* in Ireland. Our molecular data suggests that *Pst. variicollis* is in need of taxonomic revision and the geographic source of the beetle in New Zealand may not be Tasmania. *Eadya* sp. 2 was rarely collected and attacked *Pst. aegrota* elliotti and *P. charybdis*.

Most species of Eadya present in Tasmania are not host specific to one beetle species alone, but demonstrate some host plasticity across the genera *Paropsisterna* and *Paropsis*. This study is an excellent example of collaborative phylogenetic and biological control research prior to the release of prospective biological control agents, and has important implications for the *Eucalyptus* industry worldwide.

Bennett PI, Hood IA, Stone JK (2018) The genetic structure of populations of the Douglas-fir Swiss needle cast fungus *Nothophaeocryptopus gaeumannii* in New Zealand. Phytopathology (in review).

Abstract
Swiss needle cast is a foliar disease of Douglas-fir that results in premature foliage loss and reduced growth. The causal fungus, *Nothophaeocryptopus gaeumannii,* was first detected in New Zealand in 1959 and spread throughout the North and South Islands over the following decades. The contemporary genetic structure of the *N. gaeumannii* population in New Zealand was assessed by analyzing 468 multilocus SSR genotypes (MLGs) from 2,085 *N. gaeumannii* isolates collected from 32 sites in in the North and South Islands. Overall diversity was lower than that reported from native *N. gaeumannii* populations in the northwestern U.S., which was expected given that *N. gaeumannii* is introduced in New Zealand. Linkage disequilibrium was significantly higher than expected under random mating, suggesting that population structure is clonal. Populations of *N. gaeumannii* in the North and South Island were weakly differentiated, and the isolates collected from sites within the islands were moderately differentiated. This suggests that gene flow has occurred between the *N. gaeumannii* populations in the North and South Islands, and between the local *N. gaeumannii* populations within each island. Eighteen isolates of *N. gaeumannii* Lineage 2, which has previously been reported only from western Oregon, were recovered from two sites in the North Island and four sites in the South Island. The most likely explanation for the contemporary distribution of *N. gaeumannii* in New Zealand is that it was introduced on infected live seedlings through the forestry or ornamental nursery trade, as the fungus is neither seed borne nor saprobic, and the observed population structure is not consistent with a stochastic intercontinental dispersal event.

Appendix A

Technical Note

Date: 16 May 2018
Reference: Reference here

Technical Note

Laboratory tests to determine if an Australian wasp, *Eadya daenerys*, is suitable for biological control of the Eucalyptus tortoise beetle, *Paropsis charybdis*

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Summary: We conducted extensive laboratory host-range tests with female *Eadya daenerys* against the target pest *Paropsis charybdis* and also against nine other closely-related beetle species found in New Zealand. These allow us to predict how the Australian parasitoid might behave in New Zealand, and the possible consequences and potential risks it poses to other non-target beetle species.

Introduction

The Australian Eucalyptus tortoise beetle, *Paropsis charybdis,* is a major pest within New Zealand gum plantations. Present for over 100 years (Withers & Peters 2017), the pest has caused damage, defoliation, and sometimes death, to many gum trees throughout the country (Bain & Kay 1989). *Paropsis charybdis* finds some species of gum trees to be particularly palatable, especially *Eucalyptus. nitens* (shining gum), a species grown mainly for wood and pulp and paper making (Murphy & Kay 2000), and other *Eucalyptus* species being grown for ground durable-wood and lumber production (Lin et al. 2017). The pest is responsible for economic losses within the entire forest products industry.

To manage the pest population of *Paropsis charybdis*, chemical control with aerial spraying of insecticides occurs in up to a quarter of large plantations annually. However, the costs associated with aerial spraying are prohibitive for many growers and a major barrier to increasing eucalypt plantations (Withers et al. 2013). Other undesirable outcomes, such as environmental and ecological harm and risking FSC certification could also result from long-term use of chemical insecticides. An alternative approach to insecticides is using classical biological control.

Biological control, known also as biocontrol, exploits a naturally occurring process in which a natural enemy of a target pest is introduced to an area from which it is absent, to give long-term control of the

Eucalyptus tortoise beetle larvae, the target pest

Eadya daenerys, proposed biological control agent

This programme is funded by Scion …..

target pest. In the case of *Paropsis charybdis*, four potential agents have been introduced already in previous decades. Only two of these have been helpful, and a more effective control is needed. We were particularly interested in targeting the spring larval life stage, which currently goes largely unchecked. A promising agent that does target the feeding larval life stage is the native Australian parasitoid *Eadya daenerys* (Hymenoptera: Braconidae). (This was investigated under the name *Eadya paropsidis* but a name change has now been advised).

To understand how the Australian parasitoid might behave in New Zealand, and the possible consequences and potential risks on other non-target beetle species, we conducted extensive laboratory host-range tests with female *Eadya daenerys***.** Previously, this species has only ever been reared from eucalypt-feeding *Paropsis* and *Paropsisterna* tortoise beetles in Tasmania (Rice & Allen 2009). Tests were conducted against *Paropsis charybdis* and also against other closely-related beetle species found in New Zealand that *Eadya daenerys* may never have contacted before.

Selection of closely related beetles

Beetles were selected for host range testing based on how closely related they were to **the** Eucalyptus tortoise beetle, *P. charybdis,* as relatedness is the best predictor of risk**.** *Paropsis charybdis* is a leaf beetle in the family Chrysomelidae. More specifically, tortoise beetles belong to the subfamily Chrysomelinae and within that, the tribe Chrysomelini (Leschen & Reid 2004).

After careful consideration, our laboratory tests were confined to the species listed in Table 1 (Withers et al. 2015**)**. The two most closely-related beetle species to *P. charybdis* in New Zealand a**re** *Trachymela sloanei* and *Dicranosterma semipunctata*. These were used in tests to usefully inform and help to delimit the host range of *Eadya daenerys*. Both species are pests that have invaded New Zealand from Australia in recent decades, with the former feeding on *Eucalyptus* species and the latter feeding on *Acacia* trees, particularly blackwood.

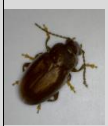
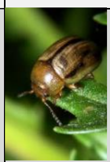
A native moderately-sized beetle, *Allocharis* near *tarsalis* (det. R. Leschen, Landcare Research, pers. comm 2018), active in spring feeding on the leaves of the subalpine shrub *Veronica* (*Hebe*) *albicans*, was collected from Kahurangi National Park in the vicinity of Mt. Peel (C. Wardhaugh, unpub. data, 2018). This was the only endemic species able to be located for host-range testing from the approximately 40 species of Chrysomelinae believed to exist in New Zealand. This species, being of moderate size and active in the spring made it very relevant to test, as most the other species are substantially smaller with some suspected to be nocturnal. Little is known about the other species and they are classified as “naturally uncommon”. Sufficient larvae of *Allocharis* were collected with iwi and DOC permission, and transported safely back to the laboratory to enable the full suite of host testing experiments with *Eadya*.

Chrysolina, the tutsan leaf beetle, and *Gonioctena olivacea***,** the broom leaf beetle, which both belong to the same beetle subfamily as the pest, were introduced to New Zealand as biological control agents for weed suppression (Hayes 2007). The former beetle was approved for release to biologically control tutsan, a serious weed in the North Island, but has not yet established in the field. The latter beetle has established as a biological control agent against scotch broom, a serious weed of roadside, productive and conservation lands in New Zealand. These two beetle species were included in our laboratory tests to **e**nsure that *Eadya daenerys* would not harm them and therefore reduce the effectiveness of their weed suppression ability.

Two species of weed biocontrol agents of the subfamily Galerucinae were also used in testing, the heather beetle (*Lochmaea suturalis)* and the alligator weed leaf beetle (*Agasicles hygrophila)*. These beetles are much smaller in body size than the tortoise beetles, but unlike our native Galerucines, some have leaf-feeding larvae that are active in spring. The heather beetle is established in the central North Island against the conservation weed heather (Ericaceae), and the alligator weed leaf beetle (*Agasicles hygrophila)* is a biocontrol agent for the semi-aquatic rooted alligator weed in Northland and the Bay of Plenty.

To ensure robust testing, we also tested two more species of more distantly-related leaf beetles that are also weed biocontrol agents with active, springtime, leaf feeding larvae. We chose to test the external feeding tradescantia leaf beetle (*Neolema ogloblini)* from the subfamily Criocerinae. It is established although not yet abundant, on tradescantia weed in some areas of the North Island. There are two more beneficial beetles in this subfamily that feed on tradescantia weed, but those larvae feed hidden inside the leaf and tip of the plant so we did not test them as we believe *Eadya daenerys* would not find them. We also tested the larvae of the green thistle leaf beetle (*Cassida rubiginosa*) established on pasture thistles, *Cirsium arvense*. It is in the subfamily Cassidinae. Interestingly, both these species of larvae carry a shield of frass (beetle poo) on their backs, called a faecal shield. We thought it possible the black colour of this shield may cause the larvae to be visually attractive to the *Eadya* wasps,

Table 1. The status of all the beetles (Coleoptera) used in the testing of *Eadya daenerys*. The darker the shading, the more closely related the beetle species is to the target pest *P. charybdis.*

	Target pest	Non-target beetles								
	Eucalyptus tortoise beetle	small tortoise beetle	blackwood tortoise beetle	veronica leaf beetle	tutsan leaf beetle	broom leaf beetle	heather beetle	alligator weed leaf beetle	green thistle leaf beetle	tradescantia beetle
Family	Chrysomelidae	Chrysomelidae	Chrysomelidae	Chrysomelidae	Chrysomelidae	Chrysomelidae	Chrysomelidae	Chrysomelidae	Chrysomelidae	Chrysomelidae
Sub-family	Chrysomelinae	Chrysomelinae	Chrysomelinae	Chrysomelinae	Chrysomelinae	Chrysomelinae	Galerucinae	Galerucinae	Cassidinae	Criocerinae
Tribe	Chrysomelini	Chrysomelini	Chrysomelini	Phyllocharitini	Gonioctenini	Gonioctenini	Luperini	**Alticini**	**Cassidini**	
Genus	*Paropsis*	***Trachymela***	***Dicranosterna***	***Allocharis***	***Chrysolina***	***Gonioctena***	***Lochmaea***	*Agasicles*	*Cassida*	*Neolema*
Species	*charybdis*	***sloanei***	***semipunctata***	**near *tarsalis***	*abchasica*	*olivacea*	*suturalis*	*hygrophila*	*rubiginosa*	*ogloblini*
					 Landcare Research	 Landcare Research		 Landcare Research	 Ben Sale	
Status	Pest	**Pest**	**Pest**	**NZ Native**	**Beneficial**	**Beneficial**	**Beneficial**	**Beneficial**	**Beneficial**	**Beneficial**
Size	7.5 x 11 mm	**5 x 8 mm**	**7 x 9 mm**	**3 x 7 mm**	**4 x 5.5 mm**	**3 x 4.5 mm**	**2.5 x 5.5 mm**	**2 x 5 mm**	**3 x 6 mm**	**2 x 5 mm**

since wild *Eadya* appear visually attracted to the young black larvae of their host in Tasmania.

To be at risk of exposure to *Eadya daenerys*, larvae of non-target beetles need to feed during the daytime externally on the leaves of their host plants for at least a portion of their lifecycle. They would also need to do this in springtime (November-December). These criteria ruled out testing a number of non-target beetles including native Galerucinae (considered the “sister group” to Chrysomelinae) that are all thought to have only root-feeding larvae, and ruled out the St John’s Wort leaf beetles (beneficial biocontrol agents) as their larvae do feed on leaves but at other times of the year and are not present in spring.

We tested tested nine non-target species in total, always using *P. charybdis* as our control pest species for comparison. All tests were conducted with *Eadya daenerys* assuming the role of a natural enemy.

The *Eadya daenerys* wasp

Eadya daenerys is a promising parasitoid because it attacks paropsine beetles from Australia. Rather than attacking the eggs of a pest (we already have two egg parasitoids in New Zealand that effect some control of *P. charybdis* populations (Mansfield et al. 2011), it attacks the larval life stage in spring. Such larval attacks have been observed repeatedly and studied in depth in eucalyptus plantations in Tasmania (Peixoto et al. 2018).

Eadya has a one-year life-cycle, with adults present only in November and December (Rice 2005). At this time, in Tasmania, there is an abundance of young larvae of *Paropsisterna agricola* which the *Eadya daenerys* feed upon. This host is not available in New Zealand but *P. charybdis* is. The role of the adult is to lay an egg in the beetle larva. That larva then eats the inside out of its host. When it has reached maturity it pops out of the larva, leaving just a skin behind. It spins a silken cocoon in the soil where it hibernates for the rest of the year before emerging in the following spring as an adult to locate a mate and reproduce itself.

No-choice physiological host range tests

No-choice tests (van Driesche and Murray, 2004) are considered to be the most thorough type of test that will reveal any possible harm to non-targets. This is because they force the parasitoid to make contact with non-target larvae by giving them no choice of anything else, and by holding the species together for a very long time without the parasitoid being able to escape.

For each of our no-choice tests against target and non-target beetle species, one female *Eadya daenerys* was introduced to a 500ml plastic cage, with honey and water provided, and a sprig of foliage on which 8 larvae of the non-target beetle were feeding. They were then left undisturbed together for exactly 24 hours, after which time the parasitoid was removed and the larvae were reared to a beetle pupa stage. Any larvae dying prematurely were frozen then dissected to look for evidence of internal parasitism that might indicate that they had been attacked or stung by the parasitoid during the test. We are certain this long duration will have created maximum possible motivation for the parasitoid to attack the non-target larvae present.

With the target pest, our results indicated that when not attacked by *Eadya daenerys*, *P. charybdis* had a survival success to pupation of 79% (depending on disease incidence in the colony, it ranged from 66% to 95% survival). This is called a negative control. But when attacked purposefully by *Eadya daenerys* (one sting observed into each larva) the survival rate to pupation of *P. charybdis* dropped to less than 10%. This is called a positive control. This demonstrates the effectiveness of the parasitoid at causing additional mortality to the pest, and is why we hope it will be safe to release in New Zealand.

For the non-target beetles, about two-thirds of the beetle larvae that we took out of each 24-hour no-choice tests successfully survived to pupate, seemingly unaffected by *Eadya daenerys,* with an average survival of 5.4 out of 8 (Table 2). The best rearing survival was achieved on the non-targets: the native veronica leaf beetle *Allocharis* (90%), the broom leaf beetle *Gonioctena* (85%) and tradescantia leaf beetle *Neolema* (85%). The worst rearing success to pupation of just 40% was achieved on the tutsan leaf beetle *Chrysolina,* a problem inherent to the species and shared with Landcare Research from whom we obtained the colony.

Apart from the target host *P. charybdis*, *Eadya daenerys* parasitoids only completed development, and emerged as a fully developed adult wasp from one non-target species, the small tortoise beetle pest *Trachymela sloanei* (Table 2). Three of the five emergent parasitoid larvae from *T. sloanei* spun cocoons, with one cocoon producing one tiny adult *Eadya daenerys* after overwintering. This confirms *Trachymela* as a host for *Eadya* (= development to adult within a species).

The only other evidence of attempted parasitism by *Eadya daenerys* parasitoids was discovered by dissecting dead non-target larvae, or by killing and dissecting prepupae (mature larvae) that had failed to pupate. Internal parasitism was found in four non-target species, all from the subfamily Chrysomelinae: the blackwood tortoise beetle pest *Dicranosterna*, the

Table 2. Outcomes of the no-choice physiological host range tests of *Eadya daenerys* to non-target beetles subsequently reared to pupation.

Species	No. Reps	Total larvae reared	No. became beetle pupae	Beetle survival (%)	No. died but contain'd Eadya	No. died unknown causes	No. Eadya emerged from
Positive control *P. charybdis* 2015	4	32	3	9.4	0	18	11
Positive control *P. charybdis* 2016	26	205	14	7.0	0	108	63
Trachymela	5	40	23	57.5	0	7	5
Dicranosterna	16	128	80	62.5	2	46	0
Allocharis	10	80	72	90.0	6*	2	0
Chrysolina	14	112	45	40.2	2	65	0
Gonioctena	12	96	82	85.4	5	9	0
Lochmaea	11	92	69	75.0	0	23	0
Agasicles	14	112	62	55.4	0	50	0
Cassida	16	128	77	60.2	0	51	0
Neolema	15	120	102	85.0	0	18	0

***These larvae failed to pupate but had not died so were killed by the researchers and discovered at that point to contain *Eadya* larvae**

native veronica leaf beetle *Allocharis,* the tutsan leaf beetle *Chrysolina* and the broom leaf beetle *Gonioctena* (Table 2). Interestingly, six *Allocharis* larvae had not died and appeared quite normal, but had remained stuck in the pre-pupal stage (non-feeding mature larvae) for longer than they should have, and failed to pupate. Twenty days after they should have pupated we became suspicious, and killed these for dissection, which is how we discovered the presence of parasitoid larvae inside them. This is referred to as being an "unsuitable physiological host" (= unable to complete development all the way to adult within a species).

Behavioural preference tests

The behaviour of individual *Eadya* parasitoids was also observed, in experimental arenas that were large clean glass petri dishes measuring 140 mm diameter x 20 mm high, and under two test conditions: either sequential no-choice tests or two-choice tests.

For the sequential no-choice tests, one female *Eadya* parasitoid was observed for 10 minutes with either eight targe,t or eight non-target, host larvae settled onto a piece of leaf or sprig of host foliage, using an A-B or B-A sequence representing whether the target larvae were presented to the parasitoid first (A) and the non-target larvae presented second (B), or vice-versa.

For the two-choice tests, one female parasitoid was observed for 25 minutes with two sprigs of foliage present in the arena; at the same time, one sprig of *E. nitens* bearing eight larvae of the target larvae *P. charybdis*, plus one of the non-target foliage bearing eight of the non-target larvae (A+B) appropriate to whichever species was being tested. Time recording began when the female *Eadya* contacted a host plant. All times spent on the plants were recorded, along with any interactions with larvae, such as attempted or successful stings (attacks), and probing of frass or other objects.

No female *Eadya* were tested repeatedly against the same non-target in the same type of test. We aimed to test 15 independent females in each test type against each non-target. Unfortunately, sometimes there were fewer than this due to either insufficient larvae or of live female *Eadya* during the testing stages. The actual number of replicates conducted ranged between 8 and 17 females for each type of test.

Analysis revealed the order of presentation (A-B vs B-A) had no significant effect on the likelihood or number of attacks by *Eadya* parasitoids. With the combined sequential test results, overall attack behaviour towards all species (target and non-targets combined) was not significantly different either between no-choice and two-choice test designs ($p=0.065$).

A significant difference in *Eadya* attack behaviour towards *Paropsis* larvae compared to each of the paired non-target larvae occurred in all except for the paired *Paropsis-Trachymela* no-choice test. This can be seen in the box-and-whisker plots (Figure 1) which show a clear overlap of boxes only for the *Paropsis-Trachymela* pair and little or no overlap for the other species pairings. The median number of attacks for the *Paropsis-Trachymela* pair was 2.5 for *Paropsis* and 1.5 for *Trachymela* while that for each other paired non-target larvae was identically zero attacks, while those towards *Paropsis* larvae ranged from two to 10 attacks.

In addition to examining the number of larval attacks, we developed a measure of excitation behaviour that the *Eadya daenerys* expressed towards the larvae present, while being in contact with that larva's host plant. We called this the 'attack rate towards larvae while on their plant' in a test:

Attack rate on plant = Number of successful attacks observed / total time spent on that hosts' plant

'Attack rate on plant' by *Eadya* towards *Paropsis* larvae was significantly greater ($p < 0.05$) than 'attack rate on plant' towards non-target larvae for the majority of non-target species (Figure 2; as indicated by the lack of overlap of boxes in the box and whisker plots). The observations during assays suggested that the majority of the time *Eadya* was in contact with non-target plants bearing non-target larvae, she just sat and rested or undertook grooming behaviour. The only exceptions where a significant difference in behaviour did not occur (and boxes in Figure 2 overlap), were found for attack rate towards *Trachymela* in the no-choice test compared to *Paropsis* (both of which involved *Eucalyptus* leaves), but also towards *Allocharis* while on *Veronica* but only in the *Paropsis*- *Allocharis* two-choice test.

To determine the likelihood of attack on non-targets in the confines of these petri dishes, it can also be useful to understand what proportion of the *Eadya* females were responsible for the attack behaviour that is summarised above in Figures 1 and 2. It was more common for a female *Eadya* to attack a blackwood tortoise beetle *Dicranosterna* larva (6/17 attacked), than a tutsan leaf beetle *Chrysolina* larva (3/16 attacked), particularly under two-choice test conditions. However, the highest proportion of *Eadya* females exhibiting attacking behaviours towards non-target species was associated with *Trachymela* in the no-choice test (5/8 attacked). In the no-choice assays, on average only 8% of female *Eadya* attacked a non-target larvae compared to 100% attacking *Paropsis*. Within two-choice assays (which did not include *Trachymela*) the average was 15% of *Eadya* females attacking a non-target, compared to 100% attacking *Paropsis*.

The on-rearing of target and non-target larvae in replicates, when attacks had been observed during behavioural observations, added a little more data to the physiological host range development data presented in Table 2. Observed attacks against tutsan leaf beetle *Chrysolina* resulted in 3/20 reared larvae being parasitized internally by *Eadya*. The same pattern as observed previously was repeated with native veronica leaf beetles *Allocharis* larvae; observed attacks resulted in another 5/19 larvae reaching pre-pupal stage but failing to pupate. These five larvae were dissected and three contained very small or encapsulated *Eadya* larvae, and one contained a well-developed *Eadya.*

Conclusions

We have herein summarised the results of laboratory host specificity testing of *Eadya daenerys* female parasitoids against two pest paropsines beetles, one native species, and six beneficial biological control agents. All species tested had springtime-active, and external leaf-feeding larvae. Physiological development through to emergence of the parasitoid only occurred in the target *Paropsis*, and the pest *Trachymela sloanei* (at 12%).

Incomplete physiological development by *Eadya daenerys* indicative of laying an egg, was discovered upon dissection at the level of 2-5% in another four non-target species that were all subfamily Chrysomelinae: in the pest *Dicranosterna*, in the native sub-alpine veronica leaf beetle *Allocharis*, in the tutsan leaf beetle *Chrysolina* and in the broom leaf beetle *Gonioctena*. *Eadya daenerys* will not form a self-sustaining population on any of these beetles.

Behavioural assays with female *Eadya daenerys* closely confined with larvae resulted in occasional observed attacks against almost all non-targets (but interestingly never against broom leaf beetle despite 3 replicates of the 24-hour no-choice tests resulting in internal parasitism). The stimulation to attack was highly significantly less towards all non-target species compared to *Paropsis* and leads us to conclude non-target attack was likely to be due to the confines of the testing environment.

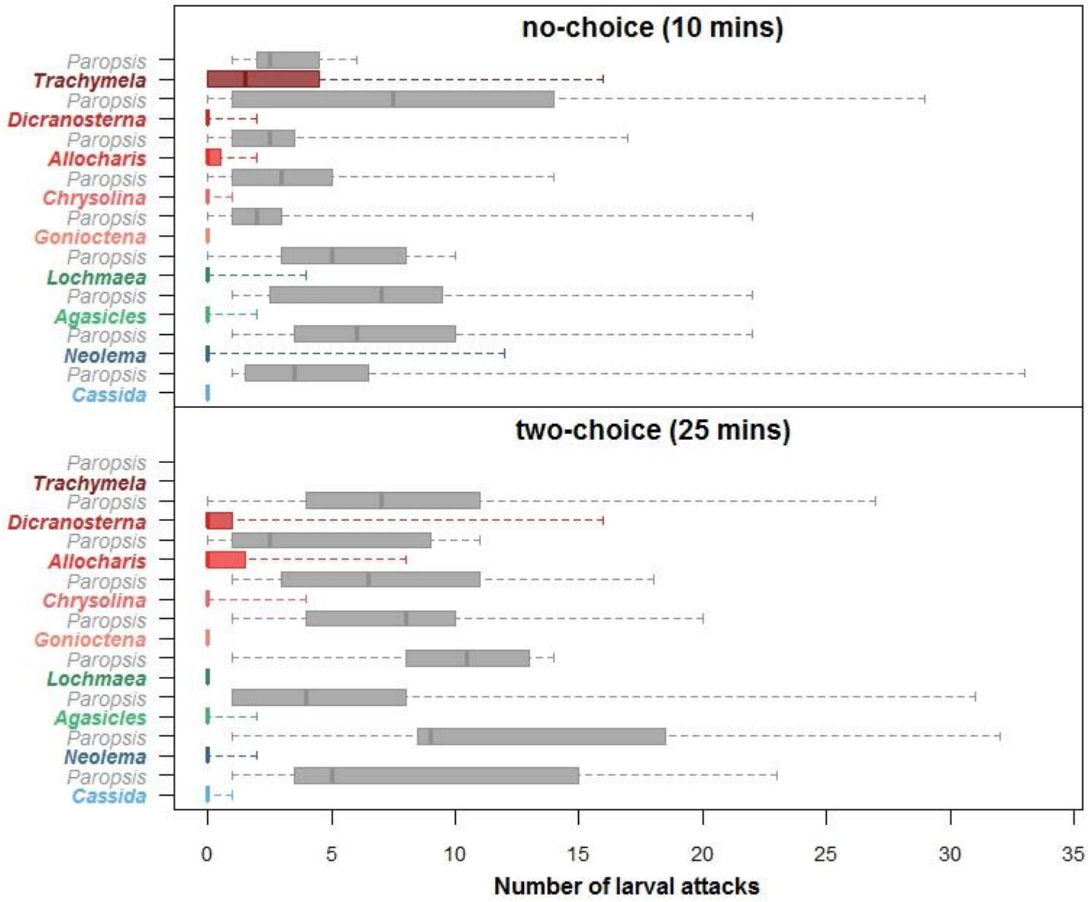


Figure 1. Box and whisker plots representing total number of successful attacks counted by each *Eadya daenerys* in no-choice sequential and two-choice tests. The *P. charybdis* target larvae paired against each non-target larval species are shown in grey.

The vertical line within each box represents the median (i.e. middle value of the ordered data), the box represents the midspread (i.e. middle 50% of values), the whiskers represent the lower and upper data quartiles, and terminate at the minimum and maximum values respectively. Where no box is visible, just a line, it means that all the data points are sitting at or close to zero.

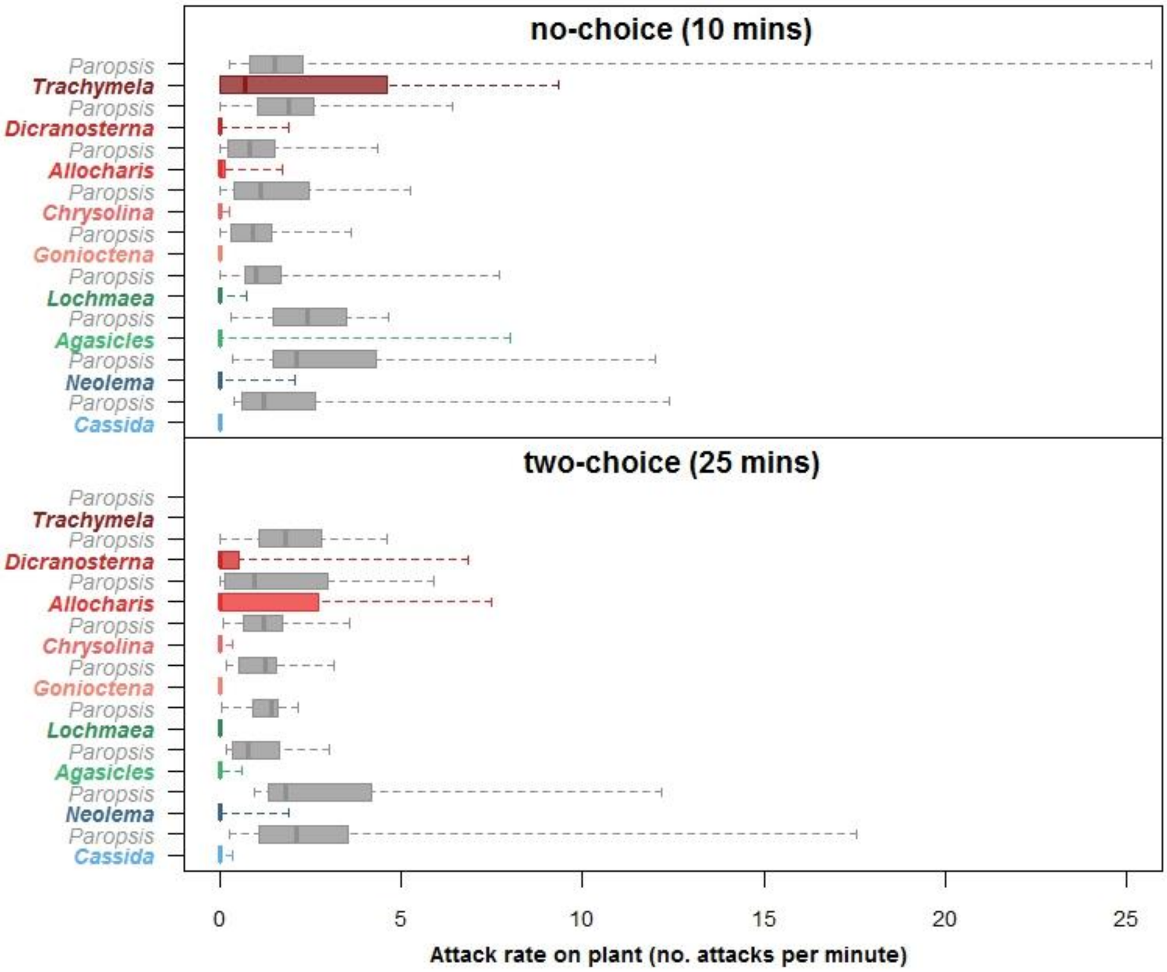


Figure 2. Box and whisker plots of *Eadya daenerys* ‘attack rate on plant’ behaviour directed against a beetle species in no-choice sequential and two-choice tests. The *P. charybdis* target larvae paired against each non-target larval species are shown in grey.

The vertical line within each box represents the median (i.e. middle value of the ordered data), the box represents the midspread (i.e. middle 50% of values), the whiskers represent the lower and upper data quartiles, and terminate at the minimum and maximum values respectively. Where no box is visible, just a line, it means that all the data points are sitting at or close to zero.

Only the other *Eucalyptus*-foliage feeding species, the small tortoise beetle pest *Trachymela* was an attractive non-target. The proportion of female *Eadya* exhibiting attraction to *Trachymela* larvae was relatively high. However, even this level of attack may not equate to them being suitable hosts, *Trachymela* larvae are nocturnal and hide during the day. The act of transferring them to leaves during the daytime disturbed them, causing them to run very rapidly around the petri dish perhaps as they sought out shelter. This made them a difficult target for *Eadya*, although they presented an attractive cue for oviposition when the *Eadya* were able to catch them.

The internal parasitism of the broom and tutsan leaf beetle larvae following exposure to *Eadya daenerys*, is likely to equate to minimal or nil impact in the field. This is because attraction to these larvae in behavioural assays was highly significantly less than that towards target larvae. It is likely that both broom and tutsan will be present in the same geographical areas of New Zealand as eucalypt trees and plantations. It is possible that, if *Eadya* establishes, it may overlap with these non-target beetle species, and could potentially encounter them if *Eadya* ***daenerys*** ever lands on these weeds to rest and groom. However, in our observations *Eadya* ***daenerys*** females were significantly more likely to actively search for paropsine larvae to attack when they were on eucalypt foliage, so we feel the risks of non-target attack occurring against broom and tutsan leaf beetles are low.

The internal parasitism of the veronica leaf beetle *Allocharis* may cause some concern. This beetle has only ever been collected between 1100 to 1300 m above sea level in Kahurangi National Park. There are no eucalypts growing in this national park, although they exist in the Motueka and Takaka river valleys approximately 50 km away. It is unknown whether *Eadya daenerys* will ever fly up into native subalpine forest habitats that are free of its host insects' plants (eucalypts). In Tasmania *Eadya daenerys* has been collected at 600 m above sea level at Moina, but that is the highest altitude at which *E. nitens* has been commercially planted there, and paropsine hosts were abundant at that site. We were unsuccessful at locating any other native sub-alpine beetle species that are medium sized and may have leaf-feeding larvae. They may be similar to *Allocharis* or may have internal feeding larvae such as the largest native Chrysomelinae beetle, *Chalcolampra speculifera*, whose larvae have been found sheltering within holes in the stem of *Olearia colensoi* (Wardle et al. 1971). Bearing all these uncertainties in mind, we consider there is minimal, although non-negligible, risk to native beetles in New Zealand from *Eadya daenerys*.

In summary, the data is consistent with field host relationship studies in Tasmania (Peixoto et al unpublished), and concludes *Eadya daenerys* is unlikely to attack any species apart from pest paropsine (Chrysomelini) species feeding on *Eucalyptus*. Therefore we propose *Eadya* is safe to release in New Zealand.

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