Biological Control of *Paropsis charybdis* Stål (Coleoptera: Chrysomelidae) and the Paropsine Threat to *Eucalyptus* in New Zealand

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ABSTRACT

Ineffective biological control of the *Eucalyptus* pest *Paropsis charybdis* Stål (Coleoptera: Chrysomelidae: Paropsini) in cold areas of New Zealand was believed to be caused a climatic mismatch of the egg parasitoid *Enoggera nassaui* Girault (Hymenoptera: Pteromalidae). Two Tasmanian strains of the parasitoid were introduced to test climate-matching theory in 2000, with approximately 7000 wasps released. Establishment of the Florentine Valley strain was detected in 2002 using the Mitochondrial (mtDNA) gene Cytochrome Oxidase I (COI) as a strain specific marker. The hyperparasitoid *Baeoanusia albifunicle* Girault (Hymenoptera: Encyrtidae) and primary parasitoid *Neopolycystus insectifurax* Girault (Hymenoptera: Pteromalidae) were detected for the first time in New Zealand.

As paropsines have proven highly invasive internationally, a risk assessment of the paropsine threat to New Zealand was undertaken by evaluating the host range of *E. nassaui* and a reproductive assessment of 23 paropsine species in the genera *Dicranosterna* Motschulsky, *Chrysophtharta* Weise, *Paropsis* Olivier, *Paropsisterna* Motschulsky and *Trachymela* Weise. *Enoggera nassaui* proved polyphagous, but bioassay results proved that *Paropsis* species were significantly more susceptible to the egg parasitoid than *Chrysophtharta* species. Resistance within *Chrysophtharta* was attributed to spine-like chorion modifications. A COI derived *Chrysophtharta* phylogeny divided the genus into two distinct groupings, which was supported by chorion morphology.

Paropsine reproductive output was tested for key parameters indicating pest potential. Pest species displayed fecundity exceeding 600 eggs at an oviposition rate above 10 eggs per day^{-1}. Several non-pest species were identified as potential pests based on these parameters. The *Chrysophtharta* phylogeny suggested a moderate relationship between genetic relatedness and reproductive output. The *Acacia* defoliating paropsine *Dicranosterna semipunctata* (Chapuis) was evaluated for its susceptibility to *E. nassaui* and reproductive output. Egg parasitism occurring in bioassay did not translate into biological suppression following a specifically targeted release of *E. nassaui*, and the fecundity and oviposition rates fell below the thresholds predicted for a pest paropsine species.

Despite establishment of Tasmanian *E. nassaui*, hyperparasitism has now rendered this control agent ineffective in New Zealand. *Neopolycystus insectifurax* offers the best hope for future biological control of paropsine species in New Zealand.

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ERRATA

Following a revision of Australian Chrysomelidae, Reid (2006) identified the genus *Chrysophtharta* Weise as a synonym of *Paropsisterna* Motschulsky. All references to *Chrysophtharta* should be taken to refer to the senior synonym.

Chapter 1. Biological Control of *Paropsis charybdis* Stål and the Paropsine Threat to *Eucalyptus* in New Zealand

1. INTRODUCTION

1.1 Background

The partial failure of a biological control system offers a rare opportunity to test a range of scientific tools and theories. Amongst these theories is one that states that strains of biological control agents exhibit particular characteristics, which can be selected to maximise the success of biological control (Doutt & DeBach 1964; Cameron *et al*. 1993; Hopper *et al*. 1993). The breakdown of the biological control system for the forestry pest *Paropsis charybdis* Stål (Coleoptera: Chrysomelidae: Paropsini) in New Zealand offered such an opportunity.

Of Australian origin, *P. charybdis* has been established in New Zealand since at least 1916 (White 1973). Classical biological control was achieved in the late 1980s using the egg parasitoid *Enoggera nassaui* Girault (Hymenoptera: Pteromalidae) from Western Australia. This was generally successful, with notable exceptions occurring in cooler, high altitude regions (Kay 1990). This partial failure was attributed to the original sourcing of the egg parasitoid from frost-free areas of Australia (Murphy & Kay 2000). The collection and release of *E. nassaui* strains tolerant of these conditions in New Zealand was proposed as a solution.

The release of 'cold tolerant' strains would allow evaluation of strain selection as a viable strategy for biological control. This not only required the collection and release of the parasitoid, but also post-release monitoring to determine if establishment, spread, and ultimately, successful biological control were achieved. Molecular techniques were expected to be able to provide a tool capable of discriminating between strains, but this method required development and validation for *E. nassaui*.

Fieldwork in Australia allowed the first critical assessment of the host range and effectiveness of *E. nassaui* against a range of paropsine hosts found in Tasmania, particularly in the genera *Chrysophtharta* Weise (**Appendix 1**) and *Paropsis* Olivier (**Appendix 2**). This knowledge would help define the host specificity of the parasitoid, which represented the only significant natural regulation of paropsine incursions in New Zealand. Australian based field work also allowed measurement of the reproductive output of paropsine species, helping to form a risk assessment of the paropsine threat to *Eucalyptus* in New Zealand. Combined, this research

sought to not only enhance an existing biological control programme, but also to improve the toolset usable in biological control.

1.1.1 Biological control of *Paropsis charybdis*

The *Eucalyptus* tortoise beetle, *Paropsis charybdis* (**Figure 1**) is one of five paropsine species currently established in New Zealand, and historically is the most consistent barrier to establishment of a commercial *Eucalyptus* resource (White 1973; Kay 1990). First detected in the Port Hills of Christchurch during 1916 (White 1973), the natural range of this insect is between the coastal and adjacent tablelands of south-eastern Australia and Tasmania (Styles 1970; Potts & de Little 1977). In Tasmania it is rare and occasionally a pest species (de Little 1989). In contrast, *P. charybdis* is capable of regular outbreaks in New Zealand, facilitated by high fecundity (Styles 1969) and a wide host range (White 1973).

Figure 1. *Paropsis charybdis* adult

Without suppression of *P. charybdis*, complete defoliation of susceptible hosts is not uncommon. Amongst the most susceptible host species are *E. globulus* Labill. and *E. nitens* (Deane & Maiden) Maiden, considered key in the development of a fast-rotation, short-fibre pulp industry in New Zealand (Wilcox 1980). *Eucalyptus nitens* is a species with increasing international importance (Tibbits *et al.* 1997) because of its fibre and growth traits (Franklin 1980; Wilcox 1980; Frederick *et al*. 1986; King & Wilcox 1988; Beadle *et al.* 1989). The excellent frost tolerance of this species (Tibbits & Reid 1987) lends itself to plantings occurring where the risk of *P. charybdis* defoliation is the greatest.

The impact of *P. charybdis* in New Zealand is considered a consequence of an absence of significant natural enemies (Styles 1970; Edwards & Suckling 1980). Classical biological control by natural enemies had previously proven effective on a range of *Eucalyptus* pests (Zondag 1977; Morales & Bain 1989; Nuttall 1989; Faulds 1990), and was considered as a

long-term solution to *P. charybdis*. The rarity of the beetle in Australia suggested effective control by natural enemies, some of which could be beneficial in New Zealand.

1.1.2 *Biological Control of* P. charybdis

Biological control for *P. charybdis* had been attempted since the 1930s (**Table 1**) by importing parasitised paropsine egg-batches or larvae from Australia. Most cultures were hyperparasitised or not amenable to rearing, leading to relatively few releases (Bain & Kay 1989).

Table 1. Summary of biological control attempts for *Paropsis charybdis* (from Bain & Kay 1989)

Date	Source	Control Agent	Family	Releases	Established / Recovered
1934 - 35	Clark 1938	*Froggattimyia tillyardi*	Tachinidae	No	
	FRI Files	*Neopolycystus insectifurax*	Pteromalidae	No	
		Aridelus sp.	Braconidae	No	
1963	FRI Files	*Aridelus* sp.	Braconidae	No	
1964	FRI Files	*Froggattimyia* sp.	Tachinidae	No	
		Aridelus sp.	Braconidae	No	
1973 - 74	FRI Files	*Froggattimyia tillyardi*	Tachinidae	No	
		Paropsivora sp.	Tachinidae	No	
		Aridelus sp.	Braconidae	No	
1974 - 75	FRI Files	*Froggattimyia tillyardi*	Tachinidae	Yes	No
		Paropsivora sp.	Tachinidae	No	
1977 - 80	Bain *et al.* 1984	*Cleobora mellyi*	Coccinelidae	Yes	Yes (partial)
1987 - 90	FRI Files	*Neopolycystus insectifurax*	Pteromalidae	Yes	No
	Kay 1990	*Enoggera nassaui*	Pteromalidae	Yes	Yes

The first success was the partial establishment of the ladybird *Cleobora mellyi* Mulsant. Its role as an important predator of *Chrysophtharta bimaculata* larvae in Tasmania (Elliott & de Little 1980; Bashford 1999) led to its evaluation in 1977. More than 3000 adult *C. mellyi* were released in the Bay of Plenty, Canterbury and the Marlborough Sounds (Bain *et al*. 1979). Later surveys found the beetle to have established only in one area of the Marlborough Sounds, where its impact on populations of *P. charybdis* is unknown.

Twenty years later, two egg parasitoids were imported from Western Australia. *Neopolycystus insectifurax* (Hymenoptera: Pteromalidae) and *E. nassaui* both proved capable of attacking *P. charybdis* eggs in the laboratory. Following mass rearing, circa 100 000 of each species were released in Nelson, Canterbury, Southland, Waikato and the Bay of Plenty in the summer of 1987/88. While *N. insectifurax* was not recovered, *E. nassaui* was found to have established in a number of localities. *Paropsis charybdis* populations went into a noticeable decline, and effective biological control of the tortoise beetle appeared to have been attained (Kay 1990).

1.1.3 *Failure of biological control*

Even prior to its successful release, concerns existed about the success of *E. nassaui* in New Zealand. The 80 *Enoggera nassaui* adults sent to New Zealand were collected from Ludlow Tuart forest, located 14 km NE of Busseltown, Perth (115°21'E 33°39'S). The climate of this area is relatively warm, with only one frost recorded over 70 years of climate data collection. Bain and Kay (1989) predicted that the sourcing of the parasitoid from a warm climate area of Australia would affect its ability to survive in colder areas in New Zealand, i.e. a climatic mismatch may occur. This became a reality in the mid to late 1990s, as *Eucalyptus* plantations in cooler, high altitude locations began to experience repeated defoliation by outbreak populations of *P. charybdis*.

Murphy and Kay (2000) measured the extent of this defoliation, and proposed a mechanism to explain the outbreaks. The trigger appeared to be the ineffectiveness of the parasitoid in spring when adult *P. charybdis* first commence oviposition after overwintering. During this period, egg parasitism by *E. nassaui* was almost undetectable, resulting in large larval populations causing extensive considerable defoliation. After pupation, surviving larvae became reproductive adults, and a second larger *P. charybdis* oviposition peak commenced in mid to late summer. Fortunately, this peak was almost 100% parasitised by *E. nassaui* and relatively few larvae occur in this period. However, the net effect of annual increases of *P. charybdis* populations would lead to persistent outbreaks unless additional regulation could be accomplished. The economic viability of commercial *Eucalyptus* forestry was once again threatened unless effective control could be established over *P. charybdis*.

1.1.4 *Restoring biological control of Paropsis charybdis*

The predicted climatic mismatch of *E. nassaui* to New Zealand conditions (Bain & Kay 1989) was considered the most likely cause of its erratic suppression of *P. charybdis* (Murphy & Kay 2000). This would explain the low parasitism rates observed in spring after the winter period, and the fact that effective control was not established until later in summer

The solution, of importing *E. nassaui* strains from climatically suitable locations in Australia, was based on practical considerations. Firstly, the parasitoid was known to operate effectively in New Zealand where climate was not restrictive. Secondly, importation of *E. nassaui* strains into New Zealand is allowable under the Hazardous Substances and New Organisms Act (1996), whereas the importation of new species as biological control agents would be considerably more difficult. Thirdly, the distribution of the parasitoid had been partially documented (Naumann 1991) which would assist in its collection.

Once released, accurate detection of whether any novel strains had established would be crucial for determining whether climate matching was an effective tool. In the absence of useful morphological characters to achieve this (Naumann 1991), molecular techniques offered the most likely solution. This would rely *a priori* on the presence of unique markers for each strain, and the availability of a method that could accurately and reliably detect them.

Accordingly, the specific tasks required to re-establish effective biological control over *P. charybdis* in New Zealand were to:

1) Collect *E. nassaui* strains from a region(s) in Australia climatically similar to cooler areas of New Zealand;
2) Import novel strains into New Zealand, and rear in quarantine facilities until official authorisation was obtained to release;
3) Release novel *E. nassaui* strains in New Zealand;
4) Develop a molecular tool that was capable of accurately discriminating between all *E. nassaui* strains in use; and
5) Test for establishment and/or spread of novel *E. nassaui* strains using the molecular tool.

1.2 Evaluation of the paropsine threat

Although importation of novel *E. nassaui* strains could resolve some problems in the biological control of *P. charybdis*, a considerable knowledge gap still existed as to the capability of this parasitoid in controlling paropsine species in general. *Paropsis charybdis* is one of five paropsine species established in New Zealand, and part of a group comprising 700 related species in Australia (Simmul & de Little 1999). All paropsine species established in New Zealand are considered rare in Australia (e.g. Styles 1969) with the exception of *P. charybdis* which is an occasional outbreak species in Tasmania (de Little 1989; Simmul & de Little 1999). None of the known Australian pest species (Greaves 1966; de Little 1989; Candy *et al.* 1992; Ohmart & Edwards 1991; Bashford 1993; Phillips 1996; Elek 1997; Elliott *et al.* 1998) have established in New Zealand, and represent an unquantified threat. The ability to predict whether *E. nassaui* would control these known pests, or any other paropsine species reaching New Zealand, would form part of a valuable risk analysis.

A robust assessment of the paropsine threat to *Eucalyptus* in New Zealand required more than the evaluation of the host range of *E. nassaui*. Assuming climate and host plant distribution were not restrictive, paropsine populations in New Zealand would be most limited by their reproductive output. Fecundity alone was considered a significant cause of the outbreak behaviour of *P. charybdis* in New Zealand (White 1973). As only a subset of all paropsine

species would be available for testing, interpretation of results would be maximised by describing the genetic relatedness of the study taxa. Such a phylogenetic framework could then theoretically be used to predict the reproductive output or vulnerability/resistance to *E. nassaui* of unstudied taxa.

Accordingly, the specific tasks required to evaluate the paropsine threat to *Eucalyptus* in New Zealand were to:

1) Evaluate the host range of *E. nassaui* from field collections;
2) Evaluate the efficacy of *E. nassaui* against a range of paropsine species;
3) Build a phylogenetic framework for the study paropsine taxa;
4) Define and measure key reproductive parameters for paropsine species;
5) Interpret these results against the phylogeny; and
6) If possible, test any predictive reproductive or susceptibility parameters against an unstudied paropsine species in New Zealand.

Section One: Biological Control of *Paropsis charybdis* Stål using Tasmanian *Enoggera nassaui* Strains

"Money, time, luck and a little bit of scientific insight"

Waage and Greathead (1988) on what makes biological control work.

Chapter 2. The Collection, Importation, and Release of Tasmanian *Enoggera nassaui* for Biological Control of *Paropsis charybdis*

1. INTRODUCTION

1.1 Biological control of *Paropsis charybdis*

Biological control of the *Eucalyptus* tortoise beetle has been attempted since the 1930s (Bain & Kay 1989). Control was not achieved until the late 1980s after the introduction of *Enoggera nassaui* (Hymenoptera: Pteromalidae) from Ludlow Tuart Forest (= Perth strain) in Western Australia (Tribe & Cillié 2000). Releases during 1987/88 resulted in establishment and a noticeable decline in tortoise beetle populations (Bain & Kay 1989; Kay 1990).

Within a decade, a decline in the effective control by *E. nassaui* was detected and attributed to a climatic mismatch with New Zealand conditions (Murphy & Kay 2000). This adverse impact was compounded by siting of commercial plantations in cold locations where *P. charybdis* outbreaks were most likely. Also susceptible was a considerable farm forestry *E. nitens* resource that had been established since biological control of *P. charybdis* had been announced (Miller *et al.* 1992). Despite this situation, the use of natural enemies still offered the best long-term control solution of *P. charybdis* in new Zealand.

1.2 Natural enemies and Biological control

Mills (1994) eloquently defined biological control as "the purposeful reconstruction of the natural enemy complex that exists in the region of the origin of the pest". Natural enemies are deliberately translocated to where their host is a pest species. The term 'classical biological control' denotes the sourcing of natural enemies from the natural distribution of the pest (Waage & Greathead 1988), distinguishing this from other methods such as inundative release (Pedigo 1989) and new association (Pimentel 1991).

Natural enemies are particularly important in controlling insect populations (van den Bosch *et al.* 1982; Price 1987). However, establishment of an insect in a novel environment frequently isolates it from control exerted by natural enemies. The majority of phytophagous species rarely or never attain outbreak because of this constant suppression (Hagen *et al.* 1971; Mason 1987). The exact impact of natural enemies can been proven by mechanical/chemical exclusion trials (Huffaker *et al.* 1968; DeBach & Huffaker 1971; Kidd & Jervis 1996) or construction of life tables (Southwood 1966; van den Bosch & Messenger 1973; Mills 1997).

Paropsis charybdis is an example of a species considered rare in its natural range (Styles 1970) and yet a significant pest in another country (White 1973). Paropsines and *Eucalyptus* appear well co-evolved in that paropsine populations appear to be more regulated by natural enemies than host-plant chemical defences (Morrow & Fox 1980; Selman 1985b; Ohmart 1996). Natural enemies can account for up to 90% of mortality (de Little 1982; Tanton & Epila 1984; Selman 1985a; de Little *et al.* 1990) although such suppression is not universal (Simmul & Clarke 1999; Nahrung & Murphy 2002). Even though insecticides are efficacious against paropsines (Baker & Latour 1962; Jackson & Poinar 1989; Harcourt *et al.* 1996; Elek *et al.* 1998), they do not represent the viable long-term solution achievable with biological control.

The cost-benefit of biological compared to chemical control of pests is compelling (Hussey 1985). Biocontrol is less likely to select for host resistance (Holt & Hochberg 1997; Holt *et al.* 1999) and should effect long-term suppression (DeBach & Rosen 1991). Insecticides and natural enemies can be combined effectively to control pests, as in integrated pest management (IPM) (Elliott *et al.* 1992; Nordlund 1996; Kogan 1998).

However, biological control is not a panacea for all pests. Practitioners have searched for common themes from successful campaigns, but as van den Bosch *et al.* (1982) noted, factors working for one scenario might not work in another, and often the causes for success are unknown. In contrast, reasons for failure are multiple and often avoidable (Beirne 1985; Hopper *et al.* 1993; Aeschlimann 1995). Certain insect orders appear more amenable to biocontrol than others. For example, success rates are 66% against Homoptera but only 7% for Coleoptera (Greathead 1995). Worldwide, the success rate is around 30%, resulting from nearly 5000 releases and 2000 agents (Greathead & Greathead 1992). In New Zealand, 75 biocontrol agents have establishment from 300 introductions, mostly directed against pasture, crop and fruit pests (Cameron *et al.* 1993).

1.3 Strains and variation

A variable known to affect the success of biological control initiatives is the use of strains of biological control agents. In this study, the term 'strain' is used interchangeably with the definition of a biotype by van den Bosch *et al.* (1982) as "a subpopulation or race of an organism adapted physiologically and behaviourally to survive under specific climatic conditions of some geographic region".

Parasitoid strains used in biocontrol are known to vary in their behaviour, preferences or adaptations (Doutt & DeBach 1964; Hopper *et al.* 1993; Smith 1996). Variation has been

recorded for host acceptance (Hassan *et al.* 1995; Henter & van Lenteren 1996; McGregor *et al.* 1998) and location rates (Henter *et al.* 1996), fecundity (Hoy 1975), insecticide tolerance (Caprio *et al.* 1991; Baker *et al.* 1998), and reproductive tactics (Wang & Smith 1996). Specifically selected strains have at times provided effective biological control after previous failures (van den Bosch *et al*. 1979; Cameron *et al.* 1993).

Climate is the main regulator of insect populations (Huffaker *et al.* 1971; Price 1987; Henson 1968; Wilson 1968; Williamson 1996). Climatic differences between source and establishment areas are frequently postulated as both a restraint on some pests (Myers 1987) and a release for others (Martinat 1987). The effects of climate on plant phenology or physiological composition (Brodbeck & Strong 1987; Mattson & Haack 1987) can also influence the population dynamics of phytophagous insects (White 1969, 1973, 1974, 1984). Biocontrol agents are similarly affected by climate and weather (Stiling 1990). For instance, parasitism is strongly influenced by short-term weather patterns (Martinat 1987; Risch 1987; Bourchier & Smith 1996), and several failed biocontrol programmes are attributed to a lack of climatic tolerance (Hopper *et al.* 1993), including the use of Western Australian *E. nassaui* to control *P. charybdis* in New Zealand (Murphy & Kay 2000).

1.4 Climate matching

The adverse effects of climate on biological control can be ameliorated by climate matching. Climate matching is a philosophy where the climates of the source and target areas for biological control agents are taken into consideration. This is considered a critical attribute for successful biocontrol programmes (Kennedy 1970; Wilson & Huffaker 1976; Wapshere 1983; Myers 1987; Roush 1990; Smith 1996; Legner & Bellows 1999), particularly for the establishment of agents (Hawkins & Cornell 1994). Coppel and Mertins (1977) advised that parasitoids are "frequently limited in effectiveness… by their greater sensitivity to cold, desiccation, heat, etc, and it is important therefore to give strong consideration to species which can withstand such conditions on a par with the host". For New Zealand conditions, Cameron *et al.* (1993) described climate matching as a "common sense approach (that) lessens the chance that a particular agent will fail to establish for climatic reasons alone".

Matching of parasitoid biotypes to their hosts is also expected to improve success of biocontrol programmes (e.g. Armstrong & Wratten 1996; Carter *et al.* 1996). Hopper *et al.* (1993) recommend that biocontrol agents should be preferentially collected from the target host, given the frequent paucity of knowledge concerning parasitoid host specificity. Failing this, their recommendation was to collect from closely related hosts, preferably within the same genus. Prior to this study, there existed no evidence to suggest that *E. nassaui* was a

natural enemy of *P. charybdis* in Australia as it was collected from Western Australian paropsines.

1.5 Reestablishment of Biocontrol for *Paropsis charybdis*

Failure of the biological control system for *P. charybdis* using *E. nassaui* was both predicted and explained on the basis of a climatic mismatch of the parasitoid. Strains that were better climatically matched provided the possibility of re-establishing effective control.

Tasmania was selected as the source area for climatically matched *E. nassaui* strains. Not only is the island considered climatically similar to much of New Zealand, both *E. nassaui* and *P. charybdis* are sympatric on the island (de Little 1979a: Naumann 1991). In addition, the taxonomy of Tasmanian *Chrysophtharta* and *Paropsis* species has been well described (de Little 1979 a, b; Selman 1983) which facilitated their study in combination with their natural enemy.

2. METHODS

2.1 CLIMEX Climate Matching

CLIMEX based climate matches were undertaken comparing Busselton (115°21'E 33°39'S) in Western Australian and the Florentine Valley (146°29'E, 42°38'S) in Tasmania against New Zealand. Maps were made using a composite of minimum and maximum temperatures for each location (D. Kriticos *pers. comm.*)

2.2 Parasitoid collection

Paropsine egg batches were collected from Tasmanian locations during 1998 to 2000 and determined to host species using the keys of de Little (1979a, b). After placement into individual Petri dishes, eggs were monitored for the emergence of parasitoids or host larvae. Parasitised eggs were identified by the presence of oviposition scars and the diagnostic patterns outlined by Tribe (2000). *Enoggera* specimens were identified to species level using the key of Naumann (1991). The Australian National Insect Collection (ANIC) identified all other parasitoid species. Parasitoid species, host paropsine, host plant and locality of collection were recorded where known.

2.3 Parasitoid rearing

Enoggera nassaui emerging from the same egg batch were considered to constitute a strain and were line reared in isolation from other strains. *Enoggera nassaui* were reared on fresh (<24 hr) *Paropsis aegrota* Boisduval eggs. Cultures were maintained by exposing adult wasps to eggs for 24 hours, after which eggs were removed and monitored for parasitism. Any paropsine larvae emerging from unparasitised eggs were removed to prevent damage to parasitised eggs. All rearing occurred in Petri dishes provided with diluted honey on a paper wick. Laboratory conditions were maintained at approximately 22°C under natural lighting.

2.4 New Zealand importation

Subsamples of each *E. nassaui* strain were sent to ANIC for identification prior to shipment to New Zealand. Upon confirmation of species identification and obtaining of permission from the Australian Quarantine and Inspection Service (AQIS), four *E. nassaui* strains (Florentine, Blue Gum Knob, Evandale 1 and Evandale 2) were transported to New Zealand in March 2000 under a 'Permit to Import Biological Products of Animal Origin' Permit number 1999007696 issued under the Biosecurity Act (1993) by the New Zealand Ministry of Agriculture and Forestry (MAF).

Shipments of both free adults and parasitised *P. aegrota* eggs were triple bagged (plastic vial, plastic bag, foam box). The four *E. nassaui* strains arrived at the New Zealand Forest

Research quarantine facilities, Rotorua, on the 24th March 2000. Live parasitoids were transferred to new vials and all packaging incinerated. Parasitoids that had not survived were labelled and stored in alcohol. Emerging paropsine larvae from unparasitised eggs were stored in alcohol and incinerated.

2.4.1 *New Zealand rearing of E. nassaui*

Enoggera nassaui rearing was undertaken in a C3 Level Quarantine Containment Facility on the Forest Research campus at 22°C under a 16:8 h light:dark photoperiod on *P. charybdis* eggs. The culture was subject to strict quarantine conditions and standards, e.g. multiple containment measures, air locks, all disposable materials were bleached, autoclaved or incinerated.

Two clear Perspex cages (1000 mm high x 700 mm wide x 800 mm deep) were used to separate adult and parasitised host-egg cultures. Access to each cage was provided via two gauze-lined arm sleeves, which were tied off after use to prevent parasitoid escape. Strains were line reared in clear plastic specimen containers (100 mm h x 40 mm d) with a screw-top lid within the Perspex cages. A 20 mm diameter hole was cut into each lid over which was secured a 50 x 50 mm fine gauze mesh to allow fresh air into each container. The strain line and generation number were recorded on each vial. Populations were periodically culled when the numbers were excessive to rearing requirements.

2.5 Release protocols

Verification of the identification and an assessment of the health of the cultures was required by MAF in order to obtain permission to release. Samples from each strain were sent to ANIC to reconfirm identification. After five generations, 30 specimens of each strain were killed in ethyl acetate and sent to BioDiscovery New Zealand Limited, where they were examined for microbial infection by Giemsa-staining. Voucher specimens of each strain were deposited with the New Zealand Arthropod Collection.

Once permission to release was obtained from MAF, the cultures were removed from the quarantine chamber to a separate rearing room within the same facility. Mass rearing was initiated using the previous rearing methods. Releases occurred in selected *E. nitens* plantations, consisting of approximately equal numbers of free adults and parasitised eggs. Releases took place in plastic takeaway containers hung from host trees at head height. Emergence holes were cut into both top and bottom of the containers, and a honey water solution provided on a paper wick.

RESULTS

2.6 CLIMEX Climate Matching

Figure 2 shows that the Western Australian climate is most similar to the northern North Island. In contrast, **Figure 3** shows the Tasmanian collection locality is more climatically similar to the central North Island and much of the South Island.

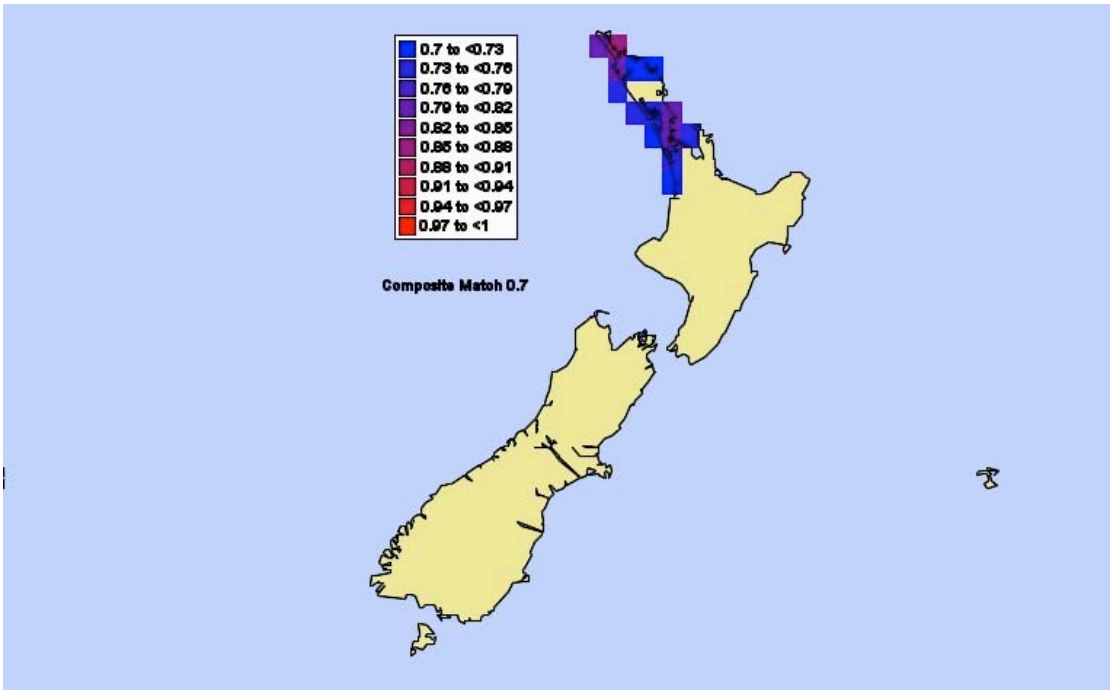


Figure 2. CLIMEX generated climate match of Busselton (Western Australia)

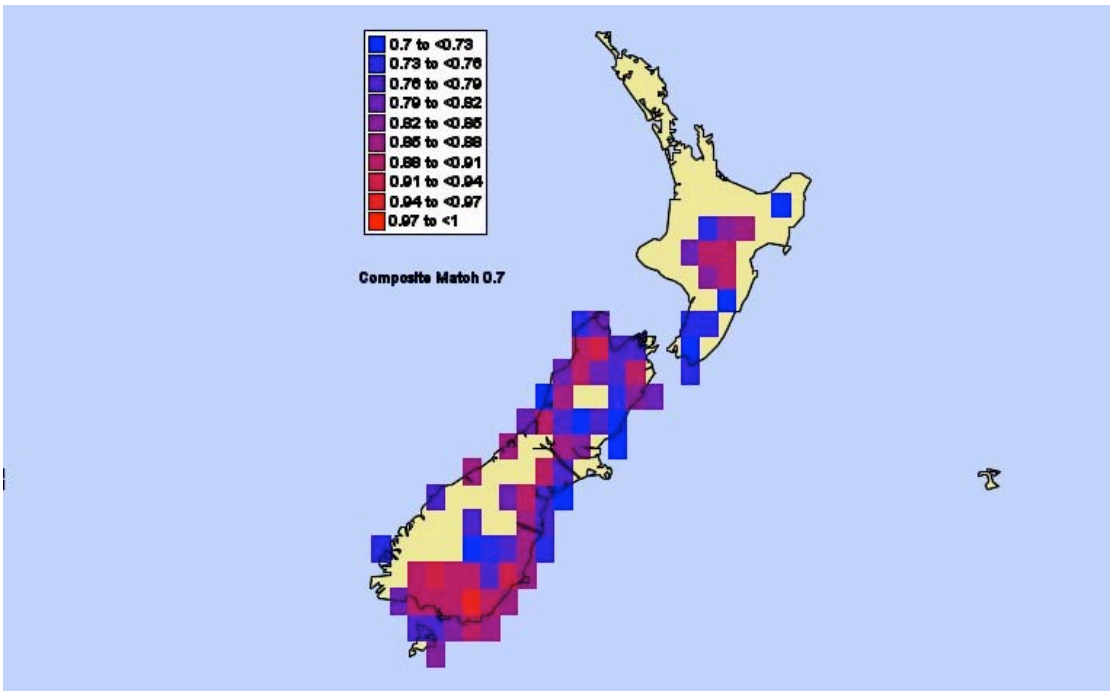


Figure 3. CLIMEX generated climate match of Florentine Valley (Tasmania)

2.7 Tasmanian *Enoggera nassaui* collection and rearing

Table 2 shows that 114 collections of egg parasitoids were made from ten paropsine species within the genera *Paropsis* and *Chrysophtharta* (**Appendix 3**). *Chrysophtharta bimaculata* yielded the most records, followed by *P. aegrota*. *Enoggera nassaui* was the most commonly collected primary parasitoid. Several *Neopolycystus* species were collected that could not be identified to species level by ANIC. Two obligate hyperparasitoids of *E. nassaui* were collected, with *Aphanomerella ovi* (Dodd) (Hymenoptera: Platygasteridae) collected twice as often as *Baeoanusia albifunicle* Girault (Hymenoptera: Encyrtidae).

Table 2. Field Collections of Tasmanian egg parasitoids on paropsine species

Host Species	Primary parasitoids		Hyperparasitoids		Totals
C = Chrysophtharta *P = Paropsis*	*E. nassaui*	*Neopolycystus*[1]	*A. ovi*	*B. albifunicle*	
C. agricola	1				**1**
C. bimaculata	13	3	16	5	**37**
C. decolorata	11		2	3	**16**
C. nobilitata	1				**1**
C. obovata	4		4	4	**12**
C. purpereo-aurea			2		**2**
P. aegrota	17	9	4	3	**33**
P. charybdis	2	4			**6**
P. debeori	1				**1**
P. porosa	5				**5**
Totals	**55**	**16**	**28**	**15**	**114**

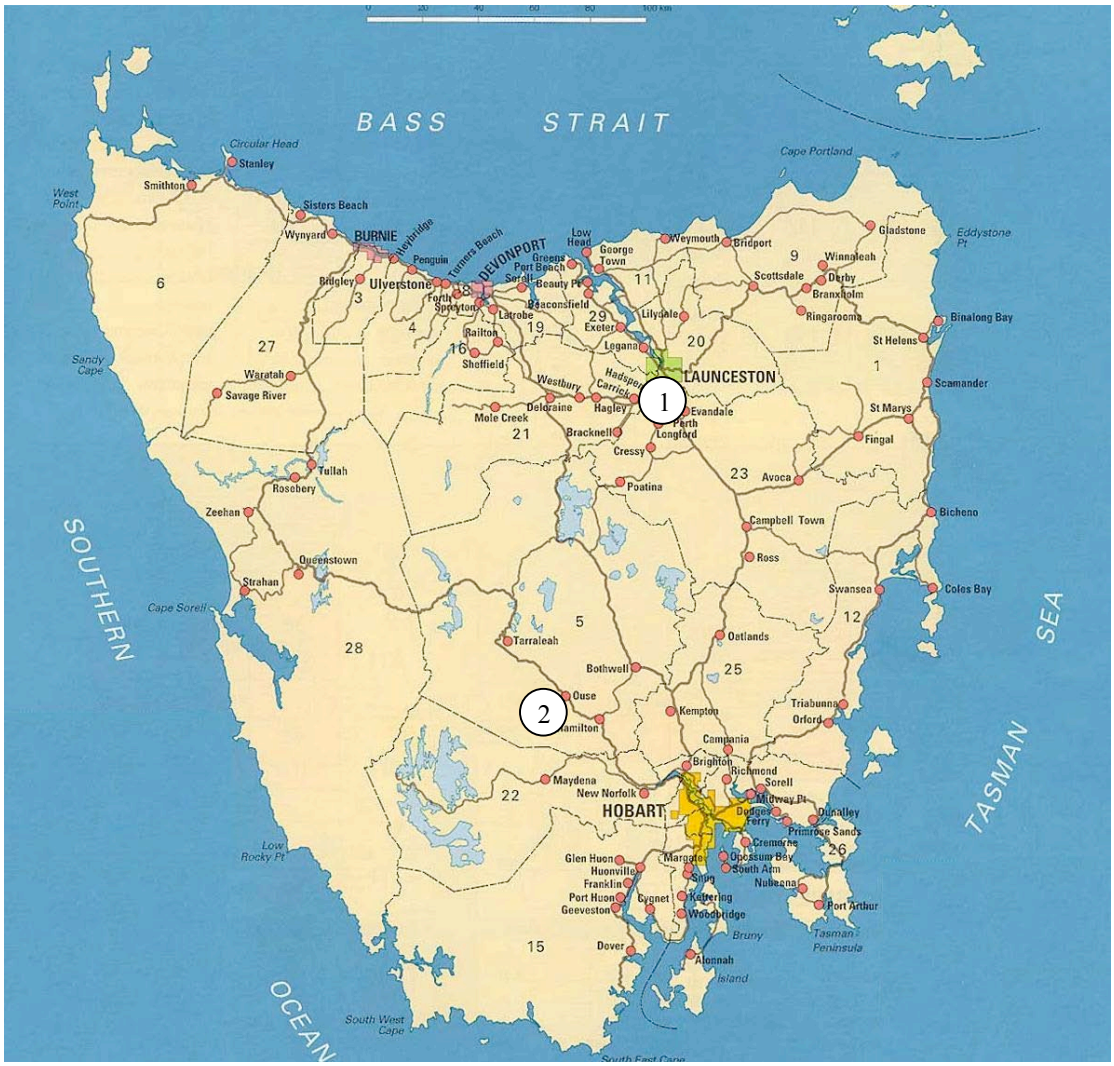
[1] Comprises at least two species

2.8 New Zealand importation and rearing

Two strains, Florentine Valley (146°29'E, 42°38'S, ex *P. aegrota*) and Evandale (147°24'E, 41°24'S, ex *P. deboeri*) were established in the quarantine facilities in New Zealand. Tasmanian source locations are shown in **Figure 4**. ANIC confirmed the identification of each strain as *E. nassaui*, and the cultures were declared free of identifiable pathogenic microorganisms

Figure 4. Sources of Tasmanian *E. nassaui* strains

1 = Evandale 2 = Florentine Valley



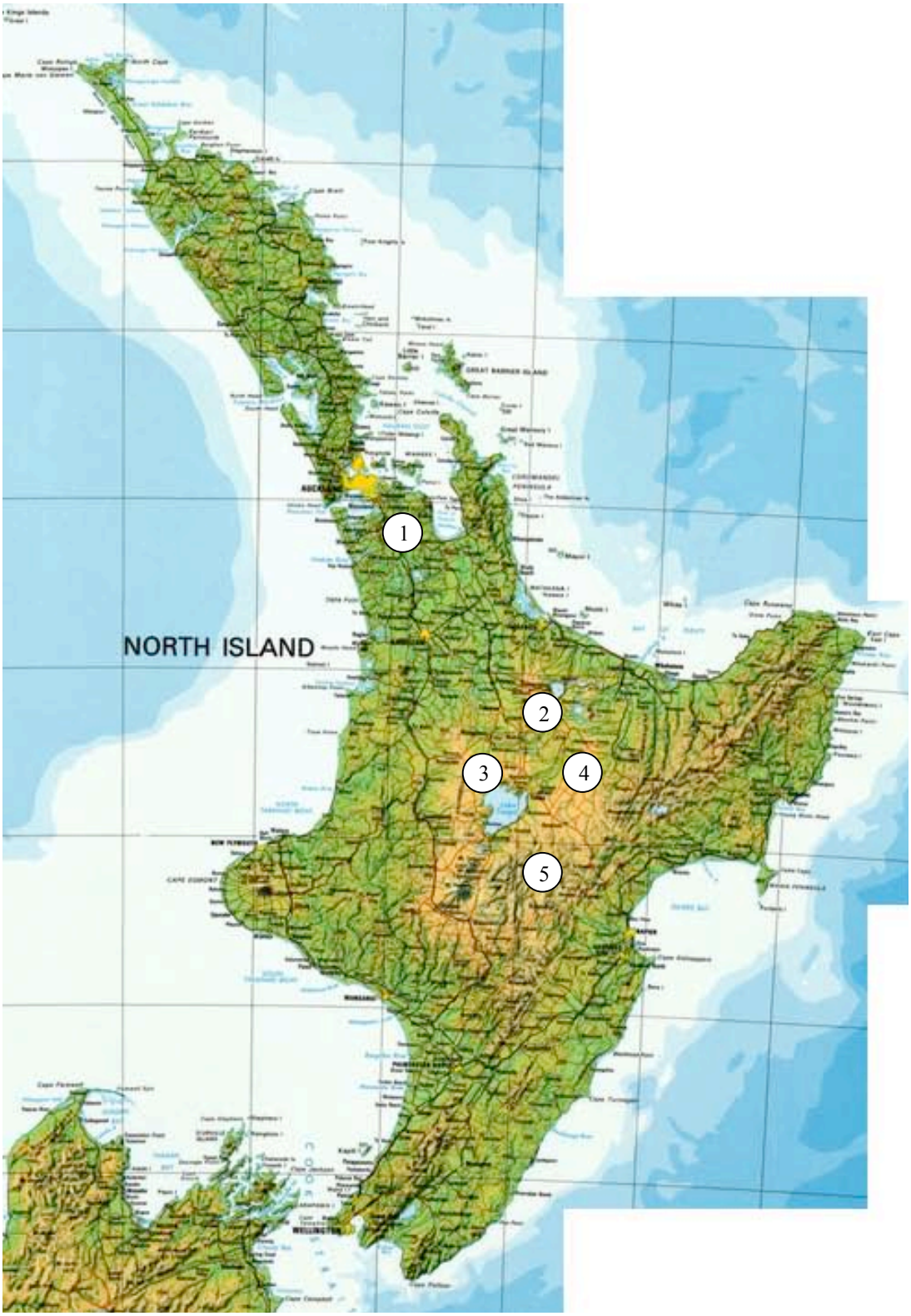
Release permission was obtained from the MAF Chief Veterinary Officer on October 6 2000. Approximately 5700 *E. nassaui* (**Table 3**) were released at four central North island locations (**Figure 5**) during November 2000. A previous release of 1500 wasps at Hunua is detailed in **Chapter 7**.

Table 3. New Zealand release locations and quantities of Tasmanian *E. nassaui* strains released

Site	Location	Florentine	Evandale	Totals
Smythes Rd	Kinleith Forest	1000	100	1100
Poronui Station	Taupo	1000	1000	2000
Cpt. 1060	Kaingaroa Forest	1200	800	2000
Kapenga	Rotorua	300	300	600
Totals		**3500**	**2200**	**5700**

Figure 5. Release sites of Tasmanian *E. nassaui* strains in the central North Island

1 = Hunua, 2 = Kapenga, 3 = Kinleith Forest, 4 = Cpt 1060, Kaingaroa Forest, 5 = Poronui.



DISCUSSION

2.9 The role of climate matching in Biological control

The collection and release of Tasmanian *E. nassaui* strains marked the first milestone in re-establishing biological control over *P. charybdis*. As the biocontrol agent occurred in New Zealand prior to 1989, allowances were available under the Hazardous Substances and New Organism (HSNO) act for importation of these strains.

The decision to import novel strains was based on the assumption that climate was a major limiting factor to the effectiveness of the current strains in New Zealand. CLIMEX mapping indicated that Tasmanian collection sites were demonstrably better suited to the New Zealand climate than Western Australian collection sites, particularly in the central North Island. Natural enemies should be sought from the region of pest origin, in order that they are phenologically adapted to the host and can survive in a similar climate (Mills 1994; Legner & Bellows 1999). Climate matching is believed particularly meritorious in New Zealand because a significant range of different climatic conditions is encountered (Cameron *et al.* 1993). However, some biological control reviews entirely neglect possible effects of climate on Biocontrol (e.g. Beirne 1985; Cock 1986) and others have criticised the use of climatic matching and strains (Clarke & Walter 1995). One main concern is the introduction of new taxonomic species resulting from multiple strain releases. However, as accepted definitions for species, sibling species, subspecies, host races, metapopulations or local populations is a particular problem in entomology, as all "conceivable intermediate states … (exist) in nature" (Symondson & Hemingway 1997), it is probable that many biological control programmes have inadvertently released a range of species.

The sourcing of *E. nassaui* from Tasmania is not meant to imply that this was the optimal climatic match to New Zealand conditions. Tasmania was chosen because it is considered both climatically similar to the central North Island of New Zealand, and also because *P. charybdis* occurs there. The Florentine Valley had previously been used as the source for *C. mellyi* because of its climatic similarity to the central North Island of New Zealand (Bain & Kay 1989).

2.10 Collection and rearing of *E. nassaui*

Enoggera nassaui proved relatively abundant in Tasmania, occurring in 55 field-collected samples spanning nine paropsine species. This represents treble the host species range and collection records recorded for the *Neopolycystus* spp. complex estimated to have consisted of at least two unidentifiable species. *Aphanomerella ovi* and *B. albifunicle* are both known

hyperparasitoids of *E. nassaui* (Tribe 2000). The two hyperparasitoid records collected from *C. purpereo-aurea* indicate that this species is also a likely host of *E. nassaui*.

Enoggera nassaui proved amenable to laboratory rearing, with a total of over 7000 Tasmanian wasps were released. However, these releases were comprised of only two strains, Florentine Valley and Evandale. This limited strain composition resulted from several failures. Firstly, the Tasmanian *E. nassaui* cultures were decimated by the two initially undetected hyperparasitoids. Their presence necessitated the destruction of all contaminated cultures, and subsequent implementation of strict hygiene practices. This included double bagging of all cultures, checking all host foliage used for rearing paropsines for paropsine egg batches (which may have contained parasitoids), and most importantly, maintaining field collections separately from laboratory cultures until they had proven clean of hyperparasitoids. The line rearing used to reduce the contamination risk also maintained strain isolation for later molecular analysis. However, the majority of strains were lost by this contamination.

A further two *E. nassaui* strains were lost within a few generations of their arrival in New Zealand. The initial shipment consisted of all four strains, sent as adults provided with honey water. These suffered high mortality due to the presence of the liquid and the transportation period (approximately three days) required to secure the cultures in quarantine. Surviving adults were used to begin a culture which then foundered when insufficient *P. charybdis* egg batches were available for rearing during the winter period.

Successful quarantine cultures were achieved by making improvements to the shipping and laboratory processes. Firstly, enquiries to MAF revealed that *E. nassaui* could be shipped to New Zealand as parasitised eggs, providing that the egg material was destroyed after parasitoid emergence. This method negated the adverse impacts of international transportation, and the parasitised *P. aegrota* cultures arrived in excellent condition. Meanwhile, the laboratory *P. charybdis* breeding population was increased and the culture stimulated into oviposition by controlling temperature and lighting conditions.

2.11 Release strategy

The quantity of parasitoids released appears to affect the likelihood of establishment. Hopper and Roush (1993) recommended releases of about 1000 individuals per site after analysing previous biocontrol programmes. Similar results were found in New Zealand (Cameron *et al.* 1993; Memmott *et al.* 1998), although some releases have succeeded with remarkably small numbers (Etzel & Legner 1999).

Releases of over 1000 *E. nassaui* were made in three of the four release sites. This provides some confidence that 'best practice' was achieved. The release total was numerically (c.f. approximately 100 000) and geospatially smaller (central North Island and South Auckland c.f. nationally) than the *E. nassaui* releases in the late 1980s. However, the total release volume was similar to the 8550 *E. reticulata* successfully released against the paropsine *Trachymela tincticollis* (Blackburn) in South Africa (Tribe & Cillié 2000).

3. SUMMARY

Biological control of *P. charybdis* in New Zealand was considered hampered by poor climatic matching of the control agent *E. nassaui* to climatic conditions in New Zealand. Importation of Tasmanian *E. nassaui* strains was proposed as a possible solution based on climate matching theory and supported by a CLIMEX based evaluation of temperature profiles.

Enoggera nassaui was the dominant primary egg parasitoid of *Paropsis* and *Chrysophtharta* species in Tasmania, comprising 48% of all records. Several unidentifiable species of the primary egg parasitoid *Neopolycystus* were also encountered, as well as *A. ovi* and *B. albifunicle* which are known obligate hyperparasitoids of *E. nassaui*. Hyperparasitism of laboratory cultures in Tasmania had an adverse impact until this was detected and subsequently prevented by stricter hygiene practices.

Two strains, from the Florentine Valley and Evandale, were successfully imported into New Zealand and established as laboratory cultures in quarantine in March 2000. Following breeding and release permission, a total of approximately 5700 wasps were released in four *Eucalyptus* forests in the central North Island of New Zealand during November 2000.

Chapter 3. Molecular Detection of *Enoggera nassaui* Strains using the Mitochondrial DNA Gene, Cytochrome Oxidase I

1. INTRODUCTION

1.1 Diagnosing strains

Two Tasmanian strains of *E. nassaui* were released in New Zealand in November 2000 to test the value of the climate-matching hypothesis for biological control. Success could only be measured with the ability to detect whether either of these strains had established in New Zealand. This necessitated a tool with sufficient resolution to accurately separate both Tasmanian strains from each either, and also from the genetic material previously released in New Zealand. Such a tool could be used to further monitor dispersal if either strain established.

Diagnostic separation of strains, biotypes or cryptic species can be achieved using characteristics such as morphology (e.g. Weseloh 1982; Otake 1987), behaviour (e.g. Shililu *et al.* 1998) or biology (e.g. Spradbery & Ratkowsky 1974; Smith & Hubbes 1986). However, these same characteristics can be misleading, inconsistent or absent (e.g. Janzon 1986; Wool *et al.* 1994; Langor & Sperling 1995; Jörg & Lampel 1996; Clark *et al.* 2001) and therefore deemed too unreliable to be used to discriminate *E. nassaui* strains. Where 'classical' methods fail, the genetic examination of a species may reveal even subtle population differences that otherwise would not be evident (e.g. Babcock & Heraty 2000; Calvert *et al.* 2001; Scheffer & Lewis 2001; Mander *et al*. 2003).

1.2 Mutations as strain specific markers

The presence of mutations provides the variation capable of accurately distinguishing between strains. Point mutations, or substitutions, result in nucleotide changes in either transition (Ti – purine to purine/ pyrimidine to pyrimidine) or transversion (Tv – purine ↔ pyrimidine) events. In insects, transitions typically outnumber transversions, although in some *Drosophila* (Diptera: Drosophilidae) species the opposite occurs (Moritz *et al.* 1987). If a detected mutation in a gene sequence is unique for a particular strain under study, this mutation can then be used as a strain-specific 'marker'. There is no requirement to understand the function or effect of these mutations.

Markers for the Tasmanian strains had to satisfy several criteria to be valuable. It needed to be shared by all individuals of a strain, as well as their offspring. That is, it could not go undergo recombination or be sex biased. Additionally, the marker needed to be relatively simple to

extract and analyse. Mitochondrial DNA (mtDNA) was identified as fulfilling these requirements.

Mitochondria contain their own DNA as a circular supercoiled double-stranded molecule (Moritz *et al.* 1987; Hoy 1994). The genes coding for its replication, transcription and translation proteins occur in the nuclear DNA (nDNA). As mtDNA generate adenosine-triphosphate (ATP) for cellular energy (Avise *et al*. 1987) they are ubiquitous in somatic and oocyte cells and occur in quantity. Because of its relatively small size of 16 - 36 kilobases (Hoy 1994) the entire mitochondrial genome has been sequenced for several insect species (Beard *et al*. 1993; Crozier & Crozier 1993). The availability of this sequence data from GenBank (Hsiao 1994) combined with published primers (Simon *et al*. 1994) meant that direct sequencing of the mtDNA to test for marker mutations was possible.

Mitochondrial DNA was preferred over nuclear DNA (nDNA) for several reasons. The higher mutation rate, possibly 3-10 times faster than for nDNA (Brown *et al*. 1979; Moritz *et al.* 1987; Watson *et al.* 1992) means that even closely related populations (e.g. strains) may have subtle, detectable differences. Additionally, mtDNA does not undergo recombination, and is maternally inherited (Hoy 1994). Hence, it fulfils the need for a unique genetic marker shared by all individuals of a strain, without chance of recombination.

1.3 Molecular methods

mtDNA was collected and sequenced from the Florentine and Evandale strains and samples collected in New Zealand to find strain-specific markers capable of discriminating between Tasmanian and New Zealand sub-populations. The Cytochrome Oxidase I (COI) gene was targeted because of its rapid mutation rate and predominance in insect molecular studies (Simon *et al*. 1994). These markers were then used to test for establishment of Tasmanian *E. nassaui* in New Zealand.

Sequencing requires a number of steps, including amplification and purification of the target gene. This is achieved using the polymerase chain reaction (PCR) method (Miyamoto & Cracroft 1991; Hsiao 1994: Simon *et al.* 1994). PCR achieves this by using two oligonucleotide primers to flank the DNA segment of interest. The primers 'Ron' and 'Nancy' used in this study were chosen because they have been previously tested on Hymenoptera (Simon *et al*. 1994). Combined with the sample DNA, nucleotides (dNTPs), DNA polymerase (usually the heat tolerant enzyme *Taq* polymerase) and cycles of heating and cooling (Mullis & Faloona 1987; Saiki *et al.* 1988), a typical multiple-cycle (25 - 35)

PCR can amplify the specific DNA fragment found between the two primers millions of times.

The PCR product is then used for sequencing. The Sanger technique (Sanger *et al.* 1977) is in principle similar to a PCR. By adding dideoxy-nucleotide (ddNTPs), nucleotides that lack the hydroxyl terminal position to which a nucleotide can attach, random lengths of the DNA fragment are produced. After fluorescent or radioactive labelling, samples randomly terminating for a specific nucleotide (Adenine, Thymine, Cytosine and Guanine) are run in adjacent wells in an electrophoretic gel matrix. The sequential reading of the bands produced provides the genetic sequence in question.

2. METHODS

2.1 Test samples

Each Tasmanian strain was analysed from the original material imported into quarantine in New Zealand in 1999. During January/February 2002, *E. nassaui* were collected from New Zealand sites where releases occurred in 2000 (Kinleith Forest, Kaingaroa Forest, Kapenga and Poronui Forest) by collecting parasitised *P. charybdis* egg batches. The control samples consisted of a South Island sample from Lyttelton, collected in 1999, and five random samples from the original Perth strain culture (circa 1989). One specimen was analysed to represent each strain or egg batch collection.

2.2 Sequencing and tree construction

Whole individuals were macerated, Proteinase K digested and cleaned using phenol/chloroform extraction (Sambrook *et al*. 1989). DNA was precipitated in equal volumes of 3M sodium acetate and isopropanol overnight at –20°C. After 25 minutes centrifuging at 12 000 rpm, the pellet was washed in 1 ml 70% ETOH for 5 minutes, air-dried and re-suspended in 100 ml Tris EDTA (pH 8) buffer.

Each 25 µl PCR reaction consisted of 3 µl Roche (10x) *Taq* polymerase buffer (+ $MgCl_2$), 2.5 µl dNTPs at 4 mM, 1 unit *Taq* (1 U/µl), 12.5 µl double distilled H_2O, 2.5 µl of the primers C1-J-1751 'Ron' and C1-N-2191 'Nancy' (Simon *et al*. 1994) at 5 µM, and 1 µl of template. Two controls were run, one with DNA from a proven laboratory sample, and one with water. PCR reactions were run on an Applied Biosystems GeneAmp PCR System 9700. After denaturation at 94°C for four minutes, samples were subjected to 30 cycles of 94°C/30 seconds, 55°C/30 seconds, 72°C/45 seconds, and a final extension phase of 4 minutes at 72ºC. Two µl of PCR product were visualised on 2% agarose gel against a 1KB Plus ladder.

Sequencing reactions (Version 3.0 Big Dye® Terminator mix Applied Biosystems) followed the manufacturer's recommendations and were run on an ABI Prism, 3100 16 capillary-array gene analyser. Sequences were aligned and a Neighbour Joining (NJ) tree generated under the Kimura 2-parameter model with 1000 bootstraps in MEGA version 2.1 (Kumar *et al*. 2001)

3. RESULTS

3.1 *Test samples*

Fifteen samples were collected at Poronui and one from Kapenga. No *P. charybdis* egg batches could be recovered from Kaingaroa Forest, or the Kinleith Forest site which had been felled the previous year because of extensive *P. charybdis* defoliation.

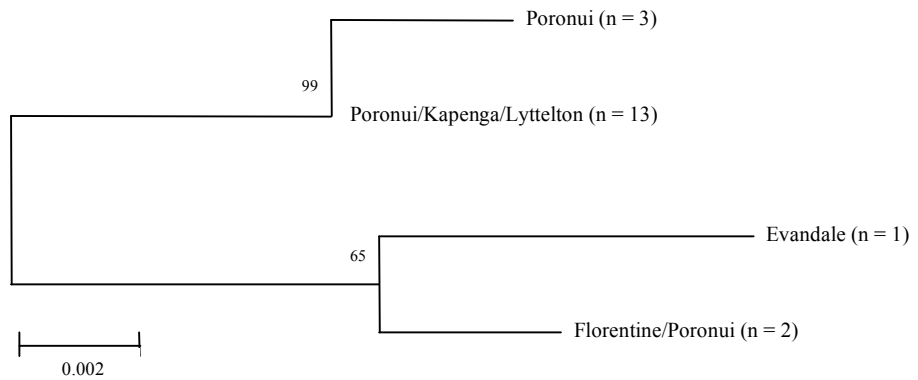
Two egg parasitoids in addition to *E. nassaui* were detected in *P. charybdis* egg batches at both the Poronui and Kapenga sites. These were identified by the author as *Baeoanusia albifunicle* Girault (Hymenoptera: Encyrtidae) and *Neopolycystus* sp. (Hymenoptera: Pteromalidae). This was later identified as *N. insectifurax* Girault (Berry 2003). This represented the first record of these species occurring in New Zealand.

3.2 Sequencing and tree construction

Sequences of approximately 370 bp were obtained for all specimens except the original Perth strain stocks (**Appendix 4**), from which mtDNA could not be extracted. The two Tasmanian strains differed from each other at three nucleotide positions. The Kapenga, Lyttelton and 11 of the Poronui samples differed from the Tasmanian strains by six nucleotides. Three Poronui samples shared a unique substitution, and the remaining Poronui sample shared the same haplotype as the Florentine Valley strain (**Figure 6**).

Figure 6. Neighbour-joining dendogram of *Enoggera nassaui* sequences using Kimura 2-Parameter substitution model

Numbers at branches represent 1000 bootstrap vales. Scale = genetic distance.



4. DISCUSSION

4.1 Establishment of a Tasmanian *Enoggera nassaui* strain

Tasmanian *E. nassaui* strains were released in an attempt to improve biological control of *P. charybdis* in cold climatic areas. Sequencing of the mtDNA COI gene proved capable of distinguishing between all the strains encountered in this study. This tool was then used to test for establishment of either Tasmanian strain.

The Tasmanian strains differed from each other in three nucleotide positions, and were separable from the 'New Zealand' genetic material by another six mutations. A one base-pair difference was also detected in the New Zealand *E. nassaui* population at Poronui. One Poronui sample shared the same haplotype as the Florentine Valley strain released over a year previously at that location. It is concluded from this result that the Florentine Valley strain has established in at least one location in New Zealand. Either the Evandale strain had not established in New Zealand, or the sample size (adversely affected by hyperparasitism) was too small to detect this strain.

4.2 Development of a molecular tool to test for establishment

Mitochondrial DNA was used to test for establishment of Tasmanian *E. nassaui* because of certain beneficial characteristics. The lack of recombination meant that all members of a particular strain would carry exactly the same mtDNA as the maternal line that founded it. The relatively high mutation rate of the molecule also provided the likelihood that sufficient unique point mutations would be present in each strain. mtDNA is generally considered superior to nDNA for population or taxon-specific markers because of these characteristics (Roderick 1996). Although only one sample of each strain was analysed, the known mtDNA characteristics of fidelity/lack of recombination, combined with the deliberate line rearing of strains should have been sufficient to maintain the purity of genetic differences within a strain. However, more confidence would have been obtained if multiple samples of each strain had been sampled.

The COI gene provided sufficient mutations to discriminate between the known *E. nassaui* strains. Not only were the Tasmanian strains discernable based on three mutations, but also an individual base pair substitution was evident within the New Zealand genetic population. Such small differences may not have been detectable using other molecular methods or less rapidly evolving genes.

Direct sequencing of mtDNA was used to obtain the strain specific marker. Although allozymes are commonly employed to discriminate between insect strains (Castañera *et al.*

1983; Blackman & Spence 1992; Jörg & Lampel 1996; Figueroa *et al.* 1999), they tend to underestimate levels of genetic differentiation (Berlocher 1984; Menken & Raijmann 1996). RAPD (Randomly Amplified Polymorphic DNA) techniques have also been used (Zhang and Hewitt 1996) particularly where morphology has been inadequate (e.g. Black *et al.* 1992; Hoy 1994; Shililu *et al.* 1998; Townson *et al.* 1999). Sequencing was preferred because it examines the genetic code directly rather than by interference (Langor & Sperling 1995; Roderick 1996; Reyes & Ochando 1998; Roehrdanz 2001). It has also been suggested that the small effective population size involved using mtDNA (due to haploid nature and maternal inheritance) is superior to nDNA when a population or taxon-specific marker is required (Roderick 1996).

Mitochondrial DNA could not be extracted from the original Perth strain samples to enable sequencing. This could have confirmed the genetic profile of the strains originally released in New Zealand, remembering that a slight genetic variant was found in one population. The storage of these specimens in an open insectary for over 12 years had resulted in a complete loss of colouration. It is expected that this treatment and the poor state of the samples had led to degraded DNA (Dean & Ballard 2001), which prevented its extraction and analysis.

Failure to confirm the Perth haplotype(s) also means that the possibility that *E. nassaui* strains have self-introduced from Australia since the 1980s releases cannot be discounted. The detection of two new egg parasitoids of *P. charybdis* in New Zealand proved that a pathway for these insects into New Zealand exists or existed. A number of self-introduced parasitoids of *Eucalyptus* pests in New Zealand have previously been documented (Berry 2003). However, the haplotype similarity of the South Island (Lyttelton) sample (collected before the Tasmanian strains were released) to the majority of the North Island samples strongly suggests that the extent material in New Zealand is relatively homogenous, and clearly differs from either Tasmanian strain.

The most parsimonious interpretation of these results is that the original Perth strain haplotype currently dominates in New Zealand, that a small amount of variation is present in this population, and that the Tasmanian Florentine Valley strain has established in at least one location.

4.3 Detection of two new egg parasitoids

Two egg parasitoids detected in this study have had a significant impact on the biological control of *P. charybdis* in New Zealand. *Baeoanusia albifunicle* is a known hyperparasitoid of *E. nassaui* (Tribe & Cillié 1997) and reduced wasp recovery from some locations by up to

50%. Jones and Withers (2003) confirmed the adverse impact of *B. albifunicle* on *E. nassaui* populations, and found up to 100% hyperparasitism of the biological control agent could occur. The potential benefit of *Neopolycystus insectifurax* led to commercial mass rearing and release in an attempt to improve biological suppression of *P. charybdis* (Jones & Withers 2003).

It is impossible to determine exactly when *B. albifunicle* or *N. insectifurax* established in New Zealand, or whether these resulted from separate introductions. Populations were found from the Coromandel Peninsular to south of Taupo within a short time of its detection, suggesting establishment had occurred at least several years previously (i.e. prior to the release of Tasmanian *E. nassaui*). However, as the full distribution of this species was never surveyed, this is likely to represent only a partial record of its full distribution. The possibility that *B. albifunicle* was a major factor in the initial decline in *E. nassaui* populations in the late 1990s is excluded on the basis that no sign was found during extensive sampling of *P. charybdis* eggs by the author during this period. The recent mass releases of *N. insectifurax* similarly mean that the prior distribution and spread of this species cannot be determined.

The availability of a tool suitable for monitoring the distribution of *E. nassaui* strains may be of academic value only. The apparent reduction in ability of *E. nassaui* to control *P. charybdis* populations in all climatic areas of New Zealand has essentially nullified the potential benefit of successfully establishing a climatically matched strain. For this reason, the original expectation that dispersal of any established Tasmanian strain would be measured was abandoned.

5. SUMMARY

A diagnostic method was required to identify if either of two Tasmanian *E. nassaui* strains introduced to New Zealand had established. The unsuitability of morphological or behavioural characters led to the selection of molecular methods. Sequencing of the mtDNA COI gene was expected to provide the resolution required because of this molecules rapid mutation rate, maternal inheritance and absence of recombination.

The gene sequences were sufficient to characterise the Tasmanian strains both from each other and from the resident New Zealand population. A small amount of variation was found within the strains originally released in New Zealand. Samples of the original Perth strain stock could not be analysed because of their poor condition to confirm the original haplotypes. The haplotype of one *E. nassaui* sample collected over a year after release of the Tasmanian strains corresponded to that of the Tasmanian Florentine Valley strain. This is taken to suggest that this strain had established in New Zealand a year after release.

Two egg parasitoids of *P. charybdis* were also detected for the first time in New Zealand. *Baeoanusia albifunicle* is an obligate hyperparasitoid of *E. nassaui* and severely reduced the amount of recoverable material from the field. The impact of *B. albifunicle* in reducing the effectiveness of *E. nassaui* against *P. charybdis* suggests that little benefit will transpire from the successful introduction of cold tolerant *E. nassaui* strains, and that there would be little benefit from further analysis of this system. In contrast, *N. insectifurax* offers some hope for biological suppression of *P. charybdis*.

Overall, COI appears to be suitable for monitoring the establishment or dispersal of biological control agents where different population sources or strains are used. Further COI analysis of the original Perth strain samples would clarify the remaining issues raised in this study.

Section Two: Predicting the Paropsine Threat to *Eucalyptus* in New Zealand

"He must have an inordinate fondness for beetles."

J.B.S. Haldane, when asked what his studies revealed about the nature of the Creator.

Chapter 4. Field and Bioassay Assessment of the Host Range of *Enoggera nassaui*

"Species usually evolve from the frying pan into marginally safer fires"
(Lawton 1986)

1. INTRODUCTION

1.1 Potential versus actual host range of *Enoggera nassaui*

Enoggera nassaui (**Figure 7**) is a polyphagous egg-parasitoid (Tribe 2000) with host records from six paropsine genera and an Australian-wide distribution (Naumann 1991). Despite this, observations suggest that *P. charybdis* is the only one of five paropsines in New Zealand susceptible to *E. nassaui*.

Figure 7. *Enoggera nassaui*

Not all hosts are of equal value to parasitoids. Some are more intrinsically central than others and therefore are attacked more vigorously (Shaw 1994). A host range may combine both ecologically or phylogenetically related hosts (Brooks 1981), making it difficult to predict the importance of each susceptible host in advance. Clearly, the host range of *E. nassaui* is restricted, but to what extent and by what factors is unknown. A superior understanding of these factors would enable predictions regarding its success or otherwise against any paropsine species that were to establish in New Zealand in the future.

1.2 Egg-defence strategies

Insect eggs are particularly vulnerable to natural enemies (Hilker 1994; Hirose 1994). Eggs are sessile and frequently exposed to predators and parasitoids, with defence restricted to chemical, internal (physiological) or physical barriers. Selection pressure is expected to improve fitness and reduce vulnerability to enemies (Lawton 1986), with defensive mechanisms conferring up to a 30% advantage over non-defended individuals (Price 1987).

The egg chorion is one feature that could be expected to reduce parasitism by increasing the handling time required by parasitoids (Gross 1993).

Defensive strategies would be expected and are found in the paropsines at various life stages. Larval defences include aposematic/cryptic colouration, conspicuous larval aggregations, prominent setae (Selman 1994a), hydrogen-cyanide excretions (Moore 1967), and nocturnal (Tribe & Cillié 1997) or winter-feeding patterns (Simmul & Clarke 1999). Adults benefit from aposematic/cryptic colouration, 'drop-dead' behaviour when disturbed, and an ability to contract the body tightly against the foliage surface to prevent predation (Selman 1994a).

Despite a lack of documented evidence, the possibility exists that defensive strategies may also exist in paropsine eggs. If so, the presence or absence of these characters could be used to explain the host range of *E. nassaui*. The existence of easily identifiable egg traits that predict their susceptibility to *E. nassaui* would enable rapid assessment of the pest risk from various paropsine species by simply examining their eggs. To achieve this, required the presence of discernable paropsine egg characteristics, and an evaluation of the host range of the parasitoid from both field and laboratory methods.

2. METHODS

2.1 Field collections

Paropsine egg batches were collected from Tasmanian locations during 1998 to 2001. Eggs were determined to species using de Little (1979b) and monitored for the emergence of parasitoids. Egg batches from which at least one individual *E. nassaui* emerged were recorded as a host.

2.2 Parasitism rate bioassay

Enoggera nassaui cultures for bioassay were reared using *P. aegrota* in Tasmania and *P. charybdis* in New Zealand. *Chrysophtharta agricola*, *C. bimaculata*, *C. obovata*, *P. aegrota, P. charybdis, P. porosa and P. rubidipes* were tested in Tasmania. *Paropsis charybdis*, *Trachymela catenata* and *T. sloanei* were tested in New Zealand.

Each replicate consisted of exposing five *E. nassaui* to 30 host eggs (< 24 hours old) for a maximum of one hour under observation. If one parasitoid commenced ovipositor probing of a host egg, the other individuals were removed, the time recorded and the female exposed to the eggs for a further hour. Eggs were then removed and the numbers of parasitised eggs recorded when parasitism signs manifested (e.g. occurrence of diagnostic blotches and parasitism scars). Where no probing of the host eggs occurred in the initial hour, the eggs were removed and replaced with a control group of 30 *P. aegrota* eggs (*P. charybdis* in New Zealand). The replicate was discarded if no probing of the *P. aegrota* eggs occurred within one hour. If probing occurred with the *P. aegrota* eggs, the original host eggs were considered as having been rejected by mature female *E. nassaui*, and the parasitism rate recorded as nil. Ten replicates were completed for each host.

Assays occurred in 90 mm diameter Petri dishes at 22ºC. Parasitism rates (egg per hour^{-1}) were calculated and tested by ANOVA. A posthoc Duncan's multiple range test was used to rank species parasitism in SAS (SAS Institute 1989).

2.3 Egg chorion structure

Egg batches of *Chrysophtharta* (11 species) and *Paropsis* (7 species) were gold coated and imaged using Scanning Electron Microscopy (SEM).

3. RESULTS

3.1 Field collections

Table 4 shows *E. nassaui* was collected from 55 paropsine egg batches (**Appendix 3**). Five *Chrysophtharta* and four *Paropsis* species were recorded as hosts. *Paropsis charyb*dis was recorded for the first time as a host in Australia. *Paropsis aegrota* (17 records) and *C. bimaculata* (13 records) were the most abundant hosts.

Table 4. Field Collections of *Enoggera nassaui* from Tasmanian *Chrysophtharta* and *Paropsis*

C = Chrysophtharta P = Paropsis

Host Species	Collections
C. agricola	1
C. bimaculata	13
C. decolorata	11
C. nobilitata	1
C. obovata	4
P. aegrota	17
P. charybdis	2
P. debeori	1
P. porosa	5
Total	**55**

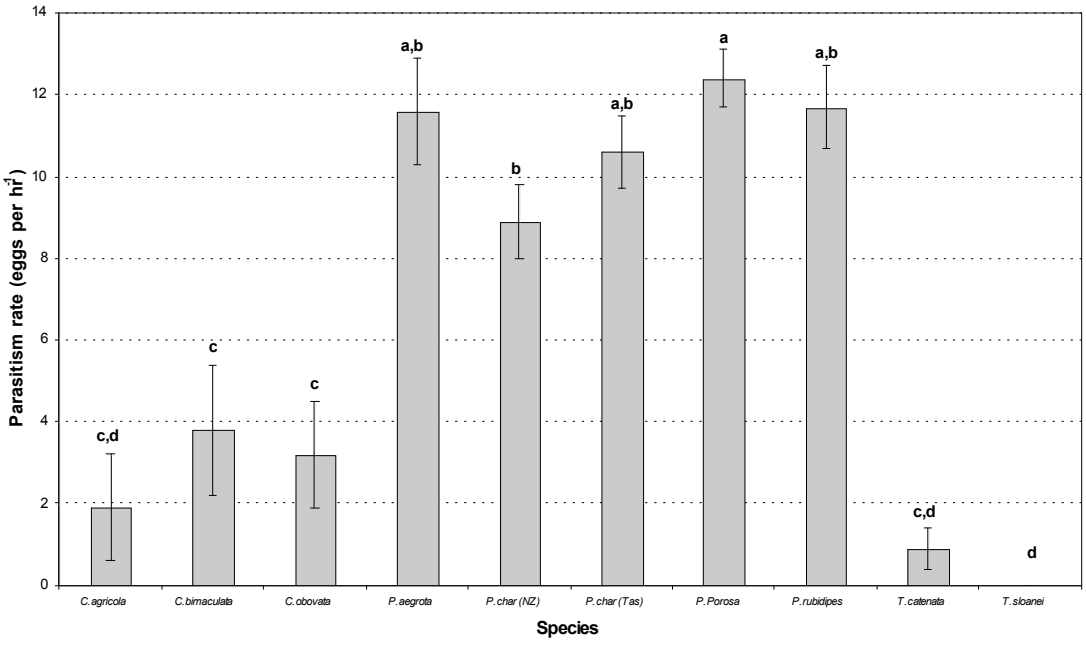
3.2 Host selection and parasitism-rate bioassay

Nine of the ten paropsine species were accepted as hosts in laboratory bioassay (**Figure 8**). All *Chrysophtharta* and *Trachymela* species had rejections, with *T. sloanei* rejected in all replicates (**Appendix 12**). No *Paropsis* replicates were rejected. Two *C. obovata* assays were discarded after the parasitoids rejected both the host and control groups.

Both host species (ANOVA $F = 25.7$, $P < 0.001$) and host genus (ANOVA $F = 112.3$, $P < 0.001$) had a significant influence on the parasitism rate. Parasitism rates on *Chrysophtharta* were significantly lower than for *Paropsis* (ANOVA $F = 113.2$, $P < 0.001$). No significant difference was detected for the performance of *E. nassaui* on *P. charybdis* when reared from either *P. aegrota* or *P. charybdis*.

Figure 8. Mean ± SE one hour parasitism rate of *Enoggera nassaui* on *Chrysophtharta*, *Paropsis* and *Trachymela* hosts (DUNCAN F = 25.7, P < 0.001)

Bars with same letter were not significantly different at p = 0.001

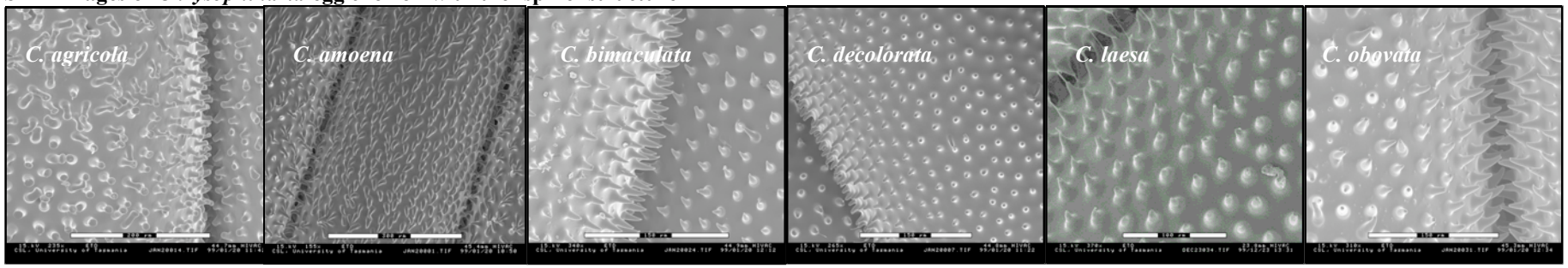


3.3 Egg chorion structure

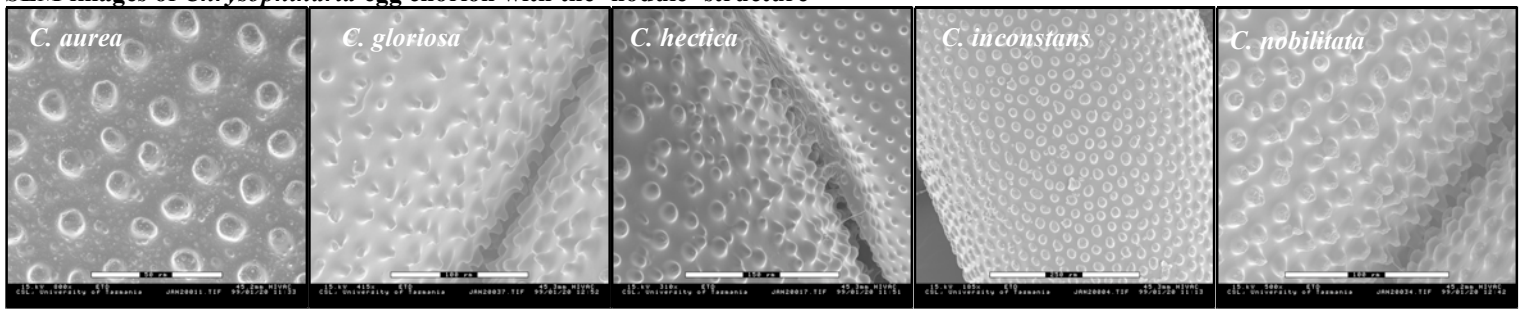
Two distinct chorion motifs were evident in *Chrysophtharta*. Six species displayed spine like protrusions (**Figure 9**) and five species had nodules. Most *Paropsis* species had a slight reticulated pattern. This was most obvious in *P. aegrota*, *P. porosa* and *P rubidipes* and less evident in *P. charybdis*, *P. deboeri* and *P. delittlei*. This pattern was absent in *P. tasmanica*, which also had distinctive longitudinal ridges.

Figure 9. Scanning Electron Micrographs of *Chrysophtharta* and *Paropsis* egg chorion

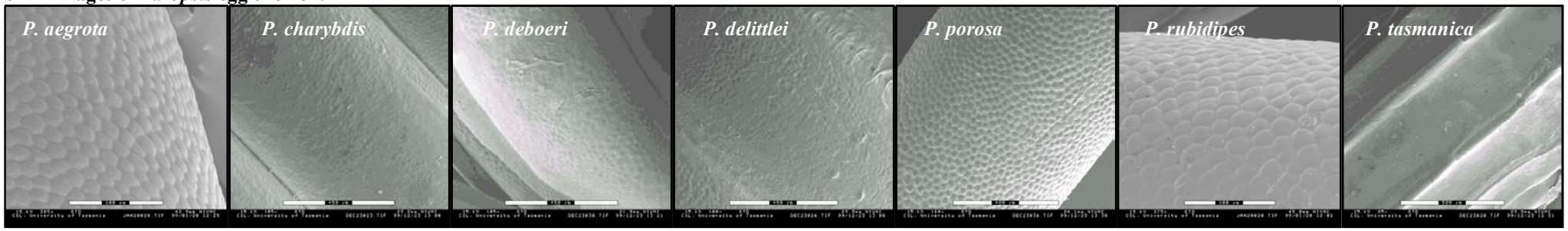
SEM images of *Chrysophtharta* egg chorion with the 'spine' structure



SEM images of *Chrysophtharta* egg chorion with the 'nodule' structure



SEM images of *Paropsis* egg chorion.



4. DISCUSSION

4.1 The host range of *Enoggera nassaui*

A broad host range would be a valuable asset for *E. nassaui* in its role as a biological control agent in New Zealand. Although known to be polyphagous (Naumann 1991), the extent of this capability had never been quantified. Field collections and laboratory bioassays were undertaken to help describe this host range, and to determine if any obvious host factors could be used to predict susceptibility to the parasitoid.

Field collections confirmed that *Chrysophtharta* and *Paropsis* species are natural hosts of the parasitoid. Significantly, the first Australian record of *P. charybdis* as a natural host was obtained. Bioassays showed *Paropsis* species were consistently susceptible to the parasitoid, and no significant intra-generic differences were found. Parasitism rates on *Chrysophtharta* and *Trachymela* species were significantly lower than for *Paropsis*, and these hosts were frequently rejected by the parasitoid in the no-choice bioassays.

Studies have shown that egg parasitism rates on paropsine are generally low in Australia. Mo and Farrow (1993) found egg parasitism of *P. atomaria* and *C. varicollis* (Chapuis) in the Australian Capital Territory totalled 6.6% and 5.2% respectively. Parasitism is completely absent on *Peltoschema* (=*Acaciacola*) *orphana* (Simmul & Clarke 1999). Bashford (1997) was confounded by the low egg parasitism rates for *C. agricola* (0%) and *C. bimaculata* (2.6%) in Tasmania. de Little *et al.* (1990) also found egg parasitism on *C. bimaculata* to be rare as did Nahrung and Murphy (2002) for *C. agricola*. Bashford (1997) noted that the high parasitism rates on *P. aegrota*, *P. charybdis* and *Paropsisterna* spp. were not reproduced in *Chrysophtharta* species, and that this may partially explain their more regular outbreaks. This contrasts strongly with parasitism rates achieved by *Enoggera* species used as classical biological control agents, where parasitism can exceed 90% (Kay 1990; Murphy & Kay 2000; Tribe & Cillié 2000).

The parasitism rate achieved by *E. nassaui* on favoured hosts was relatively high compared to the most commonly studied egg parasitoid genus, *Trichogramma* (Hymenoptera: Trichogrammatidae). For example, *Trichogramma poliae* Nagaraja parasitised between 9 and 15 eggs per day^{-1} on two lepidopteran hosts (Ahmad *et al*. 1999), and other *Trichogramma* species parasitised on average 10 *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) eggs in 24 hours (Silva & Stouthamer 1999). *Enoggera nassaui* was capable of rates exceeding 12 eggs per hour^{-1}.

The combined field and laboratory results confirm that *E. nassaui* is polyphagous, but that host acceptance and parasitism rates are variable within this range. This suggests that mechanisms may exist on host eggs that modify their suitability to *E. nassaui*. The egg chorion was suspected to be a possible influence.

4.2 Paropsine egg chorion characteristics

The possibility exists that egg chorion modifications can influence parasitism. As Nordell-Paavola *et al.* (1999) surmised, "it is evident that the structure of the chorion is of utmost importance to the species survival and is expected to be under strong selection pressure. Variation in the surface structure and colouration of the chorion undoubtedly reflects these pressures…". In spite of this, Hinton's (1981) treatise on insect eggs dedicates a chapter to defensive devices without mentioning the possibility or existence of chorion structures potentially reducing parasitism. Hilker's (1994) essay on the protection of chrysomelid eggs similarly avoids discussion of any such possibility.

Paropsine eggs are unusual in having chorion structure (de Little 1979b; Selman 1994b). Coleopteran eggs are generally soft and smooth surfaced (Crowson 1981; Lawrence & Britton 1991). The hexagonal patterns on *Diabrotica* (Coleoptera: Chrysomelidae) eggs are considered typical of insects because they correspond to the shape of the follicle cells used in chorion formation (Rowley & Peters 1972). Petitpierre and Juan (1994) found reticulated polygons, scales/warts, comma/dot shaped relief and fenestra (punctures) in 16 chrysomelid species. Under SEM, the *Paropsis* eggs examined were similarly smooth with a slight to moderate reticulation. *Paropsis deboeri* and *P. tasmanica* additionally had longitudinal ridging. In contrast, the *Chrysophtharta* eggs studied all exhibited pronounced chorion modification under SEM, ranging from nodules to spines.

4.3 Defence against parasitoids

Parasitism is a selective pressure, reducing host fitness and providing an impetus for evolution (Boulétreau 1986). Certain oviposition behaviours and egg modifications are thought to reduce egg parasitism, with an emphasis on avoidance or concealment (Gross 1993). These include shortening or excluding egg stages (Dobler & Rowell-Rahier 1996), smearing eggs with regurgitated food or excrement (Hilker 1994; Selman 1994b), insertion of eggs into crevices or holes (Crowson 1981) or egg clustering to protect basal eggs from natural enemies (Stamp 1980). Host egg size can also be a major determinant of parasitoid host range (Hirose 1994).

Similar behaviours capable of averting egg parasitism have been observed in paropsines. For instance, *T. catenata* eggs have a secretory coating (Barrett 1998). During the parasitism bioassay, *E. nassaui* were observed inspecting the eggs and exhibited typical pre-oviposition behaviours including antennal drumming. However, these parasitoids frequently left the eggs and commenced grooming to remove the exudate from their legs. Only three of ten *T. catenata* replicates were attacked by *E. nassaui*. The unsuitably small size of *T. sloanei* eggs is likely to explain their complete rejection by *E. nassaui*, even if the parasitoid could find these eggs which are normally hidden under the bark of host trees. *Chrysophtharta lignea* effectively avoids egg parasitism by laying eggs which hatch within minutes. Both *C. agricola* and *C. obovata* lay egg batches in clumps where basal eggs are completely covered. This behaviour could reasonably be expected to protect basal eggs from parasitism, but did not explain the low bioassay acceptance and parasitism rates. None of these behaviours helped explain the overall differences in parasitism susceptibility between *Paropsis* and *Chrysophtharta* hosts.

4.4 A case for chorion influence on egg parasitism

From the bioassays, a putative relationship appears to exist between the presence of egg chorion structure on *Chrysophtharta* eggs and their parasitism by *E. nassaui*. Parasitism rates were significantly lower on the spined *Chrysophtharta* eggs than on the smooth-egg *Paropsis* hosts. The presence of chorion modification such as spines is therefore implicated with reduced susceptibility, and this one character could be used to explain differences in egg parasitism previously observed between the genera (Bashford 1997). This relationship remains putative, because no method was found capable of removing the egg chorion structures without subsequent damaging of the host egg.

Although tempting to speculate that the presence of chorion spines, egg clumping or sticky chorion surfaces exist to protect eggs from parasitism, it can be problematical to allocate a defensive role to such traits. Secondary functions may be equally parsimonious (Hinton 1981; Gross 1993) and the current utility of a trait is not necessarily its original function (Pagel 1994), in which case it can be referred to as an *exaptation* (Baum & Larson 1991). For instance, scales present on the eggs of the lepidopteran *Thaumetopoea pityocampa* (Den. and Schiff.) are considered to provide protection from both the environment and parasitism (Schmidt *et al.* 1999). Extra-chorion projections may well increase parasitoid handling time, and clumping may protect internally located eggs. However, as the majority of the paropsine species tested oviposit on exposed host foliage, these characters might also have important roles controlling temperature or reducing desiccation. As it stands, there appears to be a

definite relationship between paropsine chorion structures and egg parasitism, but correlation is not necessarily causation.

The *Chrysophtharta* species tested all had eggs with spine like modifications. Unfortunately, no species with the nodule class chorion were available at the time of testing. If chorion structure reduces egg parasitism, a suitable test would be to analyse the parasitism rate of *E. nassaui* against *Chrysophtharta* species with the nodule chorion eggs. The nodule chorion structure appears to be intermediate between that of the spiny *Chrysophtharta* and smooth *Paropsis* eggs. If parasitism rates against this group were intermediate between those two groups, it would strengthen the hypothesis that chorion structures affect parasitism by *E. nassaui*, and that parasitism rates decline as chorion structure increases.

4.5 Experimental limitations

Field parasitism rates were not calculated, partially because of the large sample sizes collected (e.g. thousands of egg batches were collected for *C. agricola* alone), but also because of the difficulty in correcting parasitism rates to accurately account for the differential in exposure times for field collections, i.e. field collected eggs have invariably been exposed to parasitism for different amounts of time (Mo and Farrow 1993). There also appears to be some discrepancy between the field and laboratory parasitism results, e.g. *C. bimaculata* yielded 13 field records yet proved to be a poor host in the laboratory bioassay. Sample size is used to explain this. *Chrysophtharta bimaculata* is an abundant pest species in Tasmania and thousands of egg batches were collected in the field for recovery of parasitoids. The 13 records collected from this host represent a parasitism rate substantially less than 1% and explains this apparently inconsistent result.

The classical methodology for estimating egg parasitism is the exposure of abundant host eggs to a parasitoid for 24-hour periods, with results expressed as the numbers of eggs parasitised. The initial design used in this study was based on Tribe (2000) who exposed groups of *E. nassaui* to 30 host eggs for a period of 24 hours. However, several features of this methodology proved inappropriate for use with *E. nassaui*.

The 24 hour exposure method frequently resulted in erratic parasitism. In many cases, several egg batches in the same trial would be ignored, with eggs in some egg batches super-parasitised (as determined by oviposition scars) to the extent that parasitoid larval development failed. Therefore, accurate parasitism rates could not be determined. Increasing host densities to 50 and then 100 eggs did not alleviate this problem.

Preliminary trials were also hindered by difficulty in distinguishing parasitoid sex, an issue particular to *E. nassaui* (Naumann 1991). The logical method for identification of females is observation of oviposition. Individual parasitoids attacking eggs are obviously a) female, and b) in a physiological condition to parasitise hosts. The adverse effect is that parasitoids gain experience when attacking hosts, which may affect their later selection (Bjorksten & Hoffman 1995; Dutton *et al.* 2000). An effective bioassay for *E. nassaui* parasitism needed to identify females, assess host acceptance or rejection and provide accurate parasitism rates if hosts were accepted, whilst avoiding inducing host selection bias as a result of providing previous host experience. The bioassay developed was expected to solve these problems.

The resulting bioassay first exposed five inexperienced *E. nassaui* (sex unknown) to the target host group. Groups of adults were used as this increased the probability that at least one female was present in each assay. If the target eggs were rejected (i.e. not probed by ovipositor) within one hour, a control group of *P. aegrota* eggs were supplied for one hour. Subsequent acceptance of these control group eggs by a parasitoid confirmed that at least one female parasitoid was present and physiologically capable of attacking eggs. Ergo, the target eggs had been rejected by a female parasitoid capable of parasitism. This pattern occurred in many instances. In several instances both the test and control groups were rejected, suggesting either only males or females not ready to parasitise were present. The bioassay also provided both a means of estimating host acceptance and subsequently an hourly attack rate, which may be more relevant to field conditions than exposure to hosts for 24 hours.

Other factors known to influence host acceptance and parasitism rates were controlled where possible. All host eggs used were less than 24 hours old as they can decline in attractiveness and suitability with age (Ruberson *et al.* 1987; Hu *et al.* 1999; Godin & Boivin 2000; Honda & Luck 2000; Tribe 2000). The host from which a parasitoid is reared may condition their host range (Kudon & Berisford 1980; Ram *et al.* 1995; Henter & van Lenteren 1996) although this may be less influential than previous experience parasitising a host (Bjorksten & Hoffman 1995, Keasar *et al.* 2001). *Paropsis aegrota* was chosen as the rearing species and control group (*P. charybdis* had to be used in New Zealand through necessity) because of its ease of laboratory rearing and noted susceptibility to *E. nassaui* from field records. The influence of host rearing species appeared negligible. If influential, *E. nassaui* reared on *P. charybdis* in NZ would have been expected to have higher parasitism rates on this host than *P. aegrota* reared parasitoids on *P. charybdis* in Tasmania, but this did not occur.

A larger number of Tasmanian paropsines were initially trialed using the subsequently abandoned 24 hour parasitism bioassay. These trials showed that *E. nassaui* could parasitise

eggs from *C. aurea*, *C. laesa*, *C. nobilitata*, *P. deboeri*, *P. delittlei, P. tasmanica* and *Paropsisterna nucea*. Hence the host range of *E. nassaui* is potentially far greater than recorded in this study, but this data was of insufficient quality for statistical comparison.

5. SUMMARY

The host range of *Enoggera nassaui* is known to span a number of paropsine genera from field collections and records in Australia. However, gaps in this host range are known to occur from observations in New Zealand, and its effectiveness against specific species or genera has never been evaluated empirically.

Tasmanian field collections confirmed that *E. nassaui* is polyphagous. Five *Chrysophtharta* and four *Paropsis* species were recorded as natural hosts, and significantly the first host record for *P. charybdis* was recorded in Australia. Bioassays were undertaken on the eggs of ten *Chrysophtharta*, *Paropsis* and *Trachymela* species to determine host acceptability and parasitism rates. The egg chorion of these and other paropsine species were then examined under SEM for comparison with the results.

Nine paropsine species were successfully attacked in the bioassay, with one *Trachymela* species rejected in all replicates. *Paropsis* species were significantly more susceptible to *E. nassaui* than *Chrysophtharta* species, with *Chrysophtharta* species regularly rejected by the parasitoid. This data agreed with field observations and other studies suggesting egg parasitism on *Chrysophtharta* was generally lower than found in *Paropsis*.

Three chorion motifs were found within the study group. *Paropsis* eggs were generally smooth with slight reticulation, typical for Coleoptera. *Chrysophtharta* species exhibited greater chorion modification, with either prominent nodules or projections. The hypothesis was put forward that these modifications serve to reduce egg parasitism. This requires further study, and if correct, the presence/absence of chorion structure could be used to rapidly predict the susceptibility of a given paropsine species to *E. nassaui*.

Chapter 5. Phylogenetic Reconstruction of Tasmanian *Chrysophtharta*

1. INTRODUCTION

1.1 The purpose of phylogenetics

A study of various biological characteristics of paropsine species was undertaken to identify traits that could be used to predict their potential impact in New Zealand. A more meaningful assessment of the paropsine threat to *Eucalyptus* in New Zealand could result from an understanding of the genetic relatedness of the study taxa. This field of study is known as phylogenetics, and a phylogeny is a hypothetical genealogical tree of a group of related species (Rieppel 1994).

Traditional cladistic methods used morphological characters, or character states, to define the similarity of species to each other (Maddison 1994). DNA is now the preferred tool because it has universality, potentially large volumes of collectable data (Crozier 1993), and DNA evolution is better understood than for morphological characters (Miyamoto & Cracroft 1991). Both morphological and molecular data can be combined in cladistical analyses (e.g. Schilthuizen *et al*. 1998; Calvert *et al*. 2001). True phylogenies are rarely known (Fitch & Atchley 1987; Hillis *et al*. 1992), so the robustness/reliability of a phylogeny depends on the quality and quantity of data, and algorithms/assumptions used.

Insect phylogenies are typically constructed to interpret associations (Harvey & Pagel 1991) such as bio-geographic distribution (Juan *et al.* 1996; Becerra & Venable 1999), character traits (Stern 1998; Köpf *et al.* 1998) and host-plant relationships (Eastop 1986; Humphries *et al.* 1986; Futuyma 1994; Becerra 1997; Mardulyn *et al.* 1997; Janz & Nylin 1998). A *Chrysophtharta* phylogeny was required to allow better interpretation of fecundity and other collectable data.

1.2 Tree building methods

Traditionally, phylogeny construction/cladistics utilised morphological characters, or character states (presence/absence of characters) to reconstruct genealogical relationships among organisms (Maddison 1994). Characters differ in their utility for phylogenetic reconstruction. The presence of *synapomorphies*, or shared derived characteristics, provides evidence for phylogenetic ancestry. Primitive features maintained in groups (*plesiomorphies*) are generally ignored as they provide little information about relationships (Miyamoto & Cracroft 1991; Scotland 1993). Convergent characteristics, similar features arising independently in differently lineages, confuse the use of morphological characteristics.

A phylogeny is presented as a dendogram, or tree, with branches that correspond to the species under study (Eggleton & Vane-Wright 1994). Trees can be rooted or unrooted; rooted trees suggest the temporal or ancestral position of species, while unrooted trees provide information on distances between taxa but no indication of ancestry (Weir 1990). Outgroup species, a taxon or taxa distantly related to the study group, are generally included in a phylogeny to help root the tree.

A number of tree building options are available, and substitution models accounting for mutation rates and behaviour further refine the trees. Tree building methods include the 'Distance' methods, such as unweighted pair-group method with arithmetic averaging (UPGMA) and Neighbour Joining (NJ). These are relatively simple and calculate the distance of each species from aligned sequences (Nei 1991). Neighbour joining methods are amongst the fastest and simplest of minimum evolution methods (Nei 1991).

Camin and Sokal (1965) proposed the use of parsimony principles, or maximum parsimony (MP), that the simplest, most economical solution should be used in tree development. The tree is determined by using the least amount of changes to explain the observed nucleotide differences (Crozier 1993; Williams 1993). An exhaustive tree search evaluates all possible bifurcating trees for the data set (Kitching 1993). This is problematical for large taxon, where astronomical numbers of possible trees are generated. MP methods also have difficulty resolving branch lengths, and ignore characters unique to a taxon from analysis even though they provide useful data (Nei 1987). Maximum likelihood (ML) (Felenstein 1981) improves upon MP by using all available data. Although ML is considered more powerful than many other techniques, it requires stricter assumptions about the rate and type of evolutionary change (Hoy 1994).

Examples of substitution models include the Jukes and Cantor (1969) Distance (JC69) or Kimura (1980) 2-Parameter (K2P) models. JC69, one of the earliest and simplest models, assumes all substitutions are independent, all positions in the sequence are subject to change with equal probability and that any substitutions replace randomly with any nucleotide. In contrast, K2P treats transversions and transitions independently.

1.3 Selection of the target gene

Careful consideration must be given to the gene(s) used in phylogenetic studies. Closely related species require fast evolving genes to provide sufficient information; deeper lineages or inter-generic comparisons may require slower evolving genes. For instance, Hsiao (1994)

found that the conserved ribosomal 12S and 16S mtDNA genes were identical within conspecific beetles from the same geographic region. In contrast, the mtDNA gene COI evolves relatively fast (Crozier *et al*. 1989) which can result in high within-genus differences, *i.e*. 21% for the leaf beetle genus *Ophraella* (Funk *et al*. 1995).

Mitochondrial DNA is commonly used for phylogenetic reconstruction (Hillis 1987). According to Avise *et al*. (1987), mtDNA contains most of the ideal characteristics for a molecular phylogenetic tool. As mitochondria are ubiquitous in animals it provides an expedient source of comparable homologous DNA sequence (Harrison 1989). The presence of conserved primers (Simon *et al*. 1994) means that specific genes can be targeted across species without prior knowledge of their nucleotide composition.

Insect phylogenies are commonly built using the Cytochrome Oxidase I (COI) gene (Caterino *et al*. 2000). COI has resolved phylogenetic relationships for a number of insect groups (e.g. Kruse & Sperling 2001; Litzenberger & Chapco 2001; Manfrin *et al*. 2001) including Coleoptera (Juan *et al*. 1995, 1996; Funk 1999; Termonia *et al*. 2001). COI has proven particularly useful where morphological characters have confounded analyses (Cognato & Sperling 2000; Maus *et al*. 2001) and has provided variability sometimes lacking in nuclear genes (Andreev *et al*. 1998).

1.4 An independent phylogeny test; Chorion analysis

Phylogenies can be constructed utilising several data sources (Schilthuizen *et al.* 1998; Calvert *et al.* 2001). Independent information can be used to corroborate a hypothesised phylogeny because ecological/morphological traits can reflect phylogeny (Thompson 1994; Miller & Wenzel 1995).

The egg chorion phenotype is an indirect manifestation of the nuclear genetic code, because amino acid sequences define the structural proteins that define the chorions' characteristic shapes or patterns (Petitpierre & Juan 1994). The chorion structure is independent of mtDNA. Chorion comparisons have proven useful in separating closely related or cryptic insect species (Rowley & Peters 1972; Nokkala & Nokkala 1994; Petitpierre & Juan 1994; Nordell-Paavola *et al*. 1999). Therefore, the *Chrysophtharta* egg chorion structure could be used as an independent test of any hypothesised tree.

1.5 Phylogenetic aims

Development of a Tasmanian *Chrysophtharta* phylogeny was considered a useful process that would enhance interpretation of comparative data from other studies. mtDNA sequence data

was analysed using various tree building methods, and the egg chorion structure used to validate the chosen model.

2. METHODS

2.1 Sample collection and DNA Extraction

Tasmanian *Chrysophtharta* were collected from the field and identified from the keys of de Little (1979a,b) and Selman (1983). Larvae from eggs and field collected larvae were reared to maturity as required in the laboratory. All specimens were labelled and stored in 70-96% ETOH prior to shipment to New Zealand. *Paropsisterna nucea*, *Dicranosterna semipunctata*, *Paropsis aegrota* and *P. rubidipes* were included as outgroup species.

A leg or small ($2mm^2$) piece of tissue was removed from individuals and macerated, Proteinase K digested and cleaned using phenol/chloroform extraction (Sambrook *et al*. 1989). DNA was precipitated in equal volumes of 3M sodium acetate and isopropanol overnight at –20°C. After 25 minutes centrifuging at 12 000 rpm, the pellet was washed in 1 ml 70% ETOH for 5 minutes, air-dried and re-suspended in 100 ml Tris EDTA (pH 8) buffer. Presence and quality of DNA were checked on a 2% agarose gel.

2.2 PCR

Each 25 µl PCR reaction consisted of 3 µl Roche (10x) *Taq* polymerase buffer (+ $MgCl_2$), 2.5 µl dNTPs at 4 mM, 1 unit *Taq* (1 U/µl), 12.5 µl double distilled H_2O, 2.5 µl of the primers C1-J-1751 'Ron' and C1-N-2191 'Nancy' (Simon *et al*. 1994) at 5 µM, and 1 µl of template. Reactions were run on an Eppendorf Mastercycler Gradient 5331 with positive and negative controls. Samples were initially denatured at 94˚C for four minutes, then subjected to 30 cycles of denaturing at 94˚C/30 seconds, annealing at 50˚C/30 seconds and strand extension for 72˚C/45 seconds. After a final extension phase of 4 minutes at 72˚C, samples were stored at 4˚C. Two µl of PCR product were checked on a 2% agarose gel against a Pst I ladder, stained with ethidium bromide (2mg/ml) and visualised under UV light.

2.3 DNA Precipitation

PCR products were purified to remove sequence-inhibiting reagents. To 20 µl of PCR product was added 20 µl 4M ammonium acetate and 40 µl isopropanol, left at room temperature for 30 minutes, and spun for 30 minutes at 12 000 rpm. The supernatant was discarded and the pellet washed in 800 µl of 70% ETOH for 5 minutes. After spinning at 12000 rpm for 10 minutes, the alcohol was decanted, the pellet air dried then re-suspended in 20 µl ddH_2O. Two µl of product were quantified on 2% agarose gel.

2.4 Sequencing Reactions

Sequencing reactions were performed using the Amplicycle® sequencing kit (Applied Biosystems). Cycle sequencing used an initial denaturing at 94˚C/3 minutes, 24 cycles of denaturing at 94˚C/60 seconds, annealing at 55˚C/60 seconds and extension at 72°C/60 seconds. A series of extension steps were performed to completely terminate strands using 10 cycles of 95°C/30 seconds and 70°C/60 seconds. Four µl of Formamide stop solution terminated reactions, which were stored at 4°C until use.

Samples were denatured for 5 minutes at 94˚C prior to electrophoreses on a 6% denaturing polyacrylamide gel (60 ml PAGE, 540 µl ammonium persulfate and 30 µl TEMED) for 3-6 hours at 6000volts/70 watts in 1 x TBE buffer. Short and long run gels were run to maximise readable sequence. Gels were transferred to Whatman paper, vacuum dried for one hour at 80˚C and exposed to X-ray film (Kodak BioMax™) from 24 to 72 hours. Sequences were read manually and saved as text files.

2.5 Tree building

Sequences were checked against the GenBank® nucleotide sequence database using BLAST searches to confirm homology with Coleoptera COI. Sequence data was aligned using ClustalW v1.7 (Thompson *et al*. 1994) and trees run under NJ, UPGMA and MP methods using both JC69 and K2P substitution models with 10 000 bootstraps in MEGA version 2.1 (Kumar *et al*. 2001).

2.6 Independent chorion analysis

The chorion state for each study taxon was defined as noduled or spined and mapped against the chosen tree.

3. RESULTS

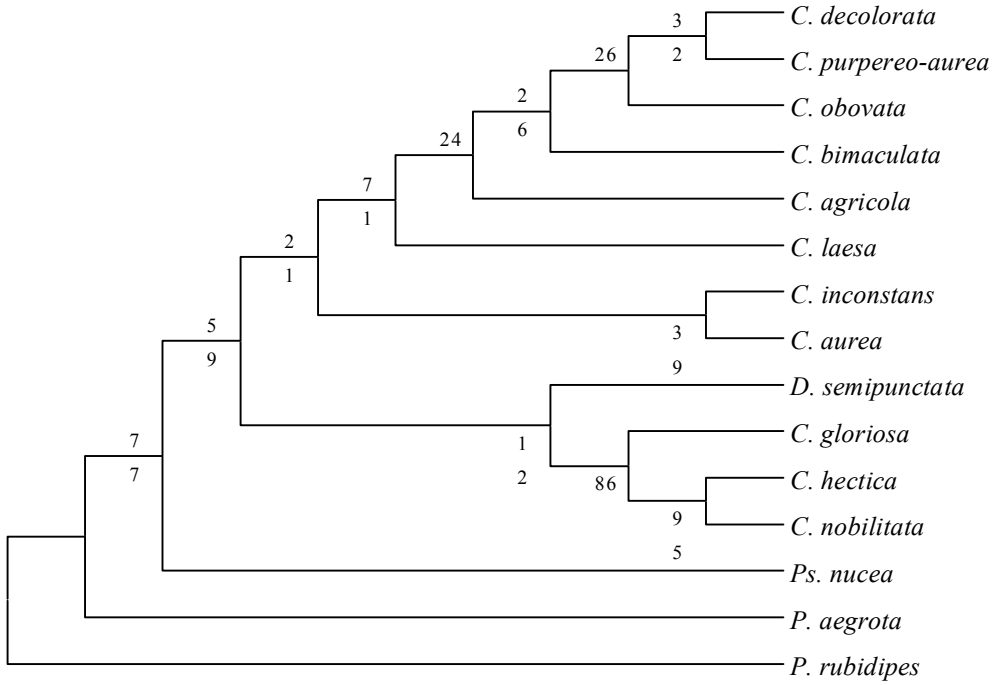
3.1 DNA sequencing

Up to 400 bp of readable sequence were obtained (**Appendix 5**) for 11 of the 14 *Chrysophtharta* species and the outgroup species. Suitable sequences were not obtained from *C. amoena*, *C. lignea*, and *C. philomela*. *Chrysophtharta* sequences were AT rich (63.8%), and transitions (33) outnumbered transversions (24).

3.2 Tree building

All tree building methods separated the outgroup species from *Chrysophtharta* with the exception of the MP tree (**Figure 10**) which placed *D. semipunctata* within *Chrysophtharta*. This tree also separated the two *Paropsis* species, so was not considered robust. *Paropsisterna* appears more closely related to *Paropsis* than *Dicranosterna* under all trees.

Figure 10. *Chrysophtharta* phylogeny using MP and K2P substitution model



The UPGMA (**Figure 11**) and NJ trees (**Figures 12** and **13**) had similar topologies with little influence from the substitution models. The major differences were that the UPGMA method consistently separated out *C. aurea* and *C. inconstans* from the other *Chrysophtharta* divisions, and placed *C. obovata* and *C. purpereo-aurea* as closely related species. The preferred trees were under the NJ method as they placed *C. agricola* and *C. obovata* closer

than the UPGMA trees (see Discussion). Of the two, the favoured tree (**Figure 13**) used the JC69 substitution model, as it provided the most favourable placement of *C. agricola* with *C. obovata*. This tree divides *Chrysophtharta* into two major divisions. The 'upper' division houses six species (*C. agricola*, *C. bimaculata*, *C. decolorata*, *C. laesa*, *C. obovata* and *C. purpereo-aurea*). The 'lower' division includes five species (*C. aurea*, *C. inconstans*, *C. hectica*, *C. gloriosa* and *C. nobilitata*).

Figure 11. *Chrysophtharta* phylogeny using UPGMA and K2P substitution model

Scale = genetic distance.

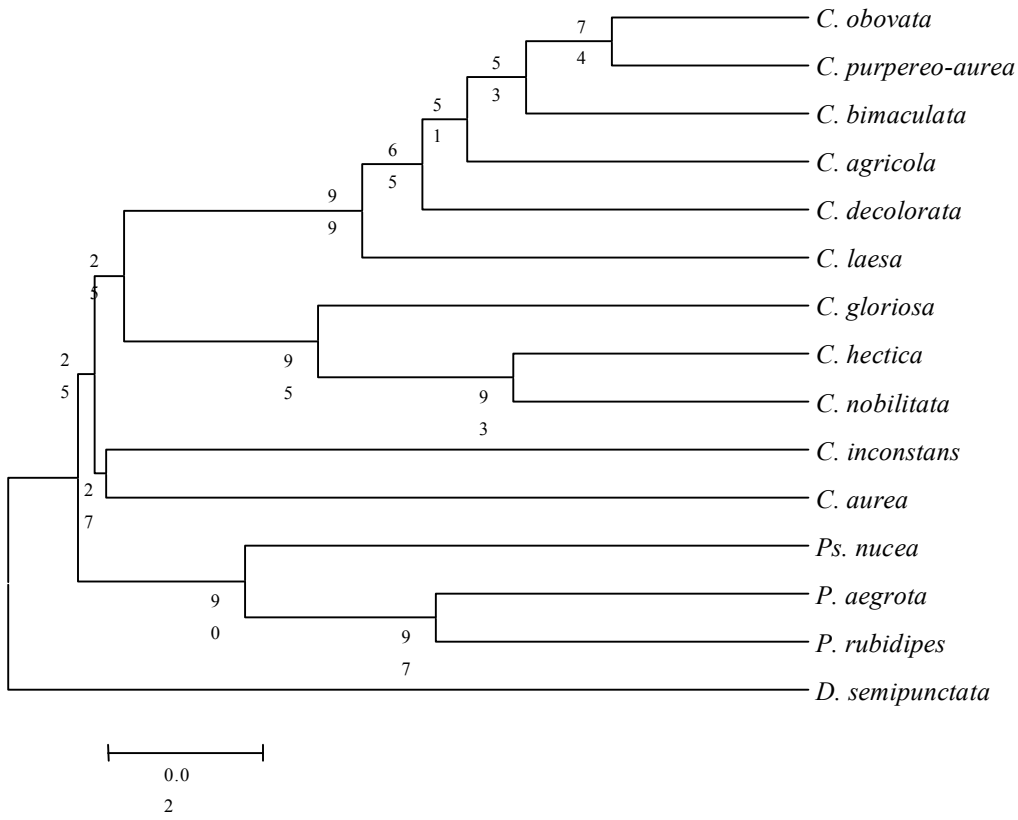


Figure 12. *Chrysophtharta* phylogeny using NJ methods and K2P substitution model

Scale = genetic difference.

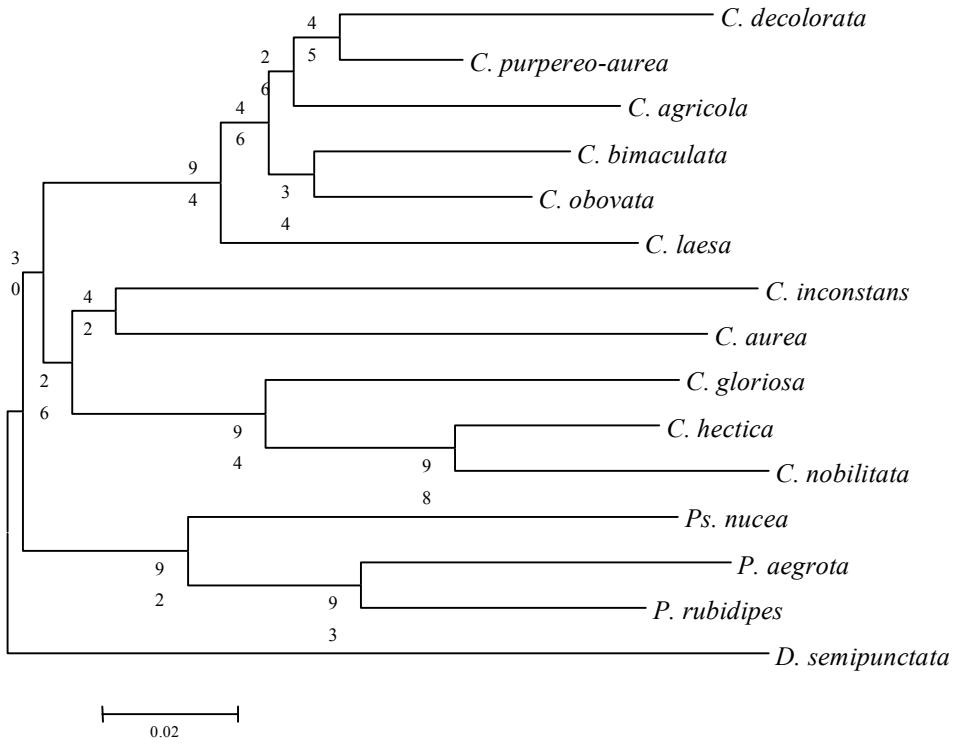
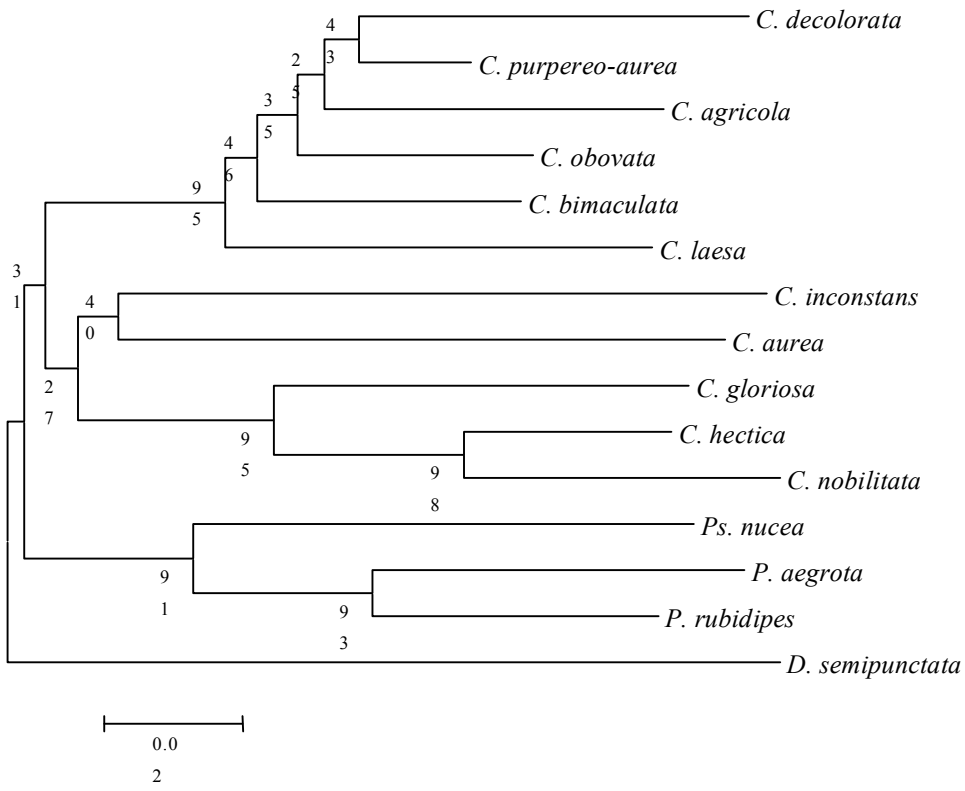


Figure 13. The chosen *Chrysophtharta* phylogeny, using NJ and JC69 substitution model

Scale = genetic distance.

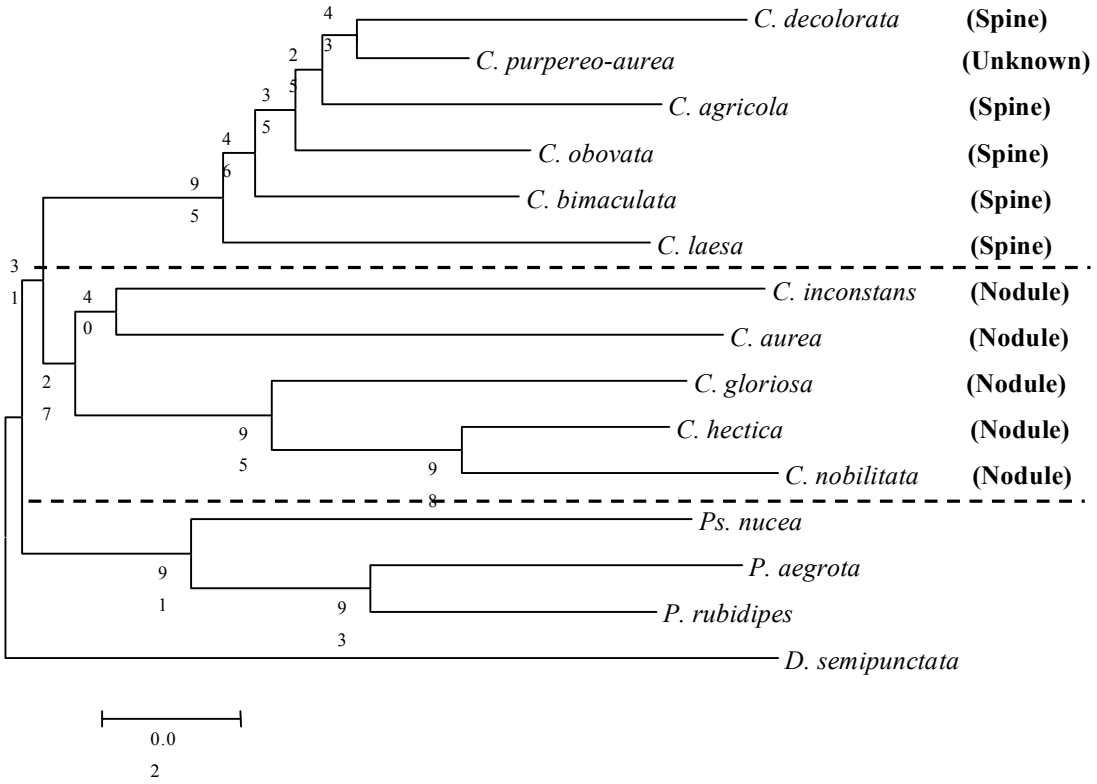


3.3 Independent chorion analysis

Figure 14 shows the character state of egg chorion morphology mapped against the chosen *Chrysophtharta* phylogeny. This process corroborated the separation of the studied *Chrysophtharta* into two divisions. The 'upper' division contained species exhibiting the spine chorion morphology. The chorion morphology of *C. purpereo-aurea* is unknown. The five species in the 'lower' division all have egg chorions in the nodule class.

Figure 14. Chorion analysis of *Chrysophtharta* phylogeny topology

Trait in brackets indicates egg chorion morphology. Scale = genetic distance. Dotted line indicates hypothetical divisions in the tree, the upper, lower and outgroup divisions.



4. DISCUSSION

4.1 The *Chrysophtharta* phylogeny

A *Chrysophtharta* phylogeny was built to allow evaluation of paropsine life history traits in the context of taxa relatedness. mtDNA sequences provided the phylogenetic data and were corroborated with egg chorion morphology as an independent test of tree topology. The final tree contained 11 *Chrysophtharta* species with four outgroup species from the genera *Paropsis*, *Paropsisterna* and *Dicranosterna*.

Several tree building methods were used. The MP tree was not considered suitable as it placed *Dicranosterna* within *Chrysophtharta.* The NJ and UPGMA methods built similar trees with subtle topological differences, and were little influenced by the substitution models. The final NJ tree with JC69 substitution model was selected because it best resolved the expected pattern where {*C. agricola* + *C. obovata*} and {*C. nobilitata* + *C. gloriosa*} are closely related pairings (de Little 1979b). *Chrysophtharta nobilitata* and *C. gloriosa* were consistently paired regardless of tree or substitution method. This pairing, along with that of the two *Paropsis* species, had consistently high bootstrap values. Other relationships within the phylogeny appear less well resolved, with relatively low bootstrap values.

A notable feature of the tree was that it partitioned the *Chrysophtharta* species into two distinct clades. This partition is supported by the chorion morphology analysis. Of the ten species with known chorion structure, the five species with spined eggs were separated from the five species with nodules.

4.2 Molecular methods

Mitochondrial DNA was used to construct the phylogeny because of a number of attributes. This molecule is extractable from pinned, alcohol or poorly preserved specimens, whereas allozyme techniques require stricter storage methods. Also, allozyme electrophoretic mobility cannot be safely used to construct or infer a phylogeny (Avise *et al.* 1987).

Sequence data was used to drive the phylogeny. Molecular data are considered superior to morphological data because characters are more clearly defined, and are available in larger quantities (Friday 1994). Zhang and Hewitt (1996) described DNA sequencing as the molecular technique giving “the highest resolution without ambiguity”. Morphological characteristics other than the egg chorion were not considered useful to helping construct the phylogeny as Selman (1985a) has already determined dead paropsine adults lack useful taxonomic characteristics.

DNA sequences are now relatively easy to obtain for phylogeny construction (gene trees), resolution of species, cryptic groups or populations. This removes potentially erroneous interpretation of phenotypes, but is subject to particular limitations (e.g. repeat site mutations, frequency of transitions vs. transversions, different mutation rates along lineages). Although nucleotide mutation rates do not appear as consistent as predicted by a molecular clock (Kimura 1968; King & Jukes 1969), they are more regular than morphological change, consequently providing superior data for phylogeny construction (Nei 1987). However, single-gene phylogenies may not reflect the true ancestry of a population of species (Crozier 1993), because single genes do not necessarily evolve in concert with the total genome.

However, there are several problems peculiar to mtDNA that can affect phylogeny construction, such as hetroplasmy, nuclear mtDNA copies and lineage sorting. Hetroplasmy can occur where mtDNA mutations within an individual lead to multiple mtDNA genotypes, although this is expected to be rare. Non-functional nuclear copies of mtDNA sequences have been detected in some insects (Zhang & Hewitt 1996), which could be confused with genuine mtDNA sequences. Lineage sorting is a stochastic process where by chance some females only have male offspring, and subsequently do not contribute mtDNA to subsequent generations. This 'self pruning tree' could make it unlikely that mtDNA from more than two founding lineages will be present in a population after several generations (Avise *et al*. 1987).

Particular patterns of maternal lineage sorting may result in the closer similarity of mtDNA of some individuals to other species than for conspecifics (Avise 1994). mtDNA is also sensitive to founder events or bottlenecks, increasing the probability of fixing a single lineage in small populations (Roderick 1996). These problems all appear to be rare but their possibility of occurring should not be ignored.

4.3 Chorion validation of tree topology

The egg chorion morphology was mapped against the chosen tree as an independent test of its topology. *Chrysophtharta* species in the study group have egg chorion features classed as either nodules or spines. Mapping of this character against the chosen tree replicated the major division into two clades. Hence, the egg chorion character appears to corroborate the gross topology of the chosen tree.

A robust tree topology should be able to resolve either the character state of a species in the tree without chorion information, or define the location (in terms of the two major clades) of a species for which the egg chorion morphology is known. For instance, the egg chorion of *C. purpereo-aurea* is unknown. According to its position in the tree, its eggs would have spines.

Chrysophtharta amoena is also missing from the tree, but known to have spiny eggs (**Figure 9**). If the tree was robust, it would be placed somewhere in the appropriate clade.

4.4 Experimental limitations

Initially, phylogenies were planned for both *Paropsis* and *Chrysophtharta*. However, extracting and sequencing difficulties meant that the *Paropsis* phylogeny had to be abandoned. Only the two species with clean sequences (*P. aegrota* and *P. rubidipes*) could be used and were consigned as outgroup species for the *Chrysophtharta* tree.

Single-gene phylogenies may not reflect the true ancestry of a population of species because single genes do not necessarily evolve in concert with the total genome (Crozier 1993). Ideally, the *Chrysophtharta* phylogeny would have been undertaken using both multiple genes and multiple individuals of each species. However, because of the difficulties encountered in sequencing one gene for individual specimens of each species, this enhancement could not be realistically undertaken within the scope of this study.

5. SUMMARY

A Tasmanian *Chrysophtharta* phylogeny was constructed to allow future testing of empirical data against genealogical patterns. A 400bp partial sequence of the mtDNA COI gene provided the tree building data. Sequences were obtained for 11 of 14 *Chrysophtharta* species and combined with outgroup species from *Paropsis*, *Paropsisterna* and *Dicranosterna*.

A number of tree building methods were used, with Neighbour Joining under a JC69 substitution model providing the most parsimonious tree. This was based on correct placement of outgroup species, and expected resolution of several closely related taxa. The tree divides *Chrysophtharta* into two major clades. The chorion morphology state (spines, nodules) was mapped against the chosen phylogeny as an independent test of tree topology. This corroborated the gross topology of the tree, with each clade comprising species with the same egg chorion state.

Chapter 6. Assessment of Paropsine Fecundity as an Indicator of Pest Potential

1. INTRODUCTION

The paropsines are speciose, invasive, and variable in their impact both in Australia and outside their normal distribution. Around 700 species exist in the paropsine chrysomelid complex (Selman 1985a; de Little 1989; Selman 1994a; Simmul & de Little 1999). Within Australia, relatively few species are pests, with *C. agricola*, *C. bimaculata*, *P. atomaria* Olivier and *Peltoschema orphana* the most notable (Carne 1966; Tanton & Khan 1978a; de Little 1989; Candy *et al.* 1992; Elliott *et al*. 1998; Simmul & Clarke 1999; Nahrung 2004).

Seven paropsine species are currently confirmed as established outside Australia. In New Zealand, *Paropsis charybdis* curtailed establishment of a viable commercial *Eucalyptus* forestry estate, whereas the other four species (*Trachymela catanata*, *T. sloanei*, *Peltoschema* sp. and *Dicranosterna semipunctata*) have had a negligible to moderate impact on hosts. *Peltoschema suturalis* was eradicated in New Zealand. Both *T. sloanei* (Miller 2000) and *C. M-Fuscum* are pests of *Eucalyptus* in California, along with *T. tincticollis* in South Africa (Tribe & Cillié 1997).

Insect species establishing outside their normal distribution often benefit from a lack of natural enemies. Assuming host plants and climate are not limiting factors, biological parameters such as reproductive output may significantly determine their impact.

1.1 A possible relationship between fecundity and pest status

Perpetuation of a species requires survival of progeny to sexual maturity. Therefore, natality rates should be higher than 'anticipated' mortality. An evolutionary choice can be made to allocate resources into either large quantities of offspring with little individual resource investment, or to fewer individuals with increased investment (Speight *et al.* 1999). These 'choices' or strategies were allocated the terms r- or K-selection respectively, based on the work of MacArthur (1960) and MacArthur and Wilson (1967). Under some interpretations, opposing life strategies are evident (Pianka 1970; Stubbs 1977) and are used to provide criteria for evaluating differing biological strategies, including fecundity and reproductive resource allocation (Parry 1981). Although thoroughly criticised (Parry 1981; Boyce 1984) and now considered passé (Reznick *et al.* 2002), this theory was initially useful in indicating the presence of different reproductive strategies among related species.

The paropsines are known to have variable impacts both in their natural distribution and where they have accidentally been established in exotic countries. For instance, Tasmanian paropsines range from outbreak/persistent pests to rare (de Little 1979b). The possibility exists that differing life strategies could be explained by reproductive output. For instance, Nothnagle and Schultz (1987) suggest that forest pest species capable of outbreaks should have higher fecundities than 'benign' relatives. However, the oviposition rate may be even more important than fecundity, as Ohmart *et al.* (1985) considered it probably the most important factor in population dynamics of *P. atomaria*. This was because females were unlikely to survive more than a few months in the field.

A molecular *Chrysophtharta* phylogeny was developed with the intent to test the genealogical component of reproductive output for this genus. Common ancestry can lead to shared life histories, behaviour and morphological traits (Thompson 1994). For instance, Dobler and Rowell-Rahier (1996) found that closely related species in the leaf beetle genus *Oreina* shared similar reproductive parameters.

Evaluation of reproductive parameters could indicate benchmark values above which pest status can be predicted, and for *Chrysophtharta*, would demonstrate whether variation in this behaviour is attributable to genetic relatedness. Therefore, the reproductive output of Tasmanian and New Zealand established paropsine species was measured, and in the case of *Chrysophtharta* species, analysed in a phylogenetic context.

2. METHODS

2.1 Pest status

Pest species were determined by literature review.

2.2 Paropsine study taxa

Chrysophtharta and *Paropsis* species, and *Paropsisterna nucea* were field collected in Tasmania as either mature beetles, larvae or egg batches. Larvae from eggs and field collected larvae were reared to maturity as required in the laboratory. Life stages were identified from the keys of de Little (1979 a, b) and Selman (1983). *Paropsis charybdis*, *T. catenata* and *T. sloanei* were collected from *Eucalyptus* in the central North Island of New Zealand. *Dicranosterna semipunctata* was also examined but is reported elsewhere (**Chapter 7**).

Up to 20 replicates were studied for each species. Each replicate consisted of a male-female pair housed in a two-tier plastic container rearing system. This system provided the beetles with a sprig of host foliage in the upper compartment (700 000 ml^3 volume). The sprig stem ran down into a smaller container filled with water to maintain foliage turgidity. Host foliage (*E. nitens*, *E. viminalis* or *E. pulchella*) was replaced when required. Males were replaced upon death or shared between females where required. Rearing occurred under natural light at 22ºC.

2.3 Reproductive measurements

Any eggs present in a replicate were removed and counted every 24 hours. Females laying less than five egg-batches in total were treated as outliers and discarded from the data set. Several paropsine species do not typically oviposit in batches (*C. aurea*, *C. lignea*, *P. porosa* and *P. rubidipes*) so the total compliment of eggs laid in a 24-hour period were treated as a single batch for analysis purposes. As *T. sloanei* typically oviposits under bark, a cork device was constructed based on Tribe and Cillié (1985).

2.4 Data analysis

Reproductive data were partitioned into total fecundity, eggs per day and egg-batch size for each species and genera. Means ± standard errors were calculated and the data graphed. Significant differences within genera were tested by ANOVA, and where detected, posthoc Tukey's HSD tests were undertaken because of unequal sample sizes. Independent t-tests were undertaken on the fecundity and oviposition rate of the species in the 'upper' and 'lower' divisions of the *Chrysophtharta* phylogeny. *Chrysophtharta* fecundity and oviposition rates were mapped against the *Chrysophtharta* phylogeny. The relationship between fecundity and oviposition rates was graphed using all species. All statistical analyses were undertaken

using SAS (SAS Institute 1989). Data for *Ps. nucea*, *T. catenata* and *T. sloanei* were not included in the statistical analysis but were provided for comparison.

3. RESULTS

3.1 Pest status

In Tasmania, *C. agricola*, *C. bimaculata*, *P. charybdis*, *P. delittlei* and *P. porosa* were identified as pest species. *Paropsis charybdis* is a pest in New Zealand. *Chrysophtharta obovata* is a pest in South Australian pest (Phillips 1996) but not in Tasmania. *Trachymela sloanei* is a pest in California, but not New Zealand. Although *P. aegrota* is cited as a pest of *Eucalyptus* in South Australia (Phillips 1996), it is only considered common and widespread in Tasmania (de Little 1979a).

3.2 Paropsine study taxa

Data was obtained for 12 *Chrysophtharta*, seven *Paropsis*, two *Trachymela* and one *Paropsisterna* species (**Appendices 6 - 11**). Both Tasmanian and New Zealand populations of *P. charybdis* were examined. Between two to 20 replicates were available for each species (**Table 5**). Data was not obtained for *C. hectica*, *C. philomela* or *P. dilatata*.

Table 5. Paropsine study species, host species, and numbers of replicates

C = Chrysophtharta, *P = Paropsis*, *Ps = Paropsisterna*, *T = Trachymela*. (P) = pest species

Paropsine species	Pest status	Host	n =
C. agricola	(P)	*E. nitens*	20
C. amoena		*E. viminalis*	12
C. aurea		*E. pulchella*	20
C. bimaculata	(P)	*E. nitens*	20
C. decolorata		*E. pulchella*	18
C. gloriosa		*E. nitens*	7
C. inconstans		*E. viminalis*	2
C. laesa		*E. nitens*	20
C. lignea		*E. nitens*	6
C. nobilitata		*E. viminalis*	20
C. obovata	(P)	*E. viminalis*	20
C. purpereo-aurea		*E. pulchella*	7
P. aegrota	(P)	*E. nitens*	20
P. charybdis (NZ)	(P)	*E. nitens*	20
P. charybdis (TAS)	(P)	*E. nitens*	17
P. deboeri		*E. viminalis*	4
P. delittlei	(P)	*E. nitens*	8
P. porosa	(P)	*E. nitens*	20
P. rubidipes		*E. nitens*	2
P. tasmanica		*E. viminalis*	6
Ps. nucea		*E. viminalis*	7
T. catenata		*E. nitens*	8
T. sloanei	(P)	*E. nitens*	20

3.3 Reproductive measurements

3.3.1 *Fecundity*

Mean *Chrysophtharta* (502.8 ± 122.8 SE) and *Paropsis* (685.4 ± 100.2 SE) fecundity did not significantly differ (t-test, P = 0.33). Fecundity levels within *Chrysophtharta* (**Figure 15**) were significantly different (ANOVA F = 24.6, P < 0.001), ranging from 105.8 ± 32.3 SE for *C. lignea* to 1428 ± 174.7 SE eggs for *C. obovata*. Differences in mean *Paropsis* fecundity (**Figure 16**) were significant (ANOVA F = 6.7, P < 0.001) ranging from 127 ± 10.4 SE (*P. delittlei*) to 1044.7 ± 139.1 SE (*P. charybdis* (Tas)). Both *Trachymela* species had fecundity of circa 400 eggs.

3.3.2 *Oviposition rate*

Mean oviposition rates were not significantly different (t-test, p = 0.59) between *Chrysophtharta* (9.72 ± 1.55 SE) and *Paropsis* (10.91 ± 0.97 SE). Oviposition rates were significantly different within *Chrysophtharta* (ANOVA F = 35.1, P < 0.001) and *Paropsis* (ANOVA F = 4.9, P < 0.001). *Chrysophtharta* oviposition rates (**Figure 17**) ranged from 1.9 ± 0.2 SE (*C. lignea*) to 19.8 ± 1.2 SE (*C. obovata*). Rates for *Paropsis* (**Figure 18**) ranged from 7.3 ± 0.0 SE for *P. rubidipes* to 14.9 ± 1.5 SE for *P. charybdis* (Tas).

3.3.3 *Mean egg batch size*

Mean egg batch size was not significantly different (t-test, p = 0.7) between *Chrysophtharta* (14.4 ± 4.4 SE) and *Paropsis* (16.1 ± 2.1 SE). Mean egg batch size (**Figure 19**) was significantly different within *Chrysophtharta* (ANOVA F = 243.6, P < 0.001) ranging from 56.6 ± 2.2 SE for *C. obovata* to 2.6 ± 0.1 SE for *C. lignea*. Mean egg batch size of *C. obovata* was significantly higher than other *Chrysophtharta* species. *Paropsis* species (**Figure 20**) also had significant differences (ANOVA F = 20.4, P < 0.001), ranging from 26.4 ± 2.7 SE for *P. deboeri* to 8.5 ± 0.1 SE for *P. rubidipes*.

3.3.4 *Relationship between fecundity and Oviposition Rate.*

A positive relationship ($R^2 = 0.79$) existed between increasing fecundity and oviposition rates (**Figure 21**).

Figure 15. Mean ± SE *Chrysophtharta* fecundity

Bars with the same letters are not significantly different at P < 0.001.

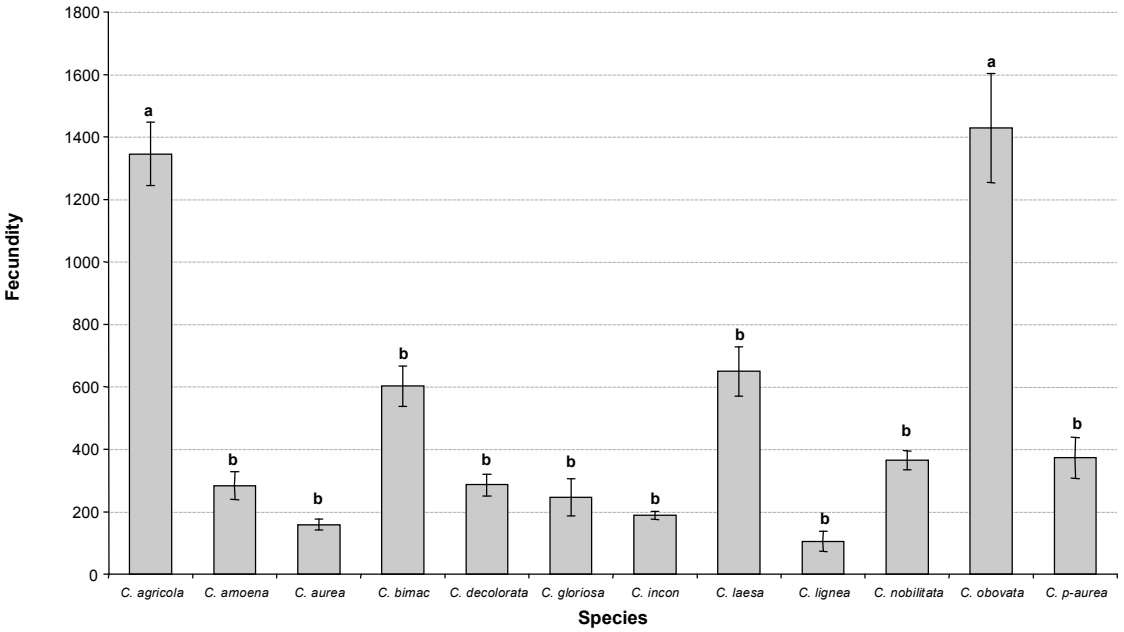


Figure 16. Mean ± SE *Paropsis* fecundity

Bars with the same letters are not significantly different at P < 0.001.

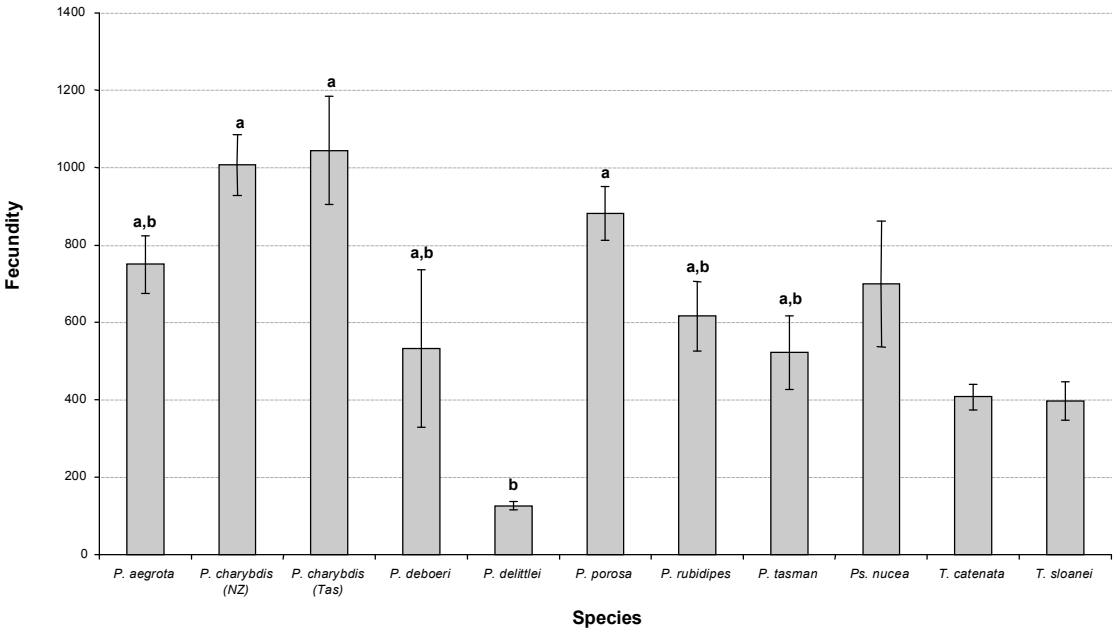


Figure 17. Mean ± SE *Chrysophtharta* oviposition rate

Bars with the same letters are not significantly different at P < 0.001.

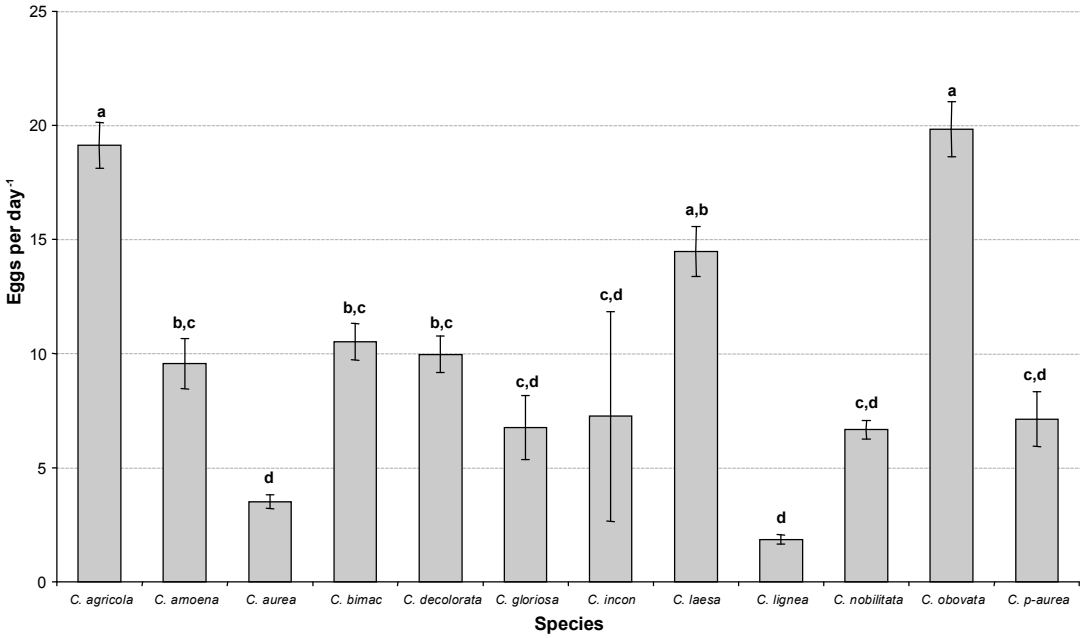


Figure 18. **Mean ± SE *Paropsis* oviposition rate**

Bar with the same letters are not significantly different at P < 0.001.

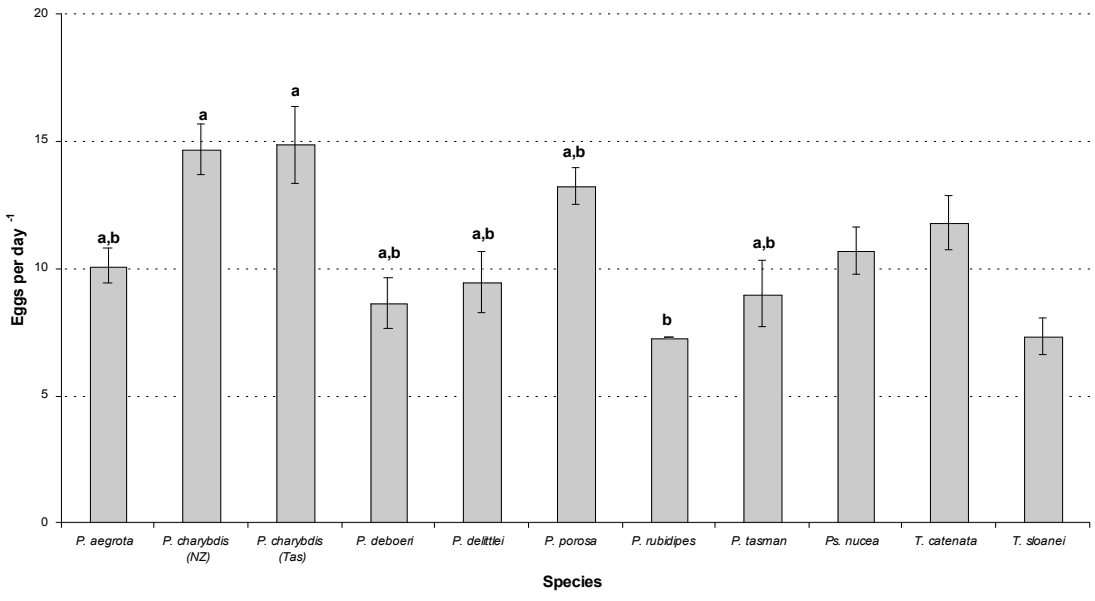


Figure 19. Mean ± SE *Chrysophtharta* egg batch size

Bars with the same letters are not significantly different at P < 0.001.

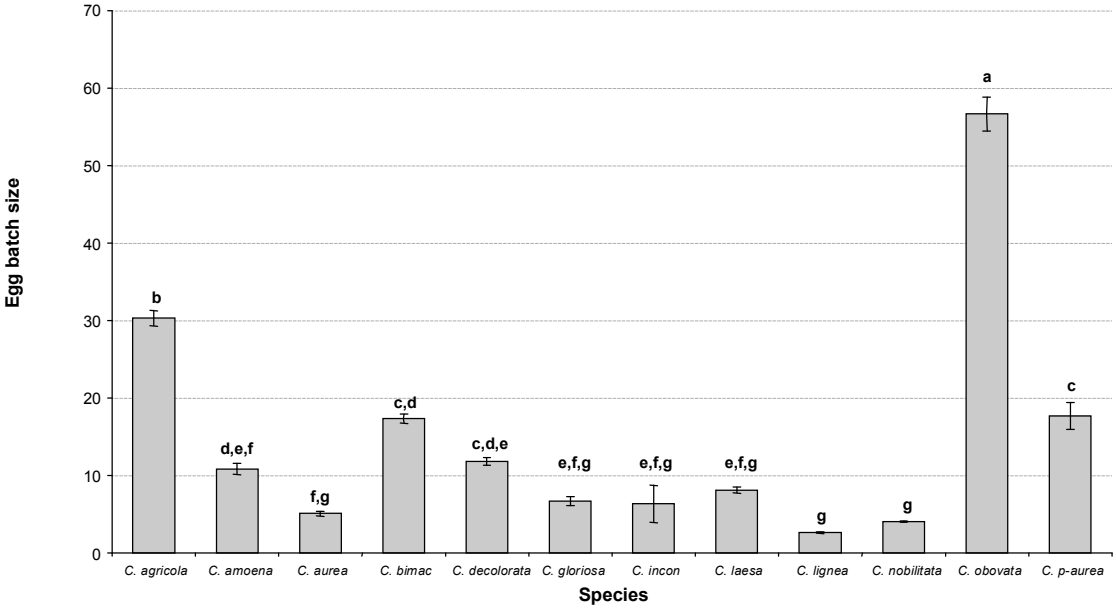


Figure 20. **Mean ± SE *Paropsis* egg batch size**

Bars with the same letters are not significantly different at P < 0.001.

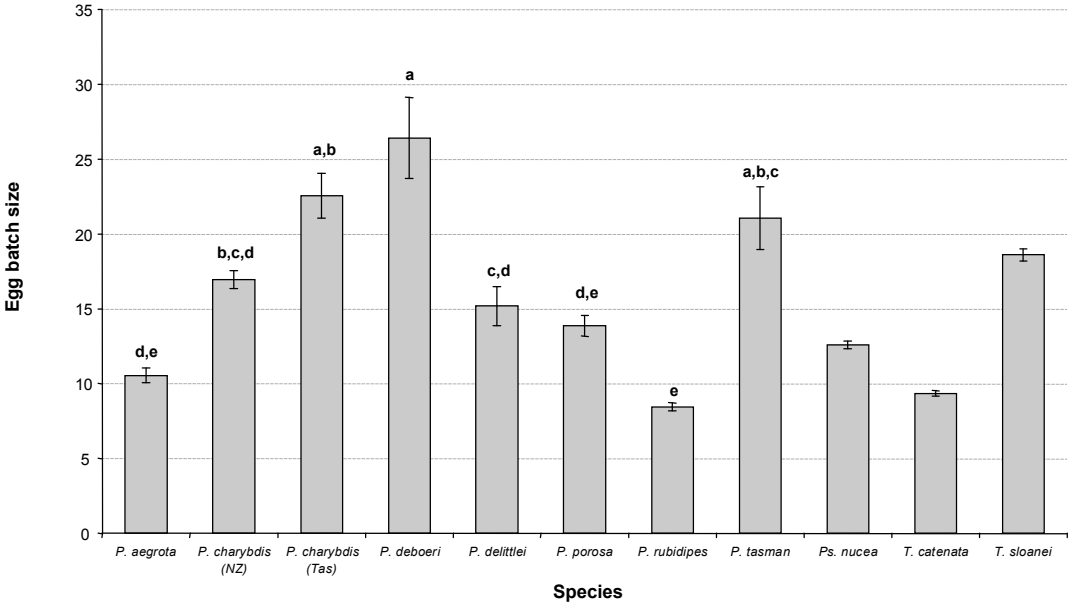
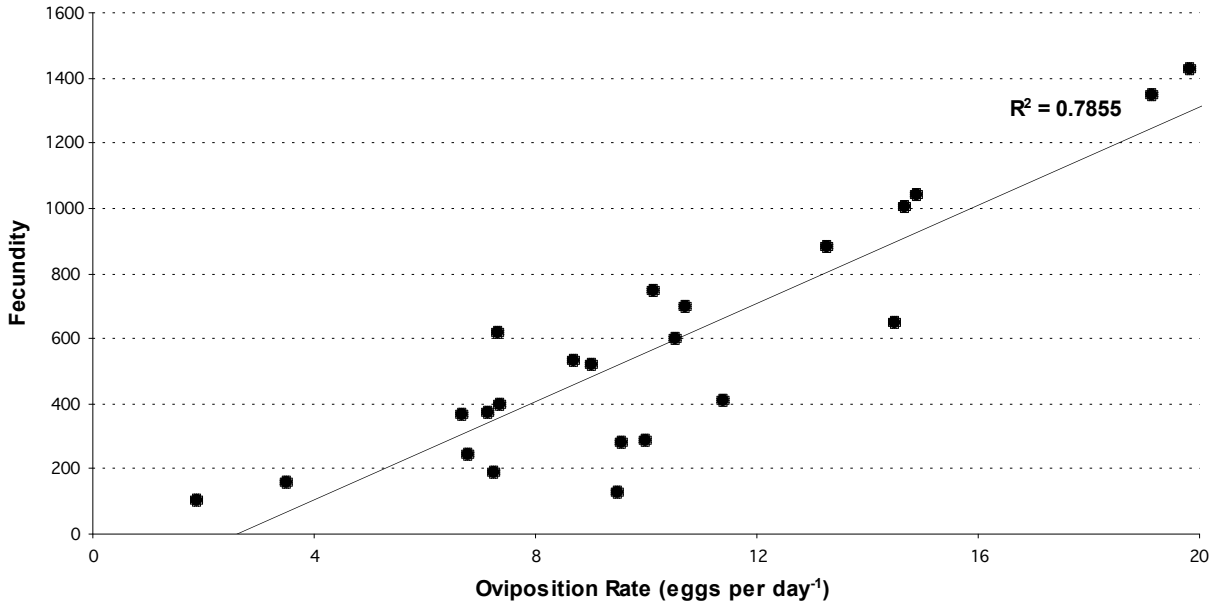


Figure 21. Correlation between fecundity and oviposition rate for paropsine species



3.3.5 *Chrysophtharta phylogenetic analysis*

Figure 22 and **Figure 23** show the *Chrysophtharta* fecundity and oviposition rate mapped against the phylogeny. Although the mean fecundity of species in the 'spine' clade (780 ±182.54 SE) was over three times that than for 'nodule' clade (239.6 ±39.5 SE), this was not significantly different (t-test, p = 0.06). The mean oviposition rate was significantly higher (t-test, p = 0.03) in the 'upper' division (13.5 ±1.9 SE) than the 'lower' (6.1 ±0.8 SE).

The two species with the significantly highest mean fecundity and oviposition rate (*C. agricola* and *C. obovata*) were closely related in the phylogeny. Other pairings with similar results for fecundity included {*C. bimaculata* + *C. laesa*} and {*C. inconstans* + *C. aurea*}. The three *Chrysophtharta* species defined as pests, *C. agricola*, *C. bimaculata* and *C. obovata* were all located in the 'spine' clade.

Figure 22. *Chrysophtharta* phylogeny and mean fecundity (± SE)

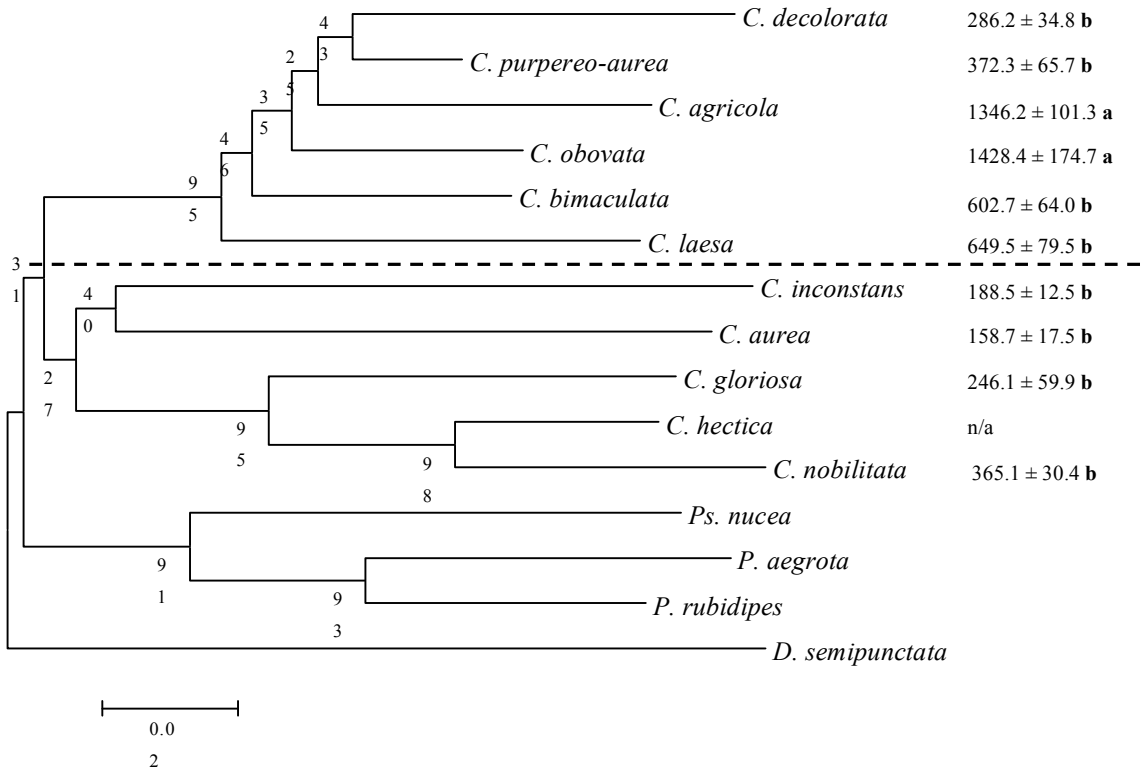
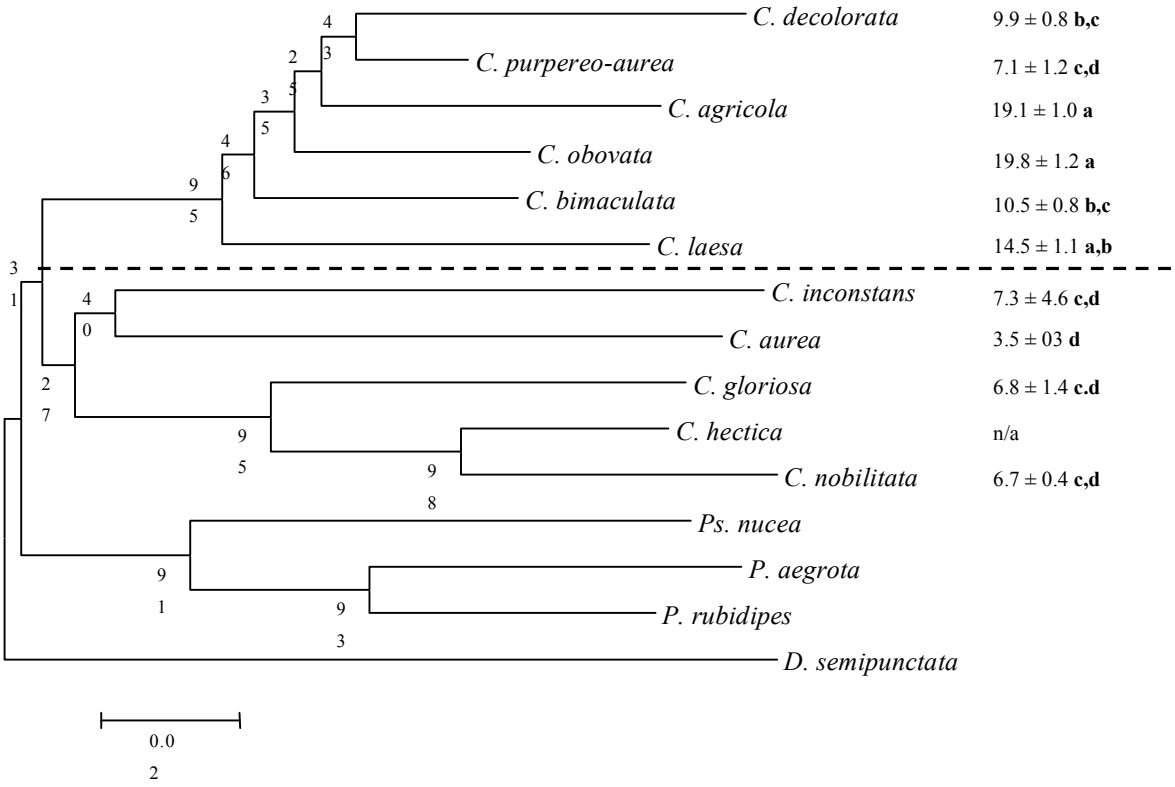


Figure 23. *Chrysophtharta* phylogeny and oviposition rate (± SE)



4. DISCUSSION

4.1 Overall features of paropsine fecundity

Paropsine reproductive output was measured to test for different reproductive strategies, and examine whether these differences could be used to describe the current pest status or pest potential of these species. Typically, the biology of pest species are examined in detail without comparisons to non-pest species (e.g. Styles 1970; Candy *et al*. 1992; de Little *et al*. 1990; Elek 1997). A comparative study of pest paropsine reproductive output against non-pest congeners provided an opportunity to clarify whether key reproductive characteristics are capable of at least partially explaining pest or outbreak behaviour.

Fecundity was examined because it was expected to substantially contribute to pest status, both in natural and exotic environments (i.e. in New Zealand). Fecundity was defined as an insect's reproductive output (Dent 1997), as egg viability was not measured (Southwood & Henderson 2000). The oviposition rate was also considered important, as species with high values could rapidly increase populations, a key attribute of outbreak behaviour. High fecundity combined with low oviposition rate might circumvent outbreak capabilities. Thirdly, egg batch sizes were measured to provide an insight into different strategies. These behaviours directly affect egg parasitism and influence subsequent larval behaviour such as aggregation (Stamp 1980). Although egg batches will not be discussed further here, the results were presented to further indicate the variation in reproductive strategies present in the study taxa.

Statistically significant differences in reproductive output were detected in the study taxa. Although mean fecundity, oviposition rate and egg batch size were not significantly different between *Paropsis* and *Chrysophtharta*, significant inter-specific differences were found within each genus. Paropsine fecundity spanned an order of magnitude, with three species capable of laying over 1000 eggs in laboratory conditions. The most fecund individual laid 3581 eggs (*C. obovata*). Predictably, the ovoviviparous *C. lignea* had the lowest fecundity value of just over 105.8 ± 32.3 SE. This is higher than the mean of 25 for the ovoviviparous beetle, *Gonioctena quinquepunctata* F. (Urban 1998), and similar to the range (70-100 eggs) of three viviparous *Oreina* chrysomelids (Dobler & Rowell-Rahier 1996).

The mean fecundity of both *Paropsis* and *Chrysophtharta* of around 500 generally exceeds that of most other chrysomelids. Although *Zygogramma bicolorata* Pallister lays over 2500 eggs (Jaynath & Bali 1996), chrysomelids rarely achieve a mean of more than several hundred eggs, e.g. 25 for *Gonioctena quinquepunctata* F (Urban 1998), 110 for *Odontota*

dorsalis (Fritz 1983), 184 for *Dicladispa gestroi* Chapuis (Delucchi 2001) and 250 for *Chrysomela viginitipunctata* Scop (Urban 1997).

4.2 Reproduction strategies of pest species

A comparative analysis of paropsine fecundity was undertaken to identify traits that act as markers for pest behaviour. Ideally, these traits would be present in pests, and absent in non-pest species, and could also be used to recognize species with pest potential.

Seven of the study taxa are considered regular pests, with several more species exhibiting irregular outbreaks with a temporary pest status. In Tasmania, *C. agricola* and *C. bimaculata* and are the two most cited pest species (Candy *et al*. 1992; de Little *et al*. 1990; Elek 1997) with *P. porosa* identified as a pest of seedlings (de Little 1989). *Paropsis charybdis* and *P. delittlei* are considered occasional outbreak species in Tasmania, with *P. charybdis* a consistent pest in New Zealand (Styles 1969). The pest status of *C. obovata* pest in South Australia (Phillips 1996) is not currently reflected in Tasmania. Despite the pest status of *T. sloanei* in California, in New Zealand it is not a notable species. Impact of this species in New Zealand is probably masked by *P. charybdis* defoliation.

Both *C. agricola* and *C. obovata* had a mean fecundity over 1000 eggs. This was significantly higher than the other *Chrysophtharta* species. These two species attained an oviposition rate of between 19 to 20 eggs per day^{-1}, the highest rate in this study. In comparison, *C. bimaculata* reproductive output was moderate, with a fecundity of 600 eggs and oviposition rate of around 10 eggs per day^{-1}. This is consistent with de Little (1983), who measured the fecundity of 13 *C. bimaculata* as 674 eggs (range 224-1706). Greaves (1966) found lower oviposition rates when he maintained 10 replicates at three temperature ranges for seven weeks. Reinterpreting this data, females held at 20 °C laid an average of 4.9 eggs per day^{-1}, increasing to 5.4 and 8.7 eggs per day^{-1} at 23.9°C and 27.2°C respectively. Greaves (1966) suggests that these oviposition rates would be unlikely to occur under field conditions.

The data for the two *P. charybdis* geographic populations was consistent. Both populations had mean fecundity in excess of 1000 eggs, with oviposition rates between 14 to 15 eggs per day^{-1}. Fecundity was slightly lower than reported from other studies. Styles (1969) suggested that *P. charybdis* typically lay 1500 to 2000 eggs over a three month period, and quotes another source as having recorded a mean of 1783 eggs from 6 females (range 1318-2102). Styles (1969) provided data for one female laying a total of 1791 eggs in 74 egg batches over 123 days, with an oviposition rate 14.6 eggs per day^{-1}. Edwards and Wightman (1984)

recorded an average of 17.3 eggs per day^{-1} for *P. charybdis,* although only five pairs were monitored for 10 days.

Paropsis porosa had a mean fecundity of 881 and an oviposition rate of 13 eggs per day^{-1}. *Paropsis aegrota*, a pest in South Australia (Phillips 1996) but not Tasmania (de Little 1979a) had a similar output, with mean fecundity of 750 eggs at 10 eggs per day^{-1}. In contrast, *P. delittlei* fecundity was just over 120, with an oviposition rate of 10 eggs per day^{-1}. This data appears to be an aberration, with this species performing inconsistently in the laboratory. The resulting data is not considered representative of this species, and should be treated as an outlier. Hence, data for *P. delittlei* will not be considered further in the discussion of reproductive output for pest paropsine species.

In general, the data suggests that a certain reproductive output threshold characterised pest or outbreak species. Of the Australian pests, *C. bimaculata* averaged over 600 eggs, *P. aegrota* over 750, *P. porosa* 800 eggs, and *C. agricola*. *C. obovata* and *P. charybdis* had mean fecundity exceeding 1000 eggs. The oviposition rates for these species ranged from 10 to 20 eggs per day^{-1}. Therefore, a reproductive output of over 600 eggs at a rate exceeding 10 eggs per day^{-1} described the pest majority of pest paropsine species examined in this study. The exception was *T. sloanei*, with a fecundity of 400 and oviposition rate below 8 eggs per day^{-1}.

4.2.1 *Non-pest paropsine fecundity*

To be a valid indicator of pest potential, reproductive values should consistently include pest species and exclude non-pests. That is, the benchmark should not regularly be exceeded by the reproductive output of rare or uncommon species. It should also be capable of consistently predicting whether a paropsine species would become a pest in an environment like New Zealand, based on reproductive output alone.

The benchmark fecundity and oviposition rates seem to exclude most non-pest paropsines, but have indicated several potential pest species. Mean fecundity exceeding 600 eggs excluded all non-pest species with the exception of *C. laesa*, *P. rubidipes* and *Ps. nucea*. The benchmark oviposition rate of 10 eggs per day^{-1} performed similarly on *Chrysophtharta* (although *C. amoena* and *C. decolorata* rates were only marginally under 10 eggs per day^{-1}). *Paropsis rubidipes* was excluded but *T. catenata* included. The combination of the two reproductive benchmarks excluded all non-pest paropsine species studied with the exceptions of *C. laesa* and *Ps. nucea*.

The biological data for *C. laesa* appears anomalous. This species is considered rare (de Little 1979a), yet fecundity is comparable to *C. bimaculata* with a substantially higher oviposition rate. The specimens used in this study were sourced from hundreds of adults collected from leaf litter as they over wintered (H. Nahrung *pers. comm*.). If *C. laesa* adults are similar to *Ps. morio* in preferring larger trees (de Little 1979b), their abundance may have been underestimated in plantation forests.

The two *Trachymela* species in New Zealand provide an important test of predictive reproductive outputs. Despite the absence of natural enemies, they are not deemed pests (despite the impact of *T. sloanei* in California). Spread has been relatively slowly through New Zealand in comparison to *P. charybdis* (N. Kay *pers. comm*.). Both species were expected to have a relatively low or moderate reproductive output. Mean fecundity was around 400 eggs, although the oviposition rate for *T. catenata* (11.4 ± 0.9 SE) was higher than *T. sloanei* (7.4 ± 0.7 SE), slightly above the 10 eggs per day^{-1} threshold.

4.3 Evaluation of phylogenetic component of *Chrysophtharta* fecundity

The fecundity and oviposition rate of *Chrysophtharta* species were mapped against the phylogeny to test the genealogical basis for fecundity and oviposition rates. Although oviposition rate differences between the hypothesised divisions were significant, mean fecundity was not.

The phylogeny provided moderate support for a genealogical basis for reproductive output. Fecundity results strongly supported the pairing of *C. agricola* and *C. obovata*, and indicated the placements of *C. bimaculata* near *C. laesa* and *C. inconstans* with *C. aurea* were appropriate. Apart from the placement of *C. agricola* with *C. obovata*, the oviposition rate was less successful in supporting the tree topology or the genealogical basis of reproductive output. Notably, the three *Chrysophtharta* species defined as pests occurred within the same phylogeny division.

4.4 Interpretation of results – the paropsine threat to *Eucalyptus* in New Zealand

Paropsine reproductive data was measured to determine if pest species exhibited levels of reproductive output above that of common or rare species. Species with these 'pest' characteristics would be more likely to become pests outside Australia based on reproductive output alone. Interpreting these results in a New Zealand context, a number of species represent a particularly high risk.

Chrysophtharta candidates for pest status in New Zealand, based on reproductive parameters alone, would be *C. agricola*, *C. bimaculata*, *C. obovata*, and possibly *C. laesa*. Of *Paropsis* species (excluding *P. charybdis*), *P. aegrota* and *P. porosa* should be considered high risk, with *P. rubidipes* considered lower risk because of its low oviposition rate. Insufficient data was available to confirm the risk posed by *P. delittlei*. The occasional outbreaks of this species in Tasmania (de Little 1979b) suggest that its reproductive output is higher than recorded here. *Paropsisterna nucea* would also be included, as its reproductive output was comparable with that of *C. bimaculata*.

4.5 Experimental limitations

A range of factors affect insect fecundity and could have influenced the reproductive results. Important variables include temperature, host food quality and population density (Jervis & Copland 1996; Leather & Awmack 1998), although influential factors may not be identifiable for all species (i.e. Coyle *et al.* 1999). Paropsine fecundity is known to be influenced by foliar nitrogen content (Ohmart *et al.* 1985), insecticide treatment (Tanton & Khan 1978b), temperature (Greaves 1966), larval nutrition and female size (Carne 1966). Only factors such as temperature, population density and host plant were controllable in this study.

Some caution must be displayed when analysing paropsine fecundity, as results appear somewhat malleable. For instance, Tribe (2000) found that *T. tincticollis* averaged 679 eggs in Western Australian, but over 1300 eggs in South Africa (Tribe & Cillié 1997). The relative similarity of the Tasmanian and New Zealand values for *P. charybdis* fecundity, and the similar results obtained for laboratory and field collected *C. agricola* egg batches (Nahrung & Murphy 2002) suggest that reproductive output is generally consistent under laboratory conditions where enough replicates are used.

Adults were field-collected, so the majority of females are expected to have commenced oviposition before collection. Contrasting this, females studied under relatively benign laboratory conditions probably live longer than their field counterparts (Ohmart *et al.* 1985), improving their reproductive life span. The use of field females may in some part explain the problems encountered in obtaining consistent reproductive data for *P. delittlei*.

The most important constraint to data analysis was replicate numbers. These ranged from 20 replicates for ten species, to two individual females for *C. inconstans* and *P. rubidipes*. This unequal sized data set necessitated the use of conservative ANOVA methods, which may have masked some significant differences.

The phylogenetic comparison seems to have suffered most from unequal datasets. Comparison of fecundity of the six 'spine' clade species against the four 'nodule' clade species provided a non-significant result, despite the 'spine' clade species averaging three times the fecundity of their counterparts. Although specimens of *C. hectica*, *C. philomela* and *P. dilatata* were collected, no egg data were obtained (only a solitary male were collected for both *C. philomela* and *P. dilatata*). Attainment of a full data set of 20 replicates for each species, including species not currently represented in the phylogeny or analysis, would solve most of these issues and allow more robust analyses of the data.

5. SUMMARY

Paropsine reproductive data was measured to determine if pest species consistently exhibited levels of reproductive output above that of common or rare species. Reproductive parameters were measured for 23 paropsine species and partitioned into mean fecundity, oviposition rate (eggs per day^{-1}) and mean egg batch size. The relevant reproductive data was also evaluated against the *Chrysophtharta* phylogeny.

Pest species were identified *a priori* to analysis. In Tasmania, the species *C. agricola*, *C. bimaculata* and *P. porosa* were identified as consistent pests, with *P. charybdis* and *P. delittlei* defined as occasional outbreak pests. *Paropsis charybdis* was recognised as a pest in New Zealand, and *P. aegrota* a pest in mainland Australia. *Trachymela sloanei* is considered a pest in California (Miller 2000) but not in New Zealand. *Chrysophtharta obovata* and *P. aegrota* were included as pest species based on their behaviour in other parts of Australia.

Mean paropsine fecundity was variable, ranging from 100 to over 1400 eggs. Oviposition rates ranged from of just over 1 to nearly 20 eggs per day^{-1}. Three identified pest species had mean fecundity in excess of 1000 eggs, with the lowest pest values of 600 eggs for *C. bimaculata* and 400 eggs for *T. sloanei*. All pest species had oviposition rates exceeding 10 eggs per day^{-1} with the exception of *T. sloanei*. The criterion of mean fecundity in excess of 600 eggs combined with an oviposition rate in excess of 10 eggs per day^{-1} excluded all non-pest species, and appears robust in indicating pest status or outbreak ability. This process identified *C. laesa* and *Ps. nucea* as potential pest species.

Application of reproductive data to the *Chrysophtharta* phylogeny proved moderately useful. Mean oviposition rates were significantly higher in spine chorion than nodule chorion species. Despite the far higher mean fecundity of the spine clade, this was not significantly different from those species in the nodule clade. The fecundity data supported the placement of *C. agricola* and *C. obovata* as related species, with moderate support for a few other pairings. The oviposition rate data was less valuable, and probably a reflection of unequal sample sizes.

Based on reproductive output, the paropsine species with the highest likelihood of becoming pests in New Zealand include *C. agricola*, *C. bimaculata*, *C. laesa*, *C. obovata*, *P. aegrota*, *P. porosa* and *Ps. nucea*.

Chapter 7. Testing the Parasitoid Host Range and Reproductive Output Hypotheses against *Dicranosterna semipunctata*

1. INTRODUCTION

1.1 Testing *E. nassaui* host range and paropsine reproductive output theories

During this study it was concluded that paropsines eggs with a smooth chorion are more susceptible to parasitism by *E. nassaui* than spined or noduled eggs. Study of paropsine reproductive data then suggested that pest species could be characterised by relatively high fecundity and oviposition rates; non-pest species generally have a lower reproductive output.

Validation of these two theories required the presence of a paropsine species in New Zealand, where both the fecundity and susceptibility to *E. nassaui* were unknown factors. If correct, visual examination of the host eggs should be sufficient to predict susceptibility or otherwise to *E. nassaui*, and study of the impact of the species, or rate of spread would indicate whether reproductive output was above or below the pest criterion.

1.2 Known biology of the *Acacia* tortoise beetle

The *Acacia* tortoise beetle *Dicranosterna semipunctata* (Chapuis) was detected in Auckland during 1996. Its natural distribution includes New South Wales and Victoria in Australia (Nicholas & Brown 2002). The main host is Tasmanian blackwood, *Acacia melanoxylan* (R. Br.), a valuable timber species with about 3000 ha of commercial plantings in New Zealand.

Dicranosterna semipunctata is not considered a serious pest in New Zealand. Dispersal from the original area of establishment was slow, and populations do not appear to increase rapidly. Once a location is infested, populations require years before levels are dense enough to defoliate hosts (M. Kay pers comm.).

Dicranosterna semipunctata eggs are green or yellow in colour, 2 - 3 mm long and have a smooth, very slightly noduled chorion. Eggs are laid individually rather than in batches, and in an unusual manner for a paropsine, are supported off the host foliage by a stalk (**Figure 24**).

Figure 24. *Dicranosterna semipunctata* egg

Specific surveys in both New Zealand and Australia have found no evidence to suggest *E. nassaui* is a natural enemy of *D. semipunctata* (M. Kay *pers. comm*.). However, Australian natural enemies surveys recovered the congener *E. polita* and an undescribed *Neopolycystus* species (Nicholas & Brown 2002). These results suggest that *E. nassaui* is not a natural enemy of *D. semipunctata*.

1.3 Predicting *D. semipunctata* susceptibility and reproductive output

The fact that *D. semipunctata* eggs lack chorion modification suggested that this species would be susceptible to *E. nassaui*. Furthermore, the low dispersal rate and low to moderate impact implied a reproductive output below that of the paropsine pest criteria, i.e. fecundity would not exceed 600 eggs, and the oviposition rate would be less than 10 eggs per day^{-1}.

To test this these two factors, *D. semipunctata* susceptibility to parasitism by *E. nassaui* as well as its reproductive output were measured and compared to results from the pest species *P. charybdis*, a pest species with known characteristics. If *E. nassaui* was found to parasitise *D. semipunctata*, field releases specifically against this paropsine were considered a viable strategy to attempt establishment of biological control.

2. METHODS

2.1 Trial conditions

Rearing and experiments were undertaken at 22°C under 16:8 light:dark photoperiod. Statistical analyses were carried out using the SAS statistical package. Mean values are presented ± SE.

2.2 Fecundity measurements

Fecundity was measured using 20 male/female pairs of field-collected adults for each species. Each pair was reared in plastic containers and fed with flush foliage (*A. melanoxylan* for *D. semipunctata* and *E. nitens* for *P. charybdis*). Eggs laid per female were recorded daily until oviposition stopped or females died. Males were replaced upon death. Mean eggs per day^{-1} and fecundity were compared by two-sample *t*-test at $P<0.05$.

2.3 Parasitism rates and host acceptance times

All *E. nassaui* were reared from *P. charybdis*. Female wasps were exposed to 30 host eggs in a Petri dish and observed for one hour. Ovipositor probing was considered to indicate host acceptance, and the time to host acceptance was recorded. A probing wasp was allowed one hour to parasitise the host eggs, which were then removed and monitored for parasitism. Ten replicates were completed for each host, with the mean acceptance times and parasitism rates compared by two-sample *t*-test at $P<0.05$.

2.4 Field release and monitoring

Releases of Tasmanian *E. nassaui* were made into a 1 ha, 9-year old stand of *A. melanoxylan* infested by *D. semipunctata*. The site was located in the Hunua valley, approximately 20 km southeast of Auckland. Releases occurred on 18 October 2000 (n = 700) and 2 November 2000 (n = 800). Releases were of approximately equal numbers of free adults and parasitised eggs of both hosts to stagger the release, but the strain type was not recorded. Adult wasps were placed onto host eggs wherever possible and their behaviour observed after release.

Prior to the initial release, and on four occasions between October 2000 and January 2001, a total of 668 *D. semipunctata* eggs were removed from the stand and monitored for parasitism.

3. RESULTS

3.1 Fecundity measurements

The fecundity and oviposition rate of *D. semipunctata* were significantly lower (t-test t = 2.0, P < 0.001) than recorded for *P. charybdis* (**Table 6**).

Table 6. Mean ± SE (range) fecundity and oviposition rates of *D. semipunctata* and *P. charybdis*

	D. semipunctata	***P. charybdis***	**P-value**
Fecundity	521 ± 76.6 (107-1270)	1007 ± 78.9 (416-1638)	P<0.001
Oviposition rate	7.6 ± 0.7 (2.7-15.1)	14.7 ± 1.0 (5-22.1)	P<0.001

3.2 Parasitism rates and host acceptance times

Although eggs of *D. semipunctata* and *P. charybdis* were accepted by *E. nassaui* in all replicates, parasitism was significantly lower on *D. semipunctata* than *P. charybdis* (t-test t = 2.1, P = 0.02) (**Table 7**). Mean acceptance time did not significantly differ between the two hosts (t-test t = 2.1, P = 0.15).

Table 7. Mean ± SE (range) *E. nassaui* parasitism rate (eggs per hour^{-1}) and time to acceptance (minutes) on *D. semipunctata* and *P. charybdis*

	D. semipunctata	***P. charybdis***	**P-value**
Parasitism rate	5.7 ± 0.8 (2-9)	8.9 ± 0.9 (5-13)	P<0.02
Acceptance time	13.6 ± 5.5 (1-59)	4.7 ± 1.9 (1-21)	P<0.15

3.3 Field-releases and monitoring

Enoggera nassaui were observed parasitising *D. semipunctata* eggs during both releases. No *E. nassaui* were recovered post-release from *D. semipunctata* eggs collected from the site.

4. DISCUSSION

That *D. semipunctata* would be susceptible to *E. nassaui*, and that it would have a reproductive output inferior to that of a pest paropsine species were predicted from several relevant observations. Firstly, the eggs lacked chorion modification, which appears to be an indicator of susceptibility to *E. nassaui*. Secondly, the relatively slow dispersal and low impact of this paropsine insinuated a moderate reproductive output. For this particular case study, the two theories appeared to be valid.

Direct comparison of parasitism rates show that *D. semipunctata* is less susceptible to *E. nassaui* than *P. charybdis*. Although no replicates were rejected, the parasitism rate was significantly lower than found on *P. charybdis*, and the time to initiate ovipositor probing was nearly twice as long. Parasitism rates were mid-way between that of the highly susceptible *Paropsis* and relatively impervious *Chrysophtharta* and *Trachymela* species previously tested. Nonetheless, the behaviour of *E. nassaui* in parasitising the eggs and subsequent development in this host was a significant finding. Unfortunately, this laboratory result did not translate into biological suppression in the field.

Both the fecundity and oviposition rate of *D. semipunctata* were significantly lower than that of *P. charybdis*. The mean fecundity of just over 500 eggs and oviposition rate less than 8 eggs per day^{-1} fell below the pest paropsine reproductive criteria. Although this beetle can reach large populations over time as they are relatively unchecked by natural enemies, this is not indicative of outbreak or consistent pest behaviour.

4.1 Attempted biological control of *D. semipunctata*

Based on host specific surveys, it is highly probable that *E. nassaui* is not a natural enemy of *D. semipunctata*. Therefore, it cannot be used as a 'classical' biological control agent in this sense. An alternative to classical biological control is new association biocontrol, where new parasite-host associations are used to control pests (Hokkanen & Pimentel 1984: Pimentel 1991).

The new association approach proposes that parasitoids and their natural hosts may be in an 'evolved balance', preventing natural enemies being effective regulators. For instance, when comparing Tasmanian and Australian Capital Territory strains of *E. nassaui*, Nahrung and Murphy (2002) found the parasitoid was more effective on a novel than home population of *C. agricola*. New association biocontrol involving a novel enemy has been suggested to provide triple the effectiveness of natural enemies (Pimentel 1991), and is 2.3 times more likely to control coleopteran pests (Hokkanen & Pimentel 1984). New association biocontrol

is not universally accepted, and its viability as a successful method rejected by Waage and Greathead (1988). Ngi-Song *et al.* (1999) support both schools of thought, but note that success of new association introductions depends strongly on the capabilities of the parasitoids used. Therefore, release of *E. nassaui* against *D. semipunctata* can be considered a test of new association theory.

A weakness of new association theory is that it does not take into account early host location steps, e.g. locating host plants where the target species occurs. Laboratory based host acceptance and parasitism trials do not always reflect field results (Kitt & Keller 1998), which is probably the result of early and essential host-location steps not being required in the laboratory (Knipling 1992; Kenis & Mills 1994). Although *D. semipunctata* was confirmed as a suitable host for *E. nassaui* in laboratory bioassay, and host eggs were observed being parasitised at the releases, no evidence of establishment was subsequently recovered. It is suspected that the parasitoid only searches *Eucalyptus* species in the field (Tribe & Cillié 2000). Therefore, adults emerging from eggs observed being parasitised during the releases probably left the site to initiate *Eucalyptus* host location elsewhere. In contrast, it is likely that the *Enoggera* species recovered from *D. semipunctata* in Australia searches *Acacia* species for hosts, and offers the best opportunity for classical biological suppression of this species in New Zealand.

5. SUMMARY

The presence of an unstudied paropsine species in New Zealand provided a unique opportunity to evaluate theories concerning both susceptibility to *E. nassaui* and reproductive output. The absence of significant chorion modification on *D. semipunctata* eggs led to a prediction that they would be susceptible to the parasitoid. The low dispersal rate of *D. semipunctata* combined with its weak to moderate impact on host *Acacia* species was then used to predict that its reproductive output would fall below thresholds used to indicate pest potential for paropsines.

Enoggera nassaui does not appear to be associated with *D. semipunctata* in either New Zealand or Australia. Despite this, the parasitoid was successful on this host in bioassay. Parasitism rates were significantly lower than on *P. charybdis*, but higher than previous results for *Chrysophtharta* and *Trachymela* hosts. A new association biological control attempt was undertaken with the release of 1500 *E. nassaui* specifically at a *D. semipunctata* population in South Auckland. Parasitism of the host was observed at release but subsequent monitoring failed to recover *E. nassaui* from this site. It is concluded that *E. nassaui* accepted the novel host in the laboratory and upon initial release because important initial host location steps had been circumvented. It is believed that *E. nassaui* only searches *Eucalyptus* for host species, and therefore control would be restricted to pests found on this genera.

The reproductive output of *D. semipunctata* was compared to *P. charybdis*. Both fecundity and oviposition rate were significantly lower than that of *P. charybdis*, and fell below the thresholds expected to indicate pest potential for a paropsine species. In the absence of natural enemies, moderate defoliation of host species is expected by *D. semipunctata*, but regular, rapid and massive outbreaks are not anticipated.

Section Three: Overall Discussion and Conclusions

The most exciting phrase to hear in science, the one that
heralds new discoveries, is not 'Eureka!' (I found it!), but 'That's funny ...'
Isaac Asimov (1920 - 1992)

Chapter 8. Discussion

1. AIMS OF THE RESEARCH

This body of research had two major goals. The first was to re-instate effective biological control of *P. charybdis* in New Zealand. This was attempted by introducing strains of *E. nassaui* that were expected to be better climatically matched to New Zealand conditions than the previous strains which were sourced from Western Australia. The second goal was to evaluate the biological characteristics of paropsine species that could be functional in predicting their pest status should they arrive in New Zealand. To achieve this goal, an analysis of both the host range of *E. nassaui* and measurement of paropsine reproductive output were undertaken. In New Zealand, if climate and host plant factors were not limiting, the major restraints on paropsine invasiveness were anticipated to be biological control by the parasitoid, and the inherent population dynamics of the paropsine species.

Both of these goals were important because paropsine species have repeatedly proven capable of reaching and establishing in New Zealand, where their presence acts as a deterrent to establishment of commercial *Eucalyptus* and *Acacia* plantations.

1.1 Section One: Biological control of *P. charybdis*

1.1.1 *Introduction of Tasmanian* E. nassaui *strains to New Zealand*

Working under the assumption that a climatic mismatch of *E. nassaui* strains in New Zealand affected the ability of the parasitoid to control *P. charybdis* in cool areas (Murphy & Kay 2000), importation of climatically suitable strains was considered a pragmatic solution with the best likelihood of success. This process was also more logistically feasible than introduction of new control agents because of the legislative environment in New Zealand. It also acknowledges that long-term sustainable solutions are more environmentally appropriate than continual applications of insecticides.

Tasmania was chosen as the source of climatically matched *E. nassaui*. This decision was based on previous activities where biological control agents for *P. charybdis* were sourced from this island (Bain *et al*. 1979), the perceived if not actual climatic similarity to New Zealand, the presence of *P. charybdis*, and access to a paropsine fauna that had been well documented and described in comparison to other areas of Australia (de Little 1979a).

Enoggera nassaui was relatively abundant and readily collectable from paropsine eggs in the field. Additional primary parasitoids in the genus *Neopolycystus* were also recovered.

However, several difficulties were encountered in both countries when attempting to establish *E. nassaui* cultures. In Tasmania the laboratory cultures were decimated by two obligate hyperparasitoids, *B. albifunicle* and *A. ovi*. This setback was remedied by implementing sanitation practices. Destruction of contaminated cultures and isolation of all field collections from laboratory cultures were sufficient to resolve this issue. Cultures were line reared as an additional safeguard that also served to maintain the genetic purity of each strain for later molecular analysis.

The next obstacle concerned the transportation and establishment of *E. nassaui* in quarantine in New Zealand. High mortality was experienced when adult parasitoids were shipped to New Zealand, both because of the time involved and practice of providing adults with liquid sustenance in their secure containers. Subsequent enquiries with the New Zealand Ministry of Agriculture and Forestry confirmed that *E. nassaui* could be sent to New Zealand as parasitised eggs, with the caveat that any non-parasitised eggs would be destroyed in quarantine. This greatly improved shipments, because transportation time was less critical and provision of liquids to feed adults was not necessary. The loss of the first quarantine cultures was attributed to lack of *P. charybdis* eggs for rearing and super-parasitism of host eggs. These issues were addressed prior to use of shipping parasitised eggs, and two strains (Florentine Valley and Evandale) were successfully established in quarantine.

After six months of quarantine rearing and examination of the health of the cultures, ministerial clearance was obtained for the release of Tasmanian *E. nassaui* in New Zealand. Over 7000 Tasmanian *E. nassaui* were eventually released in New Zealand. The majority of releases targeted *P. charybdis* populations in the central North Island, with 1500 parasitoids released against *D. semipunctata* in a separate trial in Auckland. The number of strains released was not considered ideal, but a consequence of the difficulties previously mentioned.

1.1.2 *Establishment of a Tasmanian* E. nassaui *strain*

A crucial step in re-establishment of biocontrol over *P. charybdis* was the ability to accurately assess whether either Tasmanian strains had actually established in New Zealand. Morphological features were not considered capable of providing sufficient resolution to discriminate between the strains (Naumann 1991), so molecular tools were investigated as a possible tool. Sequencing of mtDNA was proposed as the most suitable method. Sequencing offered higher accuracy and resolution than allozymes or other genetic methods, and mtDNA was selected as the study molecule in preference to nuclear DNA because it had the desirable characteristics of high abundance, maternal inheritance and absence of recombination. In theory, all individuals within a Tasmanian strain would contain exactly the same mtDNA.

This would not occur with DNA because of recombination. What was required was a mtDNA gene capable of distinguishing between the Tasmanian and New Zealand resident strains.

The COI gene was chosen because it has a high mutation rate (Brown *et al*. 1979; Crozier *et al*. 1989) and is the gene of choice for genetic study of arthropods. Because coleopteran sequence data is readily available for comparison (Hsiao 1994), specific PCR primers can be defined or obtained commercially (Simon *et al*. 1994). The COI sequences were not only able to distinguish the Tasmanian from New Zealand populations, but provided sufficient nucleotide mutations to separate the Florentine Valley and Evandale populations. This was a direct benefit of sequencing a fast evolving gene; more conserved genes or other molecular methods may not have revealed such subtle differences. Application of the technique to field collected samples obtained a year after Tasmanian *E. nassaui* were released in New Zealand detected a sample identical to the Florentine Valley strain. The logical conclusion is that the Florentine Valley strain of *E. nassaui* is now established in New Zealand. The Evandale strain was not recovered, either because it had not established, or because the sample sizes recovered were too small for its detection.

1.1.3 *Unexpected results and potential impacts*

The recovery of *E. nassaui* samples a year after the Tasmanian strains were released revealed an unexpected situation. Two egg parasitoids of *P. charybdis* were detected for the first time in New Zealand, and have subsequently had a major impact on this paropsine species and *Eucalyptus* forestry in New Zealand.

Murphy and Kay (2000) reported that the early summer peak of *P. charybdis* oviposition endured low parasitism by *E. nassaui*, but parasitism rates exceeding 90% were attained in late summer during the second wave of *P. charybdis* oviposition. Jones and Withers (2003) confirmed that *B. albifunicle* reduced the presence of *E. nassaui* and therefore adversely affected biological control of *P. charybdis*. Subsequently, a number of commercial *Eucalyptus* plantations were treated with insecticide in an attempt to reduce *P. charybdis* defoliation (Author pers. obs.).

The presence of *B. albifunicle* and its documented impact on *E. nassaui* means that there is currently little value or advantage obtained from establishment of Tasmanian *E. nassaui*. An initial goal of this study was to monitor the dispersal of any Tasmanian *E. nassaui* strain that established using mtDNA analysis. This was subsequently considered futile and discarded.

However, the appearance of *Neopolycystus insectifurax* offers some hope for the control of *P. charybdis* in New Zealand. Jones and Withers (2003) found that parasitism by *N. insectifurax* increased slowly over the season, but in at least one site was responsible for 100% parasitism of *P. charybdis* eggs. Such is the hope that *N. insectifurax* offered to *Eucalyptus* growers, it was commercially mass reared for distribution around New Zealand in 2002/03 (Author pers. obs.). Another action by concerned growers included the search for the Southern ladybird *Cleobora mellyi* which is an important predator of paropsines in Tasmania (Bashford 1999). This ladybird was only known to have established in a small area of the Marlborough sounds in the 1970s, but had not been seen since that time. A successful collection trip in 2005 led to a rearing programme and its subsequent distribution around New Zealand as a predator of both psyllids and paropsines. Releases were also made specifically targeted at *D. semipunctata* (D. Satchel *pers. comm.*).

The appearance of *N. insectifurax* caused some issues for taxonomists. *Neopolycystus insectifurax* is the name provided to the species released in New Zealand in the late 1980s, and also to a species released unsuccessfully many decades prior to this (Bain and Kay 1989). Presumably it is the same species released in South Africa by Tribe (2000) as he provided the source material for New Zealand. Upon its detection in 2002, this author compared the new species with material from the late 1980s release. They were clearly not conspecific. Berry (2003) resolved this issue by confirming the newly detected species was *N. insectifurax* after comparison with type material at ANIC, and concluded that the species released in the 1980s is an undescribed *Neopolycystus* species. It was presumed that the new species was self-established, and not a result of any historic release. The taxonomic confusion surrounding *Neopolycystus* is such that none of the species collected in Tasmania in this study could be identified to species level by ANIC.

Historic releases of *Neopolycystus* species failed to establish in New Zealand despite at times significant quantities of release material. Yet *N. insectifurax* was able to self establish, with presumably an extremely low propagule number. There may be parallels of this situation with that of parasitism of *D. semipunctata* eggs by *E. nassaui* in laboratory bioassays.

Clearly, the previous *Neopolycystus* species released in New Zealand attacked and were able to develop on *P. charybdis* eggs in the laboratory. However, as was seen with *E. nassaui*, such bioassay results are not necessarily indicative of field behaviour. It is more than possible that the *Neopolycystus* species concerned did not search *Eucalyptus* for hosts, or that its searching behaviour in the field excluded *P. charybdis* as a host. This would parsimoniously explain the failure of mass releases in contrast to a later successful self-establishment. At least

one *Neopolycystus* species has been found on an *Acacia* defoliating paropsine (Nicholas & Brown 2002). Ironically, the host was *D. semipunctata*. *Neopolycystus insectifurax* could be tested as a new association control agent for *D. semipunctata*, but the same results as found for *E. nassaui* would be expected.

1.2 Section Two: Evaluation of the paropsine threat

1.2.1 *Evaluation of the paropsine threat*

The second goal of this study was to evaluate the potential risk posed by paropsines to *Eucalyptus* in New Zealand. This was to be achieved by measuring key characteristics expected to limit paropsine populations, i.e. the host range of *E. nassaui* and paropsine reproductive output.

There is and will continue to be value is assessing the risk represented by paropsine species. Not only are they capable of destructive behaviour in their natural environment (Greaves 1966; Lowman & Heatwole 1987; Candy *et al*. 1992; Bashford 1993; Stone 1993; Elliott *et al*. 1993; Stone & Bacon 1995; Elek 1997), but have also proven to be invasive outside Australia. In addition to the five species established in New Zealand, another 13 species have been intercepted by border inspection services (Manson & Ward 1968; Richardson 1979; Keal 1981). A sixth paropsine, *Peltoschema suturalis* was detected on *Acacia* species in Wellington in 2000, but subsequently eradicated (Murphy 2004). *Trachymela tincticollis* is a pest in South Africa (Tribe & Cillié 1997), and both *T. sloanei* and *C. M-Fuscum* are in California.

The potential paropsine threat to *Eucalyptus* in New Zealand may be exacerbated by the domination of *E. nitens* in commercial plantations. This species appears highly attractive to paropsines. Of the 36 paropsine species present in Tasmania (de Little 1979a), five species had previously been observed attacking *E. nitens* (de Little 1989). These included *C. bimaculata* and *C. agricola*, as well as *P. charybdis*, *P. porosa* and *P. delittlei*. In this study, the additional species *C. decolorata*, *C. gloriosa*, *C. lignea*, *C. obovata*, *P. aegrota, P. deboeri, P. tasmanica, Paropsisterna nucea, Sterromella subcostata* and *S. trimaculata* were also found on this host, in addition to a number of unidentified *Trachymela* species. This suggests a large proportion of *Eucalyptus* defoliating paropsines would be capable of attacking this species, therefore enhancing the value of any paropsine risk assessment.

1.2.2 *Assessment of the host range of* E. nassaui

Prior to the establishment of *N. insectifurax*, natural enemy regulation of paropsine species in New Zealand was largely restricted to that achieved by *E. nassaui* (Styles 1970; Edwards &

Suckling 1980). Described as polyphagous across six paropsine genera (Naumann 1991), nonetheless this species attacked only one of five species in New Zealand. Clearly there are gaps in the host range, but no information available to suggest what if any characteristics make a paropsine species susceptible or immune to this parasitoid.

The host range of *E. nassaui* was evaluated by both field collections and laboratory bioassays. The field records confirmed the polyphagy of *E. nassaui* (Naumann 1991), with records obtained for five *Chrysophtharta* and four *Paropsis* species. The detection of *E. nassaui* from *P. charybdis* in Tasmania was the first Australian record, confirming a relationship between these two species exists naturally. The detection of *E. nassaui* on *C. agricola* was also a first for this pest species (Nahrung & Murphy 2002). A substantial number of records were attributed to *C. bimaculata*, another pest species in Tasmania (Elliott *et al*. 1993). This contrasted with the low acceptance and parasitism rates encountered in bioassay. Abundant species are easier to sample for both ecologists and parasitoids, and therefore usually reveal larger parasitoid communities, or more records, than rare species (Shaw 1994). The records from *C. bimaculata* are more attributable to the thousands of egg batches that were collected, rather than an indication that this species is highly susceptible to *E. nassaui*.

Field collections represent a conservative estimate of a parasitoids host range, and do not provide qualitative data on host acceptance of parasitoid efficacy on a host. To achieve this, laboratory bioassays were required under controlled conditions. The standard *Trichogramma* practice of 24 hour bioassays (e.g. Godin & Boivin 2000) proved entirely unsuitable, and a number of other problems identified and countered. The solution was a no choice test that could identify female wasps without compromising their performance due to providing them with pre-trial experience. Unfortunately, by the time the bioassay was developed, a reduced subset of paropsines were available for analysis.

The bioassay data showed unequivocally that the study *Paropsis* taxa were more susceptible to *E. nassaui* than the *Chrysophtharta* species examined. Of the ten species evaluated, nine species received some level of parasitism, with *Paropsis* hosts consistently receiving high parasitism rates. The fact that results for *P. charybdis* from Tasmania and New Zealand were not significantly different suggests the methodology was robust and consistent. The trial confirmed that *T. sloanei* was not a host, *T. catenata* barely so, and that host records from *C. bimaculata* did indeed represent sampling effort and not susceptibility. The parasitism rate on this host was one third of that on the best *Paropsis* host. Another seven paropsine species were accepted as hosts in preliminary observations, so the total host range of *E. nassaui* is yet to be fully quantified.

1.2.3 *Construction of a* Chrysophtharta *phylogeny*

A COI derived phylogeny was constructed for *Chrysophtharta* to provide a framework from which life history traits such as reproductive output could be evaluated. This framework should be capable of analysing other associations with paropsines, such as host ranges for paropsine parasitoids, or the plant host range of the study taxa. After sequencing, the final tree was based on a NJ tree with a JC69 substitution model. The resulting tree was chosen based on some expected placements of particular species with closely related congeners. A feature of the chosen tree was that *Chrysophtharta* appeared to separate into two distinct clades, which was corroborated by using chorion structure (see below) as an independent test of tree topology. Chorion structure may be a simple taxonomic character capable of dividing *Chrysophtharta* species into distinct groups, but this is without knowledge of the wide range of chorion structures possible present in the genus. Also notable from the phylogeny was the presence of the most notable pest species (*C. agricola* and *C. bimaculata*) in the same group.

A major restraint on the development of the phylogeny was the ability to extract clean DNA which could then be sequenced. The final tree was missing three species, including the potentially valuable *C. lignea* and *C. philomela*. Attempts to build a *Paropsis* phylogeny, which would have greatly expanded the ability to interpret the reproductive data, also proved futile and only a few species were sequenceable. This was despite multiple attempts using a range of extractions methods. In contrast, the molecular work on *E. nassaui* was achieved rapidly and worked successfully at the first attempt. Several reasons are possible for this anomaly. A number of samples were stored in 70% ethanol which is now known to be sub-optimal for DNA preservation compared to 95% ethanol or above. In addition, pigmentation corresponding to the general colour of the beetles was evident in the alcohol solution, and was evident even after phenol chloroform extraction. It is likely that this impeded subsequent reactions. This might be avoided by continual washing of the beetles in 95-99% ethanol until pigmentation is removed.

1.2.4 *Chorion modifications and their suspected role in egg defence*

The egg chorion sculpture for the study taxa was examined by SEM. This showed that in general *Paropsis* eggs had smooth chorions, and that *Chrysophtharta* eggs were ornamented. *Chrysophtharta* eggs could be arbitrarily split into nodule and spine classes. These chorion features are all visible to the naked eye, but their individual characteristics are more apparent under magnification.

Because egg ornamentation is unusual in Coleoptera (Lawrence & Britton 1991) and because egg chorion morphology is expected to evolve in response to selection pressures such as parasitism (Lawton 1986; Gross 1993), a relationship between their presence and reduced parasitism by *E. nassaui* was hypothesised but unproven. A major issue identified afterwards was the absence of *Chrysophtharta* species in the bioassay with the nodule egg chorion. It is tempting to speculate that a continuum of egg chorion modification exists in *Chrysophtharta*, where increasing chorion modification (smooth ⇒ nodule ⇒ spine) correlates with decreasing susceptibility to egg parasitism. This is not the only possible mechanism to reduce egg parasitism, and a range of behavioural (*T. sloanei*) and physiological mechanisms (*C. lignea*, *T. catenata*) were observed in the study taxa that were capable of reducing egg parasitism. At the time of testing, only *Chrysophtharta* species with spine chorions were producing sufficient eggs for testing. To speculate that the chorion modifications were defensive structures would require further trials using eggs with the nodule characteristics.

1.2.5 *Analysis of paropsine reproductive output*

A study was undertaken on the reproductive output of taxa within *Paropsis*, *Chrysophtharta*, *Paropsisterna* and *Trachymela*. The primary goal of this measurement was to determine if reproductive output, e.g. fecundity and oviposition rates, could be used as key indicators of pest potential. This is essentially a comparison of pests with non-pest species. A secondary goal achieved was an improvement in the knowledge base surrounding paropsine biology, as there is relatively sparse published data in this area, particularly for non-pest species. The *Chrysophtharta* data could also be evaluated in the context of genetic relatedness, i.e. using the phylogeny.

A key goal of this study was to evaluate the paropsine threat to *Eucalyptus* in New Zealand. Measurement of reproductive output was expected to not only help identify paropsine Tasmanian species with an unrealised pest potential, and that need to be closely monitored in the future, but more importantly, to act as a risk assessment/predictive tool for species if they were to reach New Zealand.

Paropsine species displayed a wide range of reproductive strategies. Fecundity and oviposition rates spanned an order of magnitude. The mean fecundity of *Chrysophtharta* and *Paropsis* did not significantly differ, but within genus differences were detectable. Evaluation of the data against the *Chrysophtharta* phylogeny suggests that a weak relationship between genetic relatedness and reproductive output exists; a stronger relationship is hinted out but appears to be obscured by different sample sizes, a situation which affected the overall analysis of reproductive output.

Pest species, identified from literature sources and used as a benchmark, tended to have medium to high fecundity and oviposition rates. Pest species were characterised by oviposition rates of around or above 10 eggs per day^{-1}, combined with fecundity above 600 eggs. Using this criterion, several Tasmanian species such as *C. obovata*, *C. laesa* and *Ps. nucea* appear to have a currently unrealised pest potential.

1.3 *The* D. semipunctata *'test case'*

Validation of the reproductive output and egg chorion theories necessitated a paropsine species in New Zealand where the biology was relatively unknown. Accurate assessment of its rate of distribution and 'pest' status would be valuable but not essential. *Dicranosterna semipunctata* fulfilled these standards. The twin principles of using reproductive output to predict pest behaviour, and egg chorion morphology to ascertain susceptibility to *E. nassaui* were expected to hold valid despite the fact that this is a pest of *Acacia*, not *Eucalyptus* species.

Dicranosterna semipunctata has spread slowly through New Zealand and does not exhibit outbreak behaviour. This is similar to the two *Trachymela* species in New Zealand which have moderate fecundity and are not readily parasitised by *E. nassaui*. Therefore, it was predicted that *D. semipunctata* has a moderate fecundity and oviposition rate. The eggs of the beetle were also predicted to be susceptible to *E. nassaui*. This was contrary to the lack of evidence from surveys in both New Zealand and Australia, but consistent with parasitoid acceptance of smooth chorion host eggs.

Both the reproductive parameter and host acceptance predictions proved correct. *Dicranosterna semipunctata* has modest reproductive effort, approximately half that of *P. charybdis*, and below the threshold expected of an outbreak or pest species. The parasitism results were a significant find, even if the rate parasitism rates was significantly lower than for *P. charybdis* eggs.

The resulting field releases were an attempt to establish new association control over this minor pest. This theory holds that natural enemies without evolutionary experience of a host, may be more effective than co-adapted systems. Despite the bioassay results, and observations of field parasitism during the release, the parasitoid could not be subsequently recovered from the release site. As the parasitoid is believed to utilise only *Eucalyptus* hosts during foraging/host location behaviour, it would be unlikely to encounter *D. semipunctata* on

Acacia in any other occasions than in deliberate release events. The extrapolation from laboratory trials to field results is a classic weakness of new association theory.

One observation suggests that another paropsine egg characteristic could influence egg parasitism. *Dicranosterna semipunctata* lays either green or yellow eggs, and will do so consistently throughout their reproductive cycle. Populations in different areas appear to be dominated by a particular colour, e.g. green eggs were dominant at the Hunua trial site, yellow eggs were dominant in a Coromandel population (Author pers. obs). As the laboratory culture was collected from Hunua, only two of the 20 females used for reproductive output measurements and subsequent parasitism bioassays produced yellow eggs. However, on occasions where a few yellow eggs were used in the parasitism bioassay, there appeared to be a distinct preference for *E. nassaui* to attack yellow before green eggs. Paropsines display a vast range of egg colours (de Little 1979a), and some species are highly variable for egg colour, e.g., *C. obovata* eggs range from yellow to white. The effect of egg colour as an influence on egg parasitism could be worth pursuing.

2. PREDICTING THE PAROPSINE THREAT

This study was undertaken to both improve biological control of *P. charybdis* in New Zealand, and to provide a risk analysis framework for paropsine species that are not currently, but could establish in New Zealand. Although the attempt to establish cold climate control over *P. charybdis* with Tasmanian *E. nassaui* has proven futile because of the arrival of a hyperparasitoid, there is considerable value in undertaking risk assessment because of paropsine invasiveness. Paropsine species are now more likely to be restricted in New Zealand by their reproductive output than by natural enemies.

Host records confirmed that *E. nassaui* is polyphagous in the field. It proved significantly more effective against *Paropsis* than *Chrysophtharta* species in laboratory bioassay. The *Chrysophtharta* species tested were frequently rejected and endured low parasitism when accepted. This result appears influenced by the absence or presence of chorion modification but this theory still requires some validation. A subsequent bioassay against the smooth chorion *D. semipunctata* provided some evidence to support this hypothesis.

If it were hypothetically assumed that the hyperparasitoid *B. albifunicle* and primary parasitoid *N. insectifurax* had not arrived in New Zealand, and *E. nassaui* was the sole biological restraint on paropsine population dynamics, the conclusion of a risk assessment would be that *Chrysophtharta* species represented a higher paropsine threat to *Eucalyptus* in New Zealand than *Paropsis* species. This would be particularly so for those species that have

proven relatively immune to *E. nassaui* (e.g. *C. agricola*, *C. bimaculata* and *C. obovata*). However, the reality is that *E. nassaui* is now relatively ineffective as a suppressive agent of paropsine species (Jones & Withers 2003) and the effective host range of *N. insectifurax* must be assumed to be minimal until proven otherwise.

Table 8 shows a list of paropsine species ranked by reproductive output (fecundity). Using *C. bimaculata* as the baseline species, this indicates that the greatest paropsine threat is represented by the species *C. agricola*, *C. bimaculata*, *C. laesa*, *C. obovata*, *P. aegrota*, *P. porosa*, and *Ps. nucea*. *Paropsis rubidipes* is a potential pest although the oviposition rate is lower than expected. This approach would have predicted *P. charybdis* as a pest species and identifies three currently non-pest species (*C. laesa*, *P. rubidipes* and *Ps. nucea*) as potential pest species in Australia. This data combined with the continual dispersion of paropsine species around the world suggests from a biosecurity perspective that paropsine genera rather than individual species should be targeted as regulated pests. Under current conditions, all paropsine species with a moderate to high fecundity represent a potential threat to *Eucalyptus* forestry in New Zealand.

Table 8. Ranking of paropsine threat using reproductive output

Species	Fecundity	Oviposition rate
C. obovata	1428.40	19.83
C. agricola	1346.20	19.13
P. charybdis (Tas)	1044.70	14.87
P. charybdis (NZ)	1007.00	14.68
P. porosa	881.90	13.26
P. aegrota	750.30	10.11
Ps. nucea	699.10	10.70
C. laesa	649.50	14.48
P. rubidipes	616.50	7.30
C. bimaculata	602.70	10.52
P. deboeri	532.80	8.68
P. tasmanica	522.30	9.00
T. catenata	408.25	11.39
T. sloanei	397.40	7.35
C. purpereo-aurea	372.30	7.13
C. nobilitata	365.10	6.67
C. decolorata	286.20	9.97
C. amoena	284.20	9.55
C. gloriosa	246.10	6.76
C. inconstans	188.50	7.25
C. aurea	158.70	3.51
P. delittlei	127.00	9.48
C. lignea	105.80	1.86

Chapter 9. Conclusions

Two Tasmanian strains of the egg parasitoid *Enoggera nassaui* were released in New Zealand to improve biological control of *Paropsis charybdis*. These strains were released specifically to achieve control in cool climate and high altitude regions where the Western Australian strains had proven ineffective. COI sequence data was used to characterise nucleotide mutations within each strain suitable for strain-specific markers, and evaluated against field samples collected a year after release. This method detected establishment of the Florentine Valley strain in one central North Island location. A primary egg parasitoid of *P. charybdis*, (*Neopolycystus insectifurax*) and obligate hyperparasitoid of *E. nassaui* (*Baeoanusia albifunicle*) were detected for the first time in New Zealand. *Baeoanusia albifunicle* has been associated with a dramatic decline in suppression of *E. nassaui*, and appears to have rendered the establishment of a Tasmanian strain relatively immaterial.

The host range of *E. nassaui* was investigated to characterise the extent of its polyphagy and effectiveness against a range of paropsine species. Field collections found five *Chrysophtharta* and four *Paropsis* species to be natural hosts. No choice laboratory bioassays found that while *Paropsis* hosts were highly vulnerable, *Chrysophtharta* and *Trachymela* hosts were significantly less susceptible to the parasitoid. The polyphagy of *E. nassaui* was confirmed, but the parasitoid was considered less likely to control *Chrysophtharta* than *Paropsis* hosts. The presence of significant chorion modifications ('spines' and 'nodules') on the eggs of *Chrysophtharta* species was considered a possible basis for this discrepancy in host acceptability.

A *Chrysophtharta* phylogeny was constructed using the mtDNA gene COI to test the genealogical component of reproductive. The selected tree divided the study taxa into two major clades. Mapping of the chorion ultrasculpture against the phylogeny as an independent test of topology confirmed the division, as species in each clade contained the same chorion modification class.

Reproductive parameters of *Paropsis* and *Chrysophtharta* were examined to test their contribution to outbreak behaviour or pest status. Fecundity exceeding 600 eggs and an oviposition rate over 10 eggs per day^{-1} was sufficient to describe all known pest species. Several other species were identified with the potential for pest or outbreak based on these criteria. Mapping of reproductive data against the *Chrysophtharta* phylogeny was relatively inconclusive. The three pest *Chrysophtharta* species, *C. agricola*, *C. bimaculata* and *C.*

obovata were found within the same clade, and the paired species *C. agricola* and *C. obovata* could not be statistically separated for fecundity and oviposition rates.

Dicranosterna semipunctata was used as a test case of chorion effects on parasitism and reproductive output. As the egg chorion lacked modification, it was predicted to be susceptible to *E. nassaui*. This was confirmed, although the parasitism rate was significantly lower than for the *P. charybdis* control. A low reproductive output for the beetle was predicted because it does not exhibit outbreak behaviour. Fecundity and oviposition rates were significantly lower than found for *P. charybdis*, and below the reproductive criteria used to describe a pest species. Field releases against this species did not translate into biological suppression, which would be expected if *E. nassaui* does not search *Acacia* trees for paropsine hosts.

The most likely paropsine threat to *Eucalyptus* comes from the *Chrysophtharta* genus. A number of pest species were found to have high fecundity and oviposition rates, as did several non-pest species. Combined with the lack of susceptibility to *E. nassaui*, these represent a greater threat than *Paropsis* species, which appear susceptible to egg parasitism. However, with the establishment of an obligate hyperparasitoid of *E. nassaui* in New Zealand, *Paropsis* species again represent a pest threat as seen by recurring *P. charybdis* outbreaks. Unless *Neopolycystus insectifurax* proves to be an effective, polyphagous parasitoid, even moderately fecund paropsines may have some impact. In a biosecurity context, it is concluded that paropsines should be regulated as a group rather than selecting individual species for risk assessment.

Chapter 10. References

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Chapter 11. Appendices

Appendix 1. Genus *Chrysophtharta* Weise

(Note: The taxonomy of Tasmanian *Chrysophtharta* is currently confused; species names are based on best available information. Biological notes from authors observations and de Little (1979). This genus has subsequently been revised as *Paropsisterna* Motschulsky (Reid 2006).

Chrysophtharta agricola Chapuis

PEST. Green/grey elytra with metallic flecks, two variable marks on pronotum, always with black underside. Melanistic (black) form occurs. Teneral beetles have a red edge to elytra. Grey-brown (rarely orange) eggs in clumps (20-50 eggs) on leaf tips, usually juvenile foliage. Gregarious black larvae develop yellow flanks in late instar. HOSTS: *E. ovata, E. nitens, E. viminalis, E. alrympleana, E. globulus.*

Chrysophtharta amoena Clark

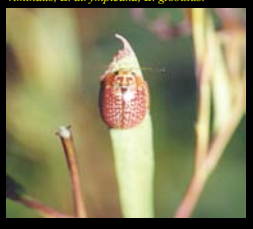
COMMON. Distinctive red/pink coloured elytra edged with dark to light green. Dark yellow eggs are deposited in tidy rows of about 10-15. Seems to prefer small seedling hosts. Larvae solitary. HOSTS: *E. ovata, E. viminalis.*

Chrysophtharta aurea Blackburn

COMMON. Brillantly coloured species prevalent on peppermints. Colours include gold, red, orange and green, often several at once. Eggs do not appear to be on hosts, are small, purple, and sausage-like, usually < 5 eggs. Larvae are distinctively narrow and solitary. HOSTS: *E. amygdalina, E. nitida, E. pulchella.*

Chrysophtharta bimaculata Olivier

PEST. Distinctive green wood-like texture on elytra, and two marks on pronotum. Eggs in 1-2 parallel rows (20-40 eggs) on the leaf surface. Beetles emerging in spring are a deep red in colour. Familiar to fishermen as can swarm over lakes in huge numbers. Occasionally black underside. Gregarious larvae. HOSTS: *E. obliqua, E. delegatensis, E. regnans, E. nitens.*



Chrysophtharta decolorata Chapuis

COMMON. Small species prevalent on peppermints. Adults with silver/copper metallic flecks on elytra, the distinctive U shape on pronotum is a key diagnostic feature. Dark grey/brown eggs in row of 10-20 eggs. Solitary larvae. HOSTS: *E. pulchella, E. amygdalina, E. viminalis.*

Chrysophtharta gloriosa Selman

RARE. Similar to *C. nobilitata*, but with four sided triangle instead of spots on elytra. Markings are metallic and variable in colour, also notable is a bright green edge to the elytra in some individuals. Eggs are bright yellow in rows of 10-15. Larvae solitary. HOSTS: *E. amygdalina, E. ovata, E. viminalis, E. nitens.*

Chrysophtharta hectica Boisduval

RARE. The typical colouration is a deep green, with the distinct feature being a metallic shading on the shoulder of elytra. Could be confused with *C. aurea* or *C. bimaculata*. Egg colour from from orange/pink to white in rows of 5-10. Solitary larvae. HOSTS: *E. delegatensis, E. dalrympleana.*

Chrysophtharta inconstans Selman

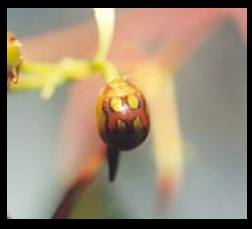
UNCOMMON. Similar to *C. amoena and C. laesa* when the elytra is stippled with red flecks, but the red shoulder bands are distinctive, as is the green underside. Bright green eggs are in small rows (5-10) on foliage. Solitary larvae. HOSTS: *E. obliqua, E. delegatensis, E. nitida*

Chrysophtharta laesa Germar

RARE. Very distinctive elytral pattern, with metallic patch on each shoulder, sometimes with red bands extending. Elytra flecked with yellow. Orange/purple/red eggs in rows of 5-20 on leaf surface. HOSTS: *E. viminalis, E. rubida, E. globulus.*

Chrysophtharta lignea Erichson

COMMON. Relatively dull species, with distinctive deep punctuation on elytra. Unusual in that female lays single eggs that hatch within minutes. Males often darker and some inconsistent patterning, occasionally spots on pronotum. Solitary larvae. HOSTS: Wide host range.



Chrysophtharta nobilitata Erichson

COMMON. Spectacular small species with brilliant gold spots on a red background. Very common on peppermints, *E. viminalis* etc. Bright yellow eggs are in small groups (5-10) on foliage. Larvae are solitary. HOSTS: *E. amygdalina, E. nitida, E. ovata, E. viminalis, E. pulchella.*

Chrysophtharta obovata Chapuis

VERY COMMON. Adults vary in colour from red to green with white flecks on elytra. Teneral beetles are a bright red/orange colour with white flecks and black underside Bright yellow larvae with black stripe form gregarious groups which may incorporate other species. Egg batches are bright yellow to white in layered clumps of up to 100 eggs. HOSTS: Wide host range.

Chrysophtharta philomela Blackburn

RARE. An extremely rare *Paropsis*-like species. Superficially similar to *Paropsis tasmanica* as has a black underside, but the pronotum marking is distinctive. Larvae and eggs unknown. HOSTS: *E. amygdalina, E. regnans.*

Chrysophtharta purpureo-aurea Selman RARE. A spectacular small species with alternating purple and gold bands. Occasionally in numbers in dry peppermint sites. Large rows (10-30) of bright yellow/brown eggs. Larvae solitary. HOSTS: *E. amygdalina, E. viminalis.*

Appendix 2. Genus *Paropsis* Olivier

Species names from de Little (1979) and Selman (1983).

Paropsis aegrota* var *Elliotti Selman

VERY COMMON. Endemic (sub-species of mainland species). Characterised by white/yellow spots on a green/brown background, and a jet black underside. Two morphological forms occur, one lighter coloured with small spots, and a darker rounded form with heavy spots. Green/yellow eggs are in spirals/whirls around leaf and shoot tips in groups of 10-20. Gregarious larve rest in lines on shoots when not feeding. HOSTS: Wide host range

Paropsis charybdis Stål

COMMON. An occasional pest in Tasmania and introduced pest in New Zealand where is known as the *Eucalytpus* tortoise beetle. As in many *Paropsis*, the colouration and pattern is highly variable but the three bands across elytra are usualy discernable. Green/yellow eggs are in 2-3 rows of 20-40 eggs usually on underside of older foliage. Larvae, yellow to pink with a dark line, initially gregarious but disperse before pupation. HOSTS: *E. ovata, E. viminalis, E. dalrympleana, E.nitens, E. globulus*

Paropsis deboeri Selman

UNCOMMON Endemic. Tasmanian version of the mainland species, *P. atomaria*. Elytral pattern common to that of *P. charybdis/P. delittlei*, but distinctly pink/purple/orange in comparison. Dark magenta eggs occur in true spirals around shoots and stems in groups of 20-40. Gregarious larvae. HOSTS: *E. viminalis, E. ovata, E. obliqua, E. nitens*

Paropsis delittlei Selman

COMMON. Endemic. Similar to *P. charybdis* but darker and grey/brown in colour. Occasional pest of Monocalyptus species such as *E. regnans/E. obliqua*. Eggs similar to those of *P. charybdis*, but are brown. Larvae have sickly appearance due to spots, gregarious then disperse in later instars. Males and females distinct from each other. HOSTS: *E. obliqua, E. delegatensis, E. regnans, E. nitens*

Paropsis dilatata Erichson

UNCOMMON. This species has a distinctive shape (gibbose) with high rise in the elytra and is large. The unusual pronotal marking is the simplest identification feature. Eggs are pinkish/mauve in small groups of eggs at leaf tip. Solitary larvae. HOSTS: *E. obliqua, E. delegatensis, E. regnans, E. nitida*

Paropsis porosa Erichson

PEST. A notorious pest of *Eucalyptus* seedlings. Similar to *P. aegrota*, but smaller, without verrucae (white spots), and has a characteristic green 3-pointed design on the black underside. The small green eggs are attached singly or in small groups to the leaf edge. Larvae are black. HOSTS: Wide host range

Paropsis rubidipes Blackburn

UNCOMMON. Endemic. Similar to *P. aegrota*, but distinguished by pink/purple colouration and a diagnostic deep magenta underside. Eggs similar to those of *P. aegrota* but are orange/green and not laid in a spiral but attached individually to leaf edge similar to *P. porosa*. Solitary larvae. HOSTS: *E. obliqua, E. delegatensis, E. nitida, E. dalyrmpleana*

Paropsis tasmanica Baly

COMMON. Endemic. The largest and most distinctive of the Tasmanian *Paropsis* species. The size and purple colouration make this clearly identifiable. Black underside. Eggs are laid in long brown/purple chains along leaf or twig edges in groups of 10-30. Larvae are colourful and large, gregarious until final instar. HOSTS: *E. obliqua, E. ovata, E. viminalis, E. regnans, E. nitens*

Appendix 3. Tasmanian egg parasitoid egg collection records

BDM records unless specified

Parasitoid species: *E = Enoggera A = Aphanomerella B = Baeoanusia*
Host species: *P = Paropsis C = Chrysophtharta*

Species	Host species	Host tree	Location	Date	Notes/Collector
E. nassaui	*C. nobilitata*	*E. pulchella*	Uni. of Tasmania	17/11/98	
E. nassaui	*C. obovata*	*E. viminalis*	Uni. of Tasmania	24/11/98	V. Patel
E. nassaui	*P. aegrota*	*E. nitens*	Blue Gum Knob	12/1/99	
E. nassaui	*P. charybdis*	*E. nitens*	Blue Gum Knob	20/1/99	V. Patel
E. nassaui	*C. decolorata*	*E. amygdalina*	Evandale	15/12/99	
E. nassaui	*C. decolorata*	*E. amygdalina*	Evandale	15/12/99	
E. nassaui	*P. aegrota*	*E. regnans*	Blue Gum Knob	16/11/99	
E. nassaui	*P. aegrota*	*E. nitens*	Blue Gum Knob	16/11/99	G. Allen
E. nassaui	*P. aegrota*	*E. nitens*	Tyenna	23/11/99	
E. nassaui	*P. aegrota*	*E. nitens*	Eleven Rd, Florentine	29/11/99	H. Nahrung
E. nassaui	*C. bimaculata*	*E. nitens*	Blue Gum Knob	13/12/99	
E. nassaui	*P. aegrota*	*E. amygdalina*	Evandale	15/12/99	
E. nassaui	*C. decolorata*	*E. ovata*	Evandale	15/12/99	
E. nassaui	*P. aegrota*	*E. viminalis*	Evandale	15/12/99	
E. nassaui	*P. deboeri*	*E. amygdalina*	Evandale	15/12/99	only 4 eggs parasitised
E. nassaui	*P. aegrota*	*E. amygdalina*	Evandale	29/2/00	
E. nassaui	*P. aegrota*	*E. viminalis*	Evandale	2/3/00	
E. nassaui	*P. aegrota*	*E. ovata*	Evandale	2/3/00	
E. nassaui	*P. aegrota*	*E. ovata*	White Rd, Florentine	1/2/00	
E. nassaui	*P. aegrota*	*E. nitens*	Blue Gum Knob	27/12/99	
E. nassaui	*C. bimaculata*	*E. regnans*	Florentine	8/3/00	A. Rice
E. nassaui	*P. aegrota*	*E. nitens*	Blue Gum Knob	6/1/00	
Neopolycystus	*P. aegrota*	*E. amygdalina*	Evandale	2/12/99	
Neopolycystus	*P. charybdis*	*E. nitens*	Ellendale	30/11/99	
Neopolycystus	*P. aegrota*	*E. amygdalina*	Evandale	15/12/99	
A. ovi	*N/A*	*E. viminalis*	Evandale	15/12/99	collected on foliage
A. ovi	*P. aegrota*	*E. amygdalina*	Evandale	15/12/99	
A. ovi	*C. bimaculata*	*E. regnans*	Eleven Rd	13/12/99	
A. ovi	*C. bimaculata*	*E. regnans*	Eleven Rd	13/12/99	
A. ovi	*C. bimaculata*	*E. regnans*	Eleven Rd	13/12/99	
A. ovi + *B. albifunicle*	*C. bimaculata*	*E. nitens*	Tim O'Shea, Florentine	22/2/00	In same batch
A. ovi	*C. bimaculata*	*E. nitens*	Eleven Rd, Florentine	22/2/00	
A. ovi	*C. bimaculata*	*E. nitens*	Eleven Rd, Florentine	22/2/00	
A. ovi	*C. bimaculata*	*E. regnans*	Florentine	8/3/00	A. Rice
A. ovi	*C. bimaculata*	*E. regnans*	Florentine	8/3/00	A. Rice
A. ovi	*C. bimaculata*	*E. regnans*	Florentine	8/3/00	A. Rice
A. ovi	*C. bimaculata*	*E. regnans*	Florentine	8/3/00	A. Rice
B. albifunicle	*C. obovata*	*E. viminalis*	Evandale	2/12/99	
B. albifunicle	*P. aegrota*	*E. amygdalina*	Evandale	2/12/99	
B. albifunicle	*C. obovata*	*E. nitida*	Evandale	2/12/99	
B. albifunicle	*C. decolorata*	*E. amygdalina*	Evandale	15/12/99	
B. albifunicle	*P. aegrota*	*E. amygdalina*	Evandale	15/12/99	
B. albifunicle	*unknown*	*E. amygdalina*	Evandale	15/12/99	
B. albifunicle	*C. bimaculata*	*E. nitens*	Tim O'Shea, Florentine	22/2/00	
Neopolycystus	*P. aegrota*	*E. amygdalina*	Evandale	2/3/00	
Neopolycystus	*P. aegrota*	*E. amygdalina*	Evandale	2/3/00	
Neopolycystus	*P. charybdis*	*E. nitens*	Eleven Rd Florentine	20/11/00	
A. ovi	*C. bimaculata*	*E. regnans*	Coles Rd. Florentine		A. Rice
Neopolycystus	*C. bimaculata*	*E. regnans*	Coles Rd. Florentine		A. Rice
E. nassaui	*C. bimaculata*	*E. regnans*	Coles Rd. Florentine		A. Rice
A. ovi	*P. aegrota*	*E. amygdalina*	Evandale	28/11/00	
Neopolycystus	*P. aegrota*	*E. amygdalina*	Evandale	28/11/00	
Neopolycystus	*P. aegrota*	*E. amygdalina*	Evandale	27/11/00	
E. nassaui	*C. decolorata*	*E. ovata*	Evandale	28/11/00	
E. nassaui	*C. decolorata*	*E. ovata*	Evandale	28/11/00	
E. nassaui	*C. decolorata*	*E. ovata*	Evandale	28/11/00	
E. nassaui	*C. decolorata*	*E. ovata*	Evandale	27/11/00	
E. nassaui	*P. aegrota*	*E. amygdalina*	Evandale	28/11/00	
Neopolycystus	*P. charybdis*	*E. nitens*	Eleven Rd, Florentine	30/11/00	

E. nassaui	*C. bimaculata*	*E. nitens*	N/A	N/A	on feeding foliage
Neopolycystus	*P. aegrota*	*E. viminalis*	Evandale	27/11/00	
Neopolycystus	*P. aegrota*	*E. amygdalina*	Evandale	28/11/00	
E. nassaui	*C. decolorata*	*E. amygdalina*	Evandale	27/11/00	
Neopolycystus	*P. charybdis*	*E. nitens*	Eleven Rd. Florentine	20/11/00	
Neopolycystus	*P. aegrota*	*E. viminalis*	Evandale	28/11/00	
E. nassaui	*P. aegrota*	*E. amygdalina*	Evandale	28/11/00	
Neopolycystus	*P. aegrota*	*E. viminalis*	Evandale	28/11/00	
E. nassaui	*P. aegrota*	*E. viminalis*	Evandale	28/11/00	
A. ovi	*C. obovata*	*E. nitida*	Evandale	27/11/00	
B. albifunicle	*C. obovata*	*E. ovata*	Evandale	28/11/00	
E. nassaui + *A. ovi*	*C. obovata*	*E. nitida*	Evandale	28/11/00	In same batch
A. ovi	*C. purp-aurea*	*E. amygdalina*	Evandale	27/11/00	
A. ovi	*C. decolorata*	*E. amygdalina*	Evandale	28/11/00	
A. ovi	*P. aegrota*	*E. amygdalina*	Evandale	28/11/00	
A. ovi	*C. purp-aurea*	*E. amygdalina*	Evandale	27/11/00	
A. ovi + *B. albifunicle*	*C. decolorata*	*E. ovata*	Evandale	28/11/00	In same batch
E. nassaui	*P. aegrota*	*E. amygdalina*	Evandale	28/11/00	
B. albifunicle	*P. aegrota*	*E. amygdalina*	Evandale	28/11/00	
B. albifunicle	*C. decolorata*	*E. amygdalina*	Evandale	28/11/00	
E. nassaui	*C. obovata*	*E. ovata*	Evandale	28/11/00	
B. albifunicle	*C. obovata*	*E. nitida*	Evandale	28/11/00	
A. ovi	*C. obovata*	*E. nitida*	Evandale	27/11/00	
A. ovi	*C. bimaculata*	*E. nitens*	Eleven Rd, Florentine	22/2/01	
A. ovi	*P. aegrota*	*E. nitens*	Eleven Rd, Florentine	18/12/00	
E. nassaui	*C. bimaculata*	*E. nitens*	Eleven Rd, Florentine	18/12/00	
E. nassaui	*C. bimaculata*	*E. nitens*	Eleven Rd, Florentine	18/12/00	
E. nassaui	*P. aegrota*	*E. viminalis*	Evandale	11/01/01	
E. nassaui	*P. aegrota*	*E. viminalis*	Evandale	11/01/01	
B. albifunicle	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	18/2/01	A. Rice
Neopolycystus	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	13/12/00	A. Rice
E. nassaui	*P. charybdis*	*E. nitens*	Blue Gum Knob	18/12/00	
Neopolycystus sp.	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	13/2/01	A. Rice
E. nassaui	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	13/2/01	A. Rice
B. albifunicle	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	13/2/01	A. Rice
E. nassaui + *A. ovi*	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	13/2/01	A. Rice – In same batch
E. nassaui	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	13/2/01	A. Rice
E. nassaui	*C. decolorata*	*E. amygdalina*	Evandale	27/11/00	
E. nassaui + *A. ovi*	*C. obovata*	*E. ovata*	Evandale	28/11/01	In same batch
E. nassaui	*C. decolorata*	*E. amygdalina*	Evandale	27/11/01	
E. nassaui	*C. decolorata*	*E. amygdalina*	Evandale	28/11/01	
E. nassaui	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	Feb 2001	A. Rice
E. nassaui	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	Feb 2001	A. Rice
A. ovi	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	Feb 2001	A. Rice
E. nassaui	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	Feb 2001	A. Rice
E. nassaui	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	Feb 2001	A. Rice
A. ovi	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	Feb 2001	A. Rice
A. ovi	*C. bimaculata*	*E. regnans*	Coles Rd, Florentine	Feb 2001	A. Rice
B. albifunicle	*C. bimaculata*	*E. nitens*	Tim Oshea, Florentine	22/2/01	A. Rice
E. nassaui	*P. porosa*	*E. nitens*	Cressey		Seedlings
E. nassaui	*P. porosa*	*E. nitens*	Cressey		Seedlings
E. nassaui	*P. porosa*	*E. nitens*	Cressey		Seedlings
E. nassaui	*P. porosa*	*E. nitens*	Cressey		Seedlings
E. nassaui	*P. porosa*	*E. nitens*	Cressey		Seedlings
E. nassaui	*C. agricola*	*E. nitens*	Florentine valley		H. Nahrung

Appendix 4. *Enoggera nassaui* COI sequence data

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Evandale        TTG GTA CAG GTA CAG GAA CTG GTT GAA CAG TTT ATC CTC CAT TAT CTT TAA GAA TAA GGC ATG GAA GGC
Florentine      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ...
Kapenga         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Lyttelton       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 1       ... .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 3       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 4       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... ...
Poronui 5       ... .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 6       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 7       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 8       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 9       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 10      ... .C. ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 11      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 12      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 13      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.
Poronui 15      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .A. ... ... .A.

Evandale        CAT CTG TTG ATT TAT CAA TTT TTT CTT TAC ATA TTG CCG GAG CTT CTT CAA TTA TAG GAT CAA TTA ATT
Florentine      ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Kapenga         ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Lyttelton       ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 1       ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 2       ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 3       ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 4       ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 5       ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 6       ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 7       ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 8       ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 9       ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 10      ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 11      ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 12      ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 13      ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 14      ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
Poronui 15      ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ... ... ... ... ... ...
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Evandale        TTA TTT CTA CTA TTT TAA ATA TAA AAA TTT ATA AAA TTG ATA ATA TTC CTT TAT TTT GTT GAG CTT TAT
Florentine      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Kapenga         ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Lyttelton       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 1       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 3       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 4       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 5       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 6       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 7       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 8       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 9       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 10      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 11      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 12      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 13      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 15      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

Evandale        TAT TAA CAG CTA TTT TAT TAT TAT TAT CAT TAC CTG TAT TAG CAG GTG CAA TTA CTA TAT TAT TAT TTG
Florentine      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .G. ... ...
Kapenga         ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Lyttelton       ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 1       ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 2       ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 3       ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 4       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .G. ...
Poronui 5       ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 6       ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 7       ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 8       ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 9       ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 10      ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 11      ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 12      ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 13      ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 14      ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
Poronui 15      ..C ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... ... ... ... ...
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Evandale        ATC GAA ACT TAA ATA CTT CTT TCT TTG ATC CTT CTG GTG GTG GGG ATC CTA TTT TAT ATC AAC ATT TAT
Florentine      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Kapenga         ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Lyttelton       ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 1       ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 2       ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 3       ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 4       ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
Poronui 5       ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 6       ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 7       ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 8       ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 9       ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 10      ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 11      ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 12      ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 13      ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 14      ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...
Poronui 15      ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T. ... .A. ... ... ... ... ... ...

Evandale        TTT GAT TTT TTG GTC ATC CTG AAG TTT
Florentine      ... ... ... ... ... ... ... ... ...
Kapenga         ... ... ... ... ... ... ... ... ...
Lyttelton       ... ... ... ... ... ... ... ... ...
Poronui 1       ... ... ... ... ... ... ... ... ...
Poronui 2       ... ... ... ... ... ... ... ... ...
Poronui 3       ... ... ... ... ... ... ... ... ...
Poronui 4       ... ... ... ... ... ... ... ... ...
Poronui 5       ... ... ... ... ... ... ... ... ...
Poronui 6       ... ... ... ... ... ... ... ... ...
Poronui 7       ... ... ... ... ... ... ... ... ...
Poronui 8       ... ... ... ... ... ... ... ... ...
Poronui 9       ... ... ... ... ... ... ... ... ...
Poronui 10      ... ... ... ... ... ... ... ... ...
Poronui 11      ... ... ... ... ... ... ... ... ...
Poronui 12      ... ... ... ... ... ... ... ... ...
Poronui 13      ... ... ... ... ... ... ... ... ...
Poronui 14      ... ... ... ... ... ... ... ... ...
Poronui 15      ... ... ... ... ... ... ... ... ...
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Appendix 5. Paropsine COI sequence data

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C. gloriosa        TCT CTA TTT TTC CTT CTA ATA AGA AGA ATT CTA GAA AGA GGA GCC GGG ACA GGT TGA ACG GTG TAT CCC
C. laesa           ... T.. ..C ..A ... T.. ... ... ... ... G.. ... ..C ..G ..A ..A ... ..C ... ..A ..T ... ...
Ps. nucea          ..A T.. ..C ..T ..A T.. ... ... ... ... G.. ... ... ..T ..T ..A ... ... ... ..A ..T ... ...
C. agricola        ... ... ... ..A ... ... ... ... ..G ... G.. ... ... ... ..A ..C ... ..C ... ..A ..T ... ...
C. bimaculata      G.. ... ... ..G ... ... ... ... ... ... G.. ... ... ... ..A ..A ... ..C ... ..A ..T ..C ...
C. decolorata      ... ... ... ..A ..C T.. ... ..G ... ... G.. ... ... ... ..T ..A ... ..C ... ... ..T ... ...
C. hectica         ... ... ... ..T ... ... ... ..G ... ... G.. ... ... ... ... ..A ... ... ... ..A ... ... ...
C. inconstans      ... ... ... C.T ..A T.. ..G ... .TG .A. G.. ... ... ... ... ..A ... ..C ... ..A ..T ..C ...
C. obovata         ... ..T ..C ..A ... T.. ... ... ... ... G.. ... ... ... ..A ..A ... ..C ... ..A ..T ..C ...
C. purp-aurea      ... ..T ..C ..A ... T.. ... ... ... ... G.. ... ... ... ..A ..A ... ..C ... ..A ..T ..C ...
D. semipunctata    ..G T.. ..C ..T ..A ... ... ... ... ... G.. ... ..G ..T ..A ..A ..T ..C ... ..A ... ... ...
C. aurea           ..A T.. ... ..A G.. ..C ... ... ..C ... G.T ... ... ..T ..T ..A ..T ..C ... ..T ..A ..C ...
C. nobilitata      ... ... ... .NN ..N ... ... ... ... ... G.. ... ... ... ... ..A ... ... ... ..A ... ... ...
P. aegrota         --- --- --- --- --- --- --- --- --- -.C G.. ..G ... ... ... ..A ..T ... ... ..A ..T ... ...
P. rubidipes       --- --- --- --- --- --- --- --- --- -.. G.. ... ... ..C ... ..A ..T ... ... ..A ..T ... ...

C. gloriosa        CCA CTT TCA GCG AAT GTT GCA CAT AGA GGA TCT TCT GTA GAC CTA GCT ATT TTT AGG CTA CAT ATA GCG
C. laesa           ... ... ... ..A ... ... ..C ... ... ..G ... ... ... ..T T.. ... ... ... ..A T.. ... ... ..C
Ps. nucea          ... ..G ... ..C ... ..A ..T ... ... ... ... ..A ..T ..T T.. ... ... ... ..A T.. ... ... ..T
C. agricola        ... ... ..G ... ... ... ..C ... ... ... ... ... ... ..T T.. ... ... ... ..A T.. ... ..G ..C
C. bimaculata      ... ... ... ..A ... ... ..C ... ... ... ..C ... ... ..T T.. ... ... ... ..A T.. ... ... ..T
C. decolorata      ... ... ... ..A ... ... ..C ... ... ... ... ... ... ..T T.. ... ... ..C ..A T.G ..C ..G ..A
C. hectica         ... ... ... ..A ... ... ... ... ... ... ... ... ... ... T.G ... ... ... ..T ... ... ... ..A
C. inconstans      ..G ... ... ..T ... ... ..C ..C ... ..T ..C ... ..T ..T ..G ... ... ..C ..A T.. ... ... ..A
C. obovata         ... ... ... ..A ... ... ..C ... ... ... ... ... ... ..T T.. ... ... ... ..A ... ... ... ..C
C. purp-aurea      ... ... ... ..A ... ... ..C ... ... ... ... ... ... ..T T.. ... ... ... ..A ..T ... ... ..C
D. semipunctata    ... ..A ... ..A ... A.. ... ... ... ..G ... ... ... ..T T.G ... ... ... ..A ..T ..C C.. ...
C. aurea           ... ..A ... ..T ... ... ..C ... ..T ... ... ... ..G ..T ..T ... ... ..C ... ..T ... ... ..C
C. nobilitata      ... ... ... ..A ... ... ... ... ... ... ... ... ... ... T.G ... ... ... ... ... ... ..G ..A
P. aegrota         ..G ..G ... ..C ... ... ..C ... ... ... ..A ... ... ..T T.. ..A ... ..C ..A T.. ..C ... ..A
P. rubidipes       ... ..G ... ..C ... ... ..C ... ... ... ..A ... ... ..T T.. ..A ... ..C ..A T.. ..C ... ..A
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C. gloriosa       GGT ATT TCA TCA ATC CTG GGT GCC ATT AAC TTC ATT ACA ACT ATT ATT AAT ATA CGA CCA ACA GGT ATA
C. laesa          ..A ..C ..T ..T ..T T.A ..A ..T ... ... ..T ... ..T ..A G.G ... ... ... ... ... ..T ... ...
Ps. nucea         ... ... ... ... ..T T.A ..A ..A ..C ..T ..T ... ..G ..C ... ... ..C ... ... ... ..T ..A ...
C. agricola       ..A ..C ... ..T ..T ..A ..A ... ... ..T ... ... ... ..A G.G ... ... ... ... ..T ... ..A ...
C. bimaculata     ..G ... ... ..T ..T T.A ... ..T ..C ..T ..T ... ..T ..A G.G ... ... ..G ... ..T ... ..A ...
C. decolorata     ..A ... ... ..T ..T ..A ..A ..T ..C ..T ..T ... ... ..A G.A ... ... ... ..G ..C ... ..A ...
C. hectica        ... ..C ... ... ..T T.A ... ..T ... ... ... ... ... ... ... ... ... ... ... ... ... ..A ...
C. inconstans     ..A ... ..C ..T ..T T.A ... ... ... ..T ... ..C ... ..A ... ... ... ..G ... ..G ... ..A ..G
C. obovata        ..A ..C ..T ..T ..T T.A ..A ... ..C ..T ..T ... ... ..G G.A ... ... ... ... ..T ... ..A ...
C. purp-aurea     ..A ..C ... ..T ..T ..A ..A ..T ..C ..T ..T ... ... ..A G.A ... ... ... ... ..T ... ..A ...
D. semipunctata   ... ... ... ... ..T T.A ... ..T ... ..T ..T ..C ..C ..A ... ... ... ..G A.. ..T ... ..G ...
C. aurea          ..A ... ..C ..T ..T ..A ..A ..A ... ... ..T ... ... ..C ... ... ... ... ... ..T ... ... ...
C. nobilitata     ..G ... ... ... ..T T.A ... ..T ... ... ... ..C ... ... ... ... ... ... ..G ..C ... ..A ...
P. aegrota        ... ... ..C ... ..T T.A ..A ..T ..C ... ... ... ... ..C ... ... ..C ... ... ... ..G ..G ...
P. rubidipes      ..A ... ..C ... ... T.A ..A ... ... ..T ... ... ... ..A ..C ... ... ... ... ... ... ..G ...

C. gloriosa       TCT ATG GAC CGT ATA CCT TTA TTT GTT TGA GCT GTA ATG ATT ACT GCT GTA TTA CTT TTA CTA TCA TTA
C. laesa          A.. ..A ... ... ... ..C ... ... ..A ..G ..A ..T ..A ... ..A ..A ..C ... ..C C.. T.. ..C C..
Ps. nucea         ..A ..A ..T ..A ... ..A ..G ... ..A ... ..A ..T ..A ... ..A ... A.C ... ..A C.T T.. ... ...
C. agricola       A.. ..A ... ..A ... ... ... ... ..A ... ..A ... G.A ... ..A ... A.T ... ... ... T.. ... ...
C. bimaculata     A.C ..A ..T ... ... ... ... ... ..A ..G ..A ..T G.A ... ..A ... ..C ... ... C.T ... ..T ...
C. decolorata     A.. ..A ... ... ... ... ... ... ..A ..G ..A ..T ..A ... ..A ... A.T ... ... C.C ... ..T ...
C. hectica        ..C ..A ... ..A ... ... ... ..C ... ... ... ... ..A ... ... ... ... ... ... C.T ... ... ...
C. inconstans     ... ..A ..T ..A ... ..C ... ... ..C ... ... ... ..A ... ... ... ..C C.. ... ... ... ... C.G
C. obovata        AT. ..A ..T ..G G.. ... ..G ... ..A ... ..A ..C ..A ... ..A ... A.C C.. ... C.T ... ..T ...
C. purp-aurea     A.. ..A ... ..A ... ... ... ... ..A ... ..A ..T ..A ... ..A ... A.T ... ... C.T ... ..T ...
D. semipunctata   AA. ... ... ... ... ..A ... ... ..A ... ... ..T T.A ... ..A ..C ..C C.T ... C.C ... ..T C.C
C. aurea          ..C ..A ... ..A ... ..A ... ... ... ... ... ... ..A ... ..A ... A.T C.T T.A C.. ..T ... ...
C. nobilitata     ... N.A ... ..A N.. ... ..N ..C ..C ... ... ..N N.N N.. ..C ... ... ... ... C.T ... ... ...
P. aegrota        ... ..A ... ..A ... ... ..G ... ... ... ... ..T ..A ... ..A ..A A.T ... T.A ..G ... ... ...
P. rubidipes      ... ..A ... ..A ... ... C.. ... ... ..G ..C ... ..A ... ..A ..A A.T ... ... ... T.. ..G ...
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C. gloriosa       CCA GTA CTA -GC AGG AGC TAT CAC TAT ACT TTT AAC TGA TCG AAA TCT AAA TAC ATC ATT CTT TG- ACC
C. laesa          ... ..T T.. -.. ... ... ... T.. ... G.. ... ... ... ... G.. .T. ... ... T.. ... T.. C.- ...
Ps. nucea         ... ... ..T -.. T.. ... A.. ... A.. .T. A.. ... ... ... ... CT. ... ... T.. T.. T.. C.- .T.
C. agricola       ... ..T T.. -.. ... C.. C.. T.. ... ... ... ... C.. C.. ... .T. ... ... T.. ... T.. ..- ...
C. bimaculata     ... ..T T.. -.. ... ... ... T.. ... ... ... ... ... C.. ... .T. ... ... T.. ... T.. ..- ...
C. decolorata     ... ..T ... -.. ... C.. ... ... ... G.. ... ... ... C.. ... .T. ... ... C.. ... T.. ..- .T.
C. hectica        ..G ... ... -.. ... ... C.. T.. ... G.. .C. ... A.. ... ... C.. G.. ... ... T.. ... ..- ...
C. inconstans     ..C ... T.. -.. C.. ... C.. T.. ... ... ... ... ... C.. ... CT. ... ... ... ... ... C.- ...
C. obovata        ... ..T T.. -.. ... ... ... T.. ... ... ... ... ... C.. T.. .T. ... ... C.. ... T.. ..- ...
C. purp-aurea     ... ..T T.. -.. ... ... C.. ... ... ... ... ... ... ... ... .T. ... ... C.. ... T.. ..- ...
D. semipunctata   ..T ..C ..T -.. C.. ... A.. ... ... .T. A.. G.. A.. C.. ... .T. ... ... C.. ... T.. C.- ...
C. aurea          ... ... T.. -.. C.. ... ... T.. A.. ... ... ... ... C.. ... .T. ... ... ... ... ... ..- .-.
C. nobilitata     ... ... T.. -.. ... G.. C.. T.. ... G.. .C. G.. A.. C.. ... ... ... ... ... TG. N.. C.T ...
P. aegrota        ... ..C ..T -.. C.. T.. A.. T.. A.. GT. A.. ... ... ... ... .T. ... ... C.. ... T.. C.- ...
P. rubidipes      ... ..T ..T -.. T.. ... A.. T.. A.. .T. A.. G.. ... ... ... .T. ... ... T.. ... T.. ..- ...

C. gloriosa       CTG CAG GAG GT- GGT GAT CCT ATT TTA TAC CAA CAT CTA TTT TGA TTT TTT GG- ACA TCC TGA AGT TTA
C. laesa          ... ... .T. ..- ... ... ..A ... ... ... ... ..C T.. ..C ... ..C ... ..- T.. C.. ... ... ...
Ps. nucea         ... ... ... ..- ..A ..C ..A ... ... ..T ... ... T.. ... ... ..C ... ..- ... C.. G.. ... ...
C. agricola       .C. .T. .T. .C- ..A ... ..A ... ... ... ..G ..C T.. ... ... ..C ... ..- G.. C.. A.. ... ...
C. bimaculata     .C. .T. .T. ..- ... ..C ... ... ... ... ... ..C T.. ... ... ... ... ..- ... ... ... ... ...
C. decolorata     ... .T. .T. .G- ... ... ..A ..C C.G ... ... ... T.. ... ... ..C ... ..- T.. C.. A.. ... ...
C. hectica        .G. ... .T. ..- ... ..C ..C ... ... ... ... ..C T.. ... ... ... ... ..- G.. ... ... ... ...
C. inconstans     .A. ... ... ..- ... ... ... G.A C.. ... ... ..C T.. ..C ... ... ... ..- T.. C.. C.. ... ...
C. obovata        .C. .T. .T. .C- ... ..C ..A ... ... ... ... ... T.. ... ... ..C ..C ..- ... ... ... ... ...
C. purp-aurea     ... .T. .T. ..- ... ... ..A ... ... ... ... ... T.. ..C ... ..C ... ..- T.. C.. C.. ... ...
D. semipunctata   ... .T. ... .A- ... ..C ... ... ... ... ... ..C T.. ... ... ... ... ..- ... C.. G.. ... ...
C. aurea          .A. ... ... .A- ..G ... ..A ... ... ... ... ... T.. ..- ... ... .-- -.- G.. CTT GA. G.- ---
C. nobilitata     .A. ... .T. .G- ... ..C ... ... C.. ... ..- ..C T.. .C. .C. --- --- --- --- --- --- --- ---
P. aegrota        .A. ... ... .G- ..A ..C ..A ... ... ... ... ... T.. ... ... ... ... ..- G.. ... A.. ... ...
P. rubidipes      .A. ... ... .A- ..A ..C ... ... ... ... ..G ..C T.. ... ... ... ... ..- G.. C.. A.. ... ...
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C. gloriosa        TAT TTT AA- -TT TTA CCA GGA
C. laesa           ... ... ..- N.. ... ... ...
Ps. nucea          ... ... ..- -.. ... ... ...
C. agricola        ... ... ..- T.. .-. ... ...
C. bimaculata      ... ... ..- T.. .-. ... ...
C. decolorata      ... ... ..- T.. .-. ... ...
C. hectica         ... ... ..- -.. ... ... ...
C. inconstans      ... ... ..- -.. ... ... ...
C. obovata         ... ... ..- T.. .-. ... ...
C. purp-aurea      ... ... ..- T.. .-. ... ...
D. semipunctata    ... ... ..- -.. ... ... ...
C. aurea           --- --- --- --- --- --- ---
C. nobilitata      --- --- --- --- --- --- ---
P. aegrota         ... ... ..- -.. ... ... ...
P. rubidipes       ... ... ..- -.. ... ... ...
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Appendix 6. *Chrysophtharta* **fecundity data**

Rep	*C. agricola*	*C. amoena*	*C. aurea*	*C. bimac*	*C. decol*	*C. gloriosa*	*C. incon*	*C. laesa*	*C. lignea*	*C. nob*	*C. obovata*	*C. purp-aurea*
1	1766	586	353	542	343	503	176	1129	193	474	3581	401
2	800	173	234	363	402	102	201	709	97	582	1587	204
3	2032	384	196	292	474	143		420	165	272	1950	645
4	1414	187	154	245	457	357		608	48	507	2299	381
5	981	134	242	615	308	337		647	26	552	1919	117
6	980	359	157	222	490	212		859		620	1773	373
7	1523	313	140	776	359	69		295		356		620
8	1054	100	175	741	178			259		408	1335	
9	756	239	230	1029	333			571		300	1177	
10	992	182	111	1032	112			1111		544	698	
11	1312	219	97	780	563			323		364	1335	
12	1642	534	125	896	253			710		325	918	
13	1878		170	458	150			316		332	1068	
14	1102		273	548	129			344		244	997	
15	682		83	1055	115			346		196	406	
16	1788		54	358	161			1421		189	2045	
17	1006		141	948	155			1165		260	890	
18	2268		55	344	169			613		208	553	
19	1364		62	548				952		287	1227	
20	1583		121	262				191		282	446	

Appendix 7. *Paropsis* and miscellaneous paropsine fecundity data

Rep	*P. aegrota*	*P. char (NZ)*	*P. char (Tas)*	*P. deboeri*	*P. delittlei*	*P. porosa*	*P. rubidipes*	*P. tasman*	*Ps. nucea*	*T. catenata*	*T. sloanei*	*D. semipunct*
1	470	1323	698	226	164	1074	706	455	1102		540	586
2	960	586	2120	563	136	1114	527	773	470		626	504
3	565	1032	1173	245	151	1185		739	1274		443	1208
4	1559	952	1906	1097	112	1504		646	1030		238	628
5	1543	1531	1684		107	1070		233	199		615	639
6	627	1415	1538		151	988		288	536		490	960
7	627	739	1132		74	437			283		257	107
8	688	1105	1607		121	975					597	122
9	1144	816	430			919					511	251
10	777	1162	271			816					985	439
11	408	1411	296			861					271	832
12	523	1628	907			1174					391	283
13	642	416	814			567					482	192
14	468	600	903			1194					205	585
15	756	971	422			861					284	131
16	833	1175	1251			894					212	1270
17	532	1191	608			726					242	425
18	872	734				293					96	468
19	371	474				570					409	620
20	640	879				415					53	167

Appendix 8. *Chrysophtharta* oviposition rate data

Rep	*C. agricola*	*C. amoena*	*C. aurea*	*C. bimac*	*C. decol*	*C. gloriosa*	*C. incon*	*C. laesa*	*C. lignea*	*C. nob*	*C. obovata*	*C. purp-aurea*
1	19.6	7.6	3.7	7.3	14.3	12.0	2.7	17.6	2.5	7.1	35.1	8.9
2	18.6	15.7	4.5	5.1	7.3	2.9	11.8	11.8	2.1	7.9	18.9	5.1
3	20.7	13.2	4.9	15.4	11.9	4.8		26.3	2.1	7.8	20.5	9.6
4	14.6	8.9	4.1	12.9	13.4	10.8		14.8	1.3	8.9	24.2	11.2
5	13.1	14.9	5.1	8.0	10.6	8.6		12.7	1.3	7.6	20.2	3.4
6	24.5	11.6	3.7	17.1	7.5	5.4		17.5		8.7	18.9	7.9
7	16.6	5.3	3.3	8.2	14.4	2.8		21.1		8.3	25.1	3.8
8	18.8	11.1	3.5	14.3	11.9			8.4		7.3	25.7	
9	14.0	8.2	5.0	10.5	15.1			16.3		7.0	17.8	
10	14.0	5.4	5.0	13.2	7.0			17.4		7.3	11.6	
11	21.9	5.6	4.9	9.3	7.5			12.9		7.1	14.4	
12	26.5	7.1	3.2	11.2	4.8			9.5		7.1	19.5	
13	20.9		3.4	10.4	7.1			5.2		3.2	21.8	
14	21.2		3.5	5.6	10.8			7.2		9.4	18.8	
15	24.4		3.5	10.8	6.8			11.9		5.0	22.6	
16	19.4		2.8	11.2	13.4			15.0		2.6	20.2	
17	11.7		2.1	9.4	9.1			14.7		6.2	13.9	
18	22.0		2.0	16.4	6.5			15.3		6.1	11.3	
19	15.3		0.9	5.6				18.0		4.9	18.9	
20	24.7		1.1	8.5				15.9		3.8	17.2	

Appendix 9. *Paropsis* and miscellaneous species oviposition rate data

Rep	*P. aegrota*	*P. char (NZ)*	*P. char (Tas)*	*P. deboeri*	*P. delittlei*	*P. porosa*	*P. rubidipes*	*P. tasman*	*Ps. nucea*	*T. catenata*	*T. sloanei*	*D. semipunct*
1	10.9	15.9	15.5	6.1	7.5	10.8	7.3	14.7	10.9		9.3	7.1
2	9.3	18.9	23.0	9.9	7.2	18.9	7.3	10.6	10.2		7.5	6.1
3	8.0	12.1	12.9	7.9	5.6	14.8		7.6	13.3		6.6	14.4
4	15.3	12.2	22.7	10.8	16.0	16.3		8.4	12.1		4.9	7.5
5	15.6	17.2	17.9		10.7	17.3		5.3	11.7		8.2	7.6
6	10.0	16.3	28.5		9.4	12.4		7.4	11.4		6.8	11.4
7	6.5	8.5	13.8		9.3	5.8			5.4		6.4	2.7
8	6.9	14.7	21.4		10.1	11.9					7.4	4.2
9	11.7	17.0	7.0			15.6					6.0	3.7
10	8.4	14.2	5.6			12.8					12.8	6.8
11	17.7	17.2	11.0			15.4					15.1	9.9
12	8.6	19.2	14.4			15.1					6.6	4.4
13	10.9	5.1	12.5			7.8					9.8	9.1
14	10.9	8.8	15.8			11.6					5.3	7.0
15	9.2	15.7	10.6			12.1					7.1	6.9
16	9.1	14.0	10.8			16.6					4.2	15.1
17	7.7	22.1	9.4			15.8					6.1	5.2
18	10.0	9.0				7.0					2.6	5.8
19	6.2	17.6				13.3					11.7	8.2
20	9.3	17.9				13.8					2.7	8.4

Appendix 10. *Chrysophtharta* mean egg batch size data

Rep	*C. agricola*	*C. amoena*	*C. aurea*	*C. bimac*	*C. decol*	*C. gloriosa*	*C. incon*	*C. laesa*	*C. lignea*	*C. nob*	*C. obovata*	*C. purp-aurea*
1	35.3	15.4	5.7	12.6	18.1	8.3	8.7	9.6	2.9	4.1	74.6	17.4
2	32.0	13.3	5.4	12.5	10.6	4.9	3.9	4.6	2.4	4.8	54.7	14.6
3	32.3	8.9	7.3	16.6	13.5	6.0		8.9	2.6	4.1	55.7	17.4
4	20.8	7.2	7.3	20.4	12.0	8.0		7.7	2.2	4.5	60.5	19.1
5	28.9	10.3	6.5	14.3	11.0	8.2		6.1	2.9	3.6	73.8	13.0
6	28.0	9.0	4.6	14.9	13.6	6.6		8.5		3.8	61.1	26.6
7	25.8	9.8	4.8	18.2	12.4	4.9		8.4		3.8	73.8	15.6
8	31.0	14.3	4.2	19.5	11.1			6.3		3.9	58.0	
9	31.5	10.0	6.6	16.8	14.5			6.8		3.5	47.1	
10	35.4	11.4	6.2	16.4	10.2			8.2		4.2	46.5	
11	32.0	9.5	6.1	18.8	10.6			6.6		4.1	53.4	
12	34.9	10.7	5.7	19.9	9.8			8.0		3.6	46.4	
13	27.6		4.6	21.8	10.7			8.8		3.8	59.3	
14	30.6		4.4	18.8	10.8			6.9		4.2	55.4	
15	34.1		3.3	17.9	10.5			10.5		4.2	40.6	
16	29.8		3	15.6	12.4			9.2		3.6	66.0	
17	21.9		4.5	17.9	10.3			7.9		4.5	52.4	
18	26.1		3.1	19.1	9.9			8.6		4.5	50.3	
19	27.8		4.1	18.8				9.1		4.0	58.4	
20	39.6		3.5	15.4				11.2		3.9	44.6	

Appendix 11. ***Paropsis*** **and miscellaneous paropsine mean egg batch size data**

Rep	*P. aegrota*	*P. char (NZ)*	*P. char (Tas)*	*P. deboeri*	*P. delittlei*	*P. porosa*	*P. rubid*	*P. tasman*	*Ps. nucea*	*T. catenata*	*T. sloanei*	*D. semipunct*
1	9.2	16.8	21.8	25.1	13.7	11.6	8.4	19.8				
2	9.1	20.9	27.5	26.8	15.1	19.2	8.5	14.1				
3	9.4	13.1	20.2	20.4	16.8	15		26.4				
4	12.5	20.0	25.8	33.4	22.4	17.7		16.2				
5	12.3	15.6	21.3		13.4	17.8		25.9				
6	8.7	15.6	34.2		15.1	12.2		24.0				
7	8.8	17.6	19.2		9.8	6.7						
8	8.3	15.8	29.2		15.1	12.3						
9	9.8	16	14.8			15.6						
10	9.8	14.9	16.9			14.3						
11	15.7	21.1	15.6			15.4						
12	9.2	15.7	17.4			15.7						
13	9.7	13.4	22.6			8.7						
14	10	13.3	22.6			13.4						
15	8.3	21.6	17.6			12.5						
16	13.2	14.9	20.9			16.9						
17	9.7	20.9	35.8			14.8						
18	12.8	16.2				8.9						
19	10.3	18.2				14.3						
20	14.2	17.6				14.3						

Appendix 12. Parasitism rates for *Enoggera nassaui*

Rep	*C. agricola*	*C. bimac*	*C. obovata*	*P. aegrota*	*P. char (NZ)*	*P. char (Tas)*	*P. porosa*	*P. rubidipes*	*T. catenata*	*T. sloanei*	*D. semipunct*
1	8	10	0	10	9	12	8	11	4	0	9
2	0	0	1	15	10	6	14	12	0	0	7
3	0	0	8	10	5	10	14	15	0	0	7
4	0	0	0	12	13	14	15	13	1	0	8
5	0	0	4	9	5	13	12	19	0	0	8
6	0	10	0	22	8	14	11	10	0	0	3
7	11	0	0	12	7	12	11	9	0	0	4
8	0	0	9	8	12	6	14	9	0	0	3
9	0	7	0	10	8	9	12	8	4	0	2
10	0	11	10	8	12	10	13	11	0	0	6
Mean ± SE	**1.9 ± 1.3**	**3.8 ± 1.6**	**3.2 ± 1.3**	**11.6 ± 1.3**	**8.9 ± 0.9**	**10.6 ± 0.9**	**12.4 ± 0.7**	**11.7 ± 1.0**	**0.9 ± 0.5**	**0**	**5.7 ± 0.8**

Appendix 13. Published paper

ATTEMPTED NEW ASSOCIATION BIOLOGICAL CONTROL OF *DICRANOSTERNA SEMIPUNCTATA* CHAPUIS (COLEOPTERA: CHRYSOMELIDAE: PAROPSINI)

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ABSTRACT

The new association theory of biological control predicts that novel enemies may be more effective in controlling pest species than their natural enemies. This theory was tested using the egg parasitoid *Enoggera nassaui* Girault (Hymenoptera: Pteromalidae) on the *Acacia* tortoise beetle, *Dicranosterna semipunctata* (Chapuis) (Coleoptera: Chrysomelidae) in New Zealand. In no choice laboratory bioassays, parasitism on the new host was significantly lower than on a natural host, *Paropsis charybdis* Stål (Coleoptera: Chrysomelidae) (5.7 cf. 8.9 eggs/h, P=0.02). The fecundity and oviposition rate of *D. semipunctata* were approximately half that of *P. charybdis*. A field release of 1500 *E. nassaui* directed against *D. semipunctata* did not result in sustained field parasitism. It is suspected that *E. nassaui* will not normally encounter *D. semipunctata* because it searches *Eucalyptus* not *Acacia* species. The ability of biological control agents to locate the target species in the field needs to be considered when evaluating new association biological control.

Keywords: *Dicranosterna semipunctata*, *Paropsis charybdis*, *Enoggera nassaui*, biological control

INTRODUCTION

The Acacia tortoise beetle, *Dicranosterna semipunctata* (Chapuis), was first detected in Auckland, New Zealand, during 1996. Its natural distribution includes New South Wales and Victoria in Australia (Nicholas & Brown 2002). The main host is Tasmanian blackwood, *Acacia melanoxylon* (R. Br.), a timber species with about 3000 ha planted in New Zealand. Biologically, *D. semipunctata* is similar to other paropsine species, with four larval instars, and a prepupal and pupal stage.

Biological control is considered a likely means for long-term sustainable control of *D. semipunctata*. Classical biological control uses natural enemies to control the target species when it becomes a pest outside its normal distribution. For instance, the paropsine *Paropsis charybdis* Stål is partially controlled in New Zealand following introduction of the Australian egg parasitoid *Enoggera nassaui* Girault (Kay 1990). However, natural enemy exploration can prove expensive, and introduction of novel organisms, even beneficial ones, is highly regulated in New Zealand.

An alternative approach is new association biocontrol, where new parasite-host associations are used to control pests (Hokkanen & Pimentel 1984; Pimentel 1991). This approach proposes that parasitoids and their natural hosts may be in an 'evolved balance', preventing natural enemies being effective regulators. As an example, when comparing Tasmanian and Australian Capital Territory strains of *E. nassaui*, Nahrung & Murphy (2002) found the parasitoid was more effective on a novel population than the home population of *Chrysophtharta agricola* (Chapuis) (Coleoptera: Chrysomelidae).

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An analysis of biocontrol cases by Hokkanen & Pimentel (1984) led to the claim that use of new or novel enemies may be 2.3 times more likely to control coleopteran pests than classical methods.

To test the effectiveness of the new association method, the parasitism by *E. nassaui* on both a novel host (*D. semipunctata*) and a natural host (*P. charybdis*) was evaluated. As specific natural enemy surveys in New Zealand and Australia have not detected *E. nassaui* with *D. semipunctata*, this is considered a new association relationship. The fecundity of *D. semipunctata* was also measured so it could be compared against *P. charybdis* and other paropsine species.

METHODS

Trial conditions

Rearing and experiments were undertaken at 22°C under16:8 h light:dark conditions.

Fecundity measurements

Fecundity was measured using 20 male/female pairs of field-collected adults for each species. Each pair was reared in plastic containers and fed with flush foliage (*A. melanoxylon* for *D. semipunctata* and *Eucalyptus nitens* for *P. charybdis*). Eggs laid per female were recorded daily until oviposition stopped or females died. Males were replaced upon death. Mean eggs per day and fecundity were compared by two-sample *t*-test at $P<0.05$.

Parasitism bioassays

Parasitism rates were examined using the no choice tests of Nahrung & Murphy (2002). All wasps had been reared from *P. charybdis* eggs. Female wasps were exposed to 30 host eggs in a Petri dish and observed for one hour. Ovipositor probing was considered to indicate host acceptance and the time to host acceptance was recorded. The wasps were then allowed one hour to parasitise host eggs. Eggs were removed and monitored for parasitism symptoms (presence of dark spots after several days). Ten replicates were completed for each host, with the mean acceptance times and parasitism rates compared by two-sample *t*-test at $P<0.05$.

Field release and monitoring

Parasitoid releases were made into a 1 ha, 9-year old stand of *A. melanoxylon* located in the Hunua Valley, approximately 20 km south-east of Auckland. *Enoggera nassaui* were released on 18 October 2000 (n =700) and 2 November 2000 (n = 800). Releases were of approximately equal numbers of free adults and parasitised eggs of both hosts to stagger the release. Adults were placed onto host eggs where possible and their behaviour observed after release. Prior to the initial release, and on four occasions between October 2000 and January 2001, a total of 668 *D. semipunctata* eggs were removed from the stand and monitored for parasitism.

Statistical analyses were carried out using the SAS statistical package. Mean values are presented ± SE.

RESULTS

Fecundity measurements

The fecundity and oviposition rate of *D. semipunctata* were significantly lower than for *P. charybdis* (Table 1).

TABLE 1: Fecundity (total eggs/female) and oviposition rate (eggs/day) of *D. semipunctata* and *P. charybdis*. Values are the mean±SE, with the range indicated in parentheses.

	D. semipunctata	*P. charybdis*	P-value
Fecundity	521±76.6 (107–1270)	1007±78.9 (416–1638)	P<0.001
Oviposition rate	7.6±0.7 (2.7–15.1)	14.7 ±1.0 (5–22.1)	P<0.001

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Parasitism bioassays

Eggs of *D. semipunctata* and *P. charybdis* were accepted by *E. nassaui* in all replicates. Parasitism by *E. nassaui* was significantly lower on *D. semipunctata* than *P. charybdis* (Table 2). Mean acceptance time did not significantly differ between the two hosts.

TABLE 2: *Enoggera nassaui* parasitism rate (eggs/h) and acceptance times (min) on *D. semipunctata* and *P. charybdis*. Values are the mean±SE, with the range indicated in parentheses.

	D. semipunctata	*P. charybdis*	P-value
Parasitism rate	5.7±0.8 (2–9)	8.9±0.9 (5–13)	P=0.02
Acceptance time	13.6±5.5 (1–59)	4.7±1.9 (1–21)	P=0.15

Field-releases and monitoring

Enoggera nassaui were observed parasitising *D. semipunctata* eggs during both releases. No *E. nassaui* were recovered from any field-collected eggs.

DISCUSSION

New association theory is claimed to be a superior alternative to classical methods (Hokkanen & Pimentel 1984) but has received strong criticism (Waage & Greathead 1988). Ngi-Song et al. (1999) found evidence supporting both schools of thought, but noted that success of new association control depends on the capabilities of the parasitoids used.

The new association theory was evaluated by introducing *E. nassaui* to *D. semipunctata* under laboratory conditions. Despite the fact that parasitism was significantly lower on the new host than a natural host, the fact that parasitism and successful development occurred was a significant finding. Unfortunately, this did not translate into biological suppression in the field.

A weakness of new association theory is that it does not take into account early host location steps, i.e. locating host plants where the target species occurs. Host acceptance and parasitism in laboratory trials do not always reflect field results (e.g. Kitt & Keller 1998), which is probably a result of early and essential host-location steps not being required in the laboratory (Knipling 1992). Although *D. semipunctata* is a suitable host for *E. nassaui*, it is suspected that the parasitoid only searches *Eucalyptus* species (Tribe & Cillié 2000). Therefore, adults emerging from eggs that were observed being parasitised during the field releases probably left the site to initiate host location.

Dicranosterna semipunctata fecundity was modest in comparison to that of *P. charybdis* but similar to published data for other paropsine species. de Little (1983) recorded a fecundity of 674 ± 127 eggs for *C. bimaculata* (Olivier) and Carne (1966) estimated *P. atomaria* (Ol.) fecundity at about 640 eggs. This fecundity level implies that even moderate suppression by *E. nassaui* would have helped reduce any economic impact of this pest.

CONCLUSIONS

The new association theory was tested using the egg parasitoid *Enoggera nassaui* as a novel enemy of *Dicranosterna semipunctata*. Parasitism occurred in laboratory trials, although the rate was significantly lower than on the natural host *Paropsis charybdis*. A field release of 1500 *E. nassaui* did not result in detectable levels of parasitism on the novel host. It is concluded that although *D. semipunctata* is a suitable host, new association biocontrol does not take into consideration the fact that the control agent may not search the host plants where the target species is located.

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Appendix 14. Published paper

DETECTION OF A TASMANIAN STRAIN OF THE BIOLOGICAL CONTROL AGENT *ENOGGERA NASSAUI* GIRAULT (HYMENOPTERA: PTEROMALIDAE) USING MITOCHONDRIAL COI

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ABSTRACT

Paropsis charybdis (Coleoptera: Chrysomelidae), an Australian pest of *Eucalyptus* in New Zealand, is subject to biological control by *Enoggera nassaui* (Hymenoptera: Pteromalidae), a solitary egg parasitoid sourced from Western Australia (= Perth Strain) in 1987. Erratic control in inland regions of New Zealand led to the introduction and release in 2000 of two Tasmanian *E. nassaui* strains to attempt expansion of the climatic range of biocontrol. Samples recovered a year later were analysed using partial sequences of the Cytochrome oxidase I (COI) mitochondrial gene to test for establishment. This method detected a haplotype corresponding with a Tasmanian (Florentine Valley) strain. However, as sequences could not be obtained from original Perth strain stocks, some doubt remains as to the genetic source of the other current *E. nassaui* populations in New Zealand. COI proved adept at distinguishing between different parasitoid populations and shows promise for similar studies.
Keywords: *Paropsis charybdis*, *Enoggera nassaui*, Cytochrome oxidase I, biological control.

INTRODUCTION

The *Eucalyptus* tortoise beetle *Paropsis charybdis* Stål is an accidentally established pest of *Eucalyptus* in New Zealand. The solitary egg parasitoid *Enoggera nassaui* Girault was obtained from Western Australia (= Perth strain) and released as a classical biological control agent in 1987 (Kay 1990). Bain & Kay (1989) were concerned that this population may not be climatically tolerant of inland New Zealand conditions, a theory supported by Murphy & Kay (2000), who found that low parasitism rates during spring were associated with high levels of *P. charybdis* defoliation in the central North Island region.

Climatic matching of biocontrol agents is considered important in New Zealand (Cameron et al. 1993) where a range of climatic conditions are encountered. Natural enemies should be sought from the region of origin of a pest to ensure they are phenologically adapted to the host and can survive in a similar climate (Legner & Bellows 1999). It was hypothesised that populations (strains) of Tasmanian *E. nassaui* could potentially provide better control of *P. charybdis* than currently occurs in cooler areas, because of a presumably better climate match and because *P. charybdis* occurs in Tasmania (de Little 1989) but not Western Australia.

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A suitable method was required to test for Tasmanian strain establishment. The morphological features of *E. nassaui* were considered insufficient for such a task (Naumann 1989), whereas molecular tools have proven useful in detecting subtle population differences in insects (e.g. Scheffer & Lewis 2001; Mander et al. 2003). Mitochondrial DNA (mtDNA) was considered suitable because it is maternally inherited and does not undergo recombination. The Cytochrome oxidase I (COI) gene was anticipated to provide sufficient genetic variation to discriminate between even closely related *E. nassaui* populations because of its rapid mutation rate (Simon et al. 1994).

METHODS

Collection and importation of Tasmanian *Enoggera nassaui* strains

Enoggera nassaui were collected from paropsine eggs in Tasmania during 1999/2000, identified using the key of Naumann (1989), and reared on *P. aegrota* var *Elliotii* Selman eggs. Adults emerging from the same egg batch were assumed siblings (i.e. from the same female wasp). To maintain mtDNA purity, each strain was maintained separately. Live adults and parasitised *P. aegrota* eggs were sent to New Zealand in May 2000, but only two strains, from the Florentine Valley (146°29'E, 42°38'S, ex *P. aegrota*) and Evandale (147°24'E, 41°24'S, ex *P. deboeri*), were established in culture. Rearing occurred at 22°C under 16:8 h light:dark on *P. charybdis* eggs. Identifications were confirmed by the Australian National Insect Collection.

Release protocols

Voucher specimens of both strains were deposited with the New Zealand Arthropod Collection prior to release at four central North Island *Eucalyptus nitens* Maiden (Deane et Maiden) plantations during November 2000 (Table 1). Releases consisted of approximately equal numbers of free adults and parasitised eggs hung in plastic containers from foliage.

TABLE 1: The numbers of Tasmanian *Enoggera nassaui* released for the Florentine Valley and Evandale strains at the four New Zealand release sites. Releases consisted of approximately similar numbers of free adults and parasitised eggs.

Site	Location	Florentine	Evandale	Total
Smythes Rd	Kinleith Forest	1000	100	1100
Poronui	Poronui Station	1000	1000	2000
Cpt. 1060	Kaingaroa Forest	1200	800	2000
Kapenga	Rotorua	300	300	600
Total		3500	2200	5700

Molecular analysis

To detect whether Tasmanian *E. nassaui* had established at the release sites, samples were collected from parasitised *P. charybdis* egg batches during January 2001. Fifteen samples collected at Poronui and one from Kapenga were compared with one specimen of each Tasmanian strain, a South Island sample (Lyttelton, collected in 1999), and five alcohol-preserved samples of the original Perth strain cultures. No samples could be recovered from Cpt 1060 and the Smythes Rd site had been felled.

Whole individuals were macerated, Proteinase K digested and cleaned using phenol/chloroform extraction (Sambrook et al. 1989). DNA was precipitated in equal volumes of 3M sodium acetate and isopropanol overnight at –20°C. After centrifuging at 12,000 rpm for 25 minutes the pellet was washed in 1 ml 70% ETOH for 5 minutes, air-dried and re-suspended in 100 ml Tris EDTA (pH 8) buffer.

Each 25 µl PCR reaction consisted of 3 µl Roche (10x) Taq polymerase buffer (+ $MgCl_2$), 2.5 µl dNTPs at 4 mM, 1 unit Taq (1 U/µl), 12.5 µl double distilled H_2O, 2.5 µl of the primers C1-J-1751 'Ron' and C1-N-2191 'Nancy' from Simon et al. (1994) at 5 µM, and

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1 µl of template. PCR and sequence reactions were run on an Applied Biosystems GeneAmp, PCR System 9700. After denaturation at 94°C for four minutes, samples were subjected to 30 cycles of 94°C/30 seconds, 55°C/30 seconds, 72°C /45 seconds, and a final extension phase of 4 minutes at 72°C. Two µl of PCR product were visualised on 2% agarose gel against a 1KB Plus ladder. Sequencing reactions (Version 3.0 Big Dye® Terminator mix Applied Biosystems) followed the manufacturer's recommendations and were run on an ABI Prism, 3100 16 capillary-array gene analyser. The approximately 370 bp sequences were aligned and a Neighbour Joining (NJ) tree generated under the Kimura 2-parameter model with 1000 bootstraps in MEGA version 2.1 (Kumar et al. 2001).

RESULTS

PCR products and sequences were obtained for all specimens except the original Perth stocks. The resulting NJ tree (Fig. 1) shows three haplotypes were present. The Lyttelton, Kapenga and 11 Poronui samples had the same haplotype, with three Poronui samples sharing a unique substitution.

The Tasmanian strains differed from each other at three positions and from the majority of other samples by six nucleotides. One Poronui sample had the same haplotype as the Florentine Valley strain.

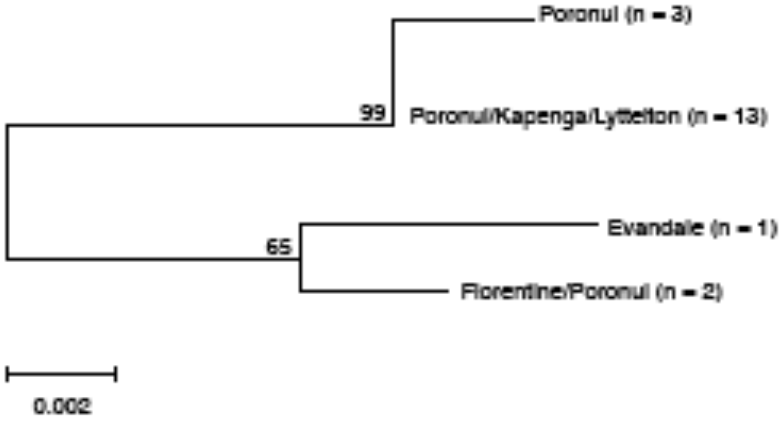

FIGURE 1: Neighbour-joining dendogram of *Enoggera nassaui* sequences using Kimura 2 Parameter. Numbers at branches represent 1000 bootstrap values. Scale = genetic distance. n = sample size.

DISCUSSION

This study was undertaken to determine whether Tasmanian *E. nassaui* strains could improve biocontrol of *P. charybdis* and to evaluate the ability of COI to discriminate between different parasitoid populations. However, two aspects of this study undermined our ability to draw clear conclusions from the results.

Firstly, recovery of *E. nassaui* was reduced by *Baeoanusia albifuniele* Girault (Hymenoptera: Encyrtidae), an obligate hyperparasitoid of *E. nassaui* (Tribe 2000). High hyperparasitism rates and competition from another self introduced egg parasitoid were recorded by Jones & Withers (2003). Because of this situation, there appears to be little future value in monitoring the effect of Tasmanian *E. nassaui* on *P. charybdis* since the parasitoid has been rendered relatively ineffective. This also means that the climate-matching hypothesis cannot be tested.

The second issue was the inability to extract DNA from the original Perth strain samples. Because two parasitoid species have now accidentally established in New Zealand from Australia, we cannot exclude the possibility that *E. nassaui* populations may also have self introduced since 1987, although the material recovered in this study

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is likely to be the Perth strain. The similarity of the Lyttelton haplotype (collected before the Tasmanian strains were released) to the North Island samples confirms that the extant material in New Zealand is different from either Tasmanian strain. Although only one sample of each Tasmanian strain was analysed, we believe that the maternal inheritance, lack of recombination and separate rearing of strains maintained any mtDNA differences within a strain, and therefore one sample was a sufficient sample size. The recovery of a sample from Porotui sharing the same haplotype as the Florentine Valley strain suggests that a Tasmanian strain did establish in at least one location in New Zealand. In this aspect, the programme appears to have been successful.

COI proved useful for the task of discriminating between *E. nassaui* populations. In addition to separating the two Tasmanian strains, it also indicated a slight genetic diversity in the population previously established in New Zealand. It therefore seems a useful tool for monitoring the establishment or dispersal of biocontrol agents where different population sources may be used. Further COI analysis of Tasmanian strains collected but not released and the original Perth strain samples would clarify the remaining issues raised in this study.

CONCLUSIONS

Two Tasmanian strains of the egg parasitoid *Enoggera nassaui* were introduced to New Zealand in an attempt to improve biological control of *Paropsis charybdis*. Cytochrome oxidase I sequences were evaluated as a method to distinguish between different populations and to test for establishment. Sufficient variation was detected to discriminate both between Tasmanian strains and between geographic races (Tasmanian and presumably Western Australian). Three haplotypes were detected in New Zealand, one of which appears to be the Tasmanian Florentine Valley strain. However, the detection of an obligate hyperparasitoid of *E. nassaui* reduced the recovery of samples and mitigates any advantage that may have been obtained from the release of Tasmanian strains.

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Appendix 15. Published paper

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Differences in egg parasitism of *Chrysophtharta agricola* (Chapuis) (Coleoptera: Chrysomelidae) by *Enoggera nassaui* Girault (Hymenoptera: Pteromalidae) in relation to host and parasitoid origin

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Abstract The first instances of egg parasitism of *Chrysophtharta agricola*, a pest of eucalypt plantations, are recorded. *Enoggera nassaui* was found parasitising *C. agricola* egg batches in Tasmania, the Australian Capital Territory (ACT), New South Wales and Victoria: this is the first record of this parasitoid species from Victoria. One instance of *Neopolycystus* sp. parasitising *C. agricola* eggs in Victoria was also recorded. Parasitism of egg batches by *E. nassaui* ranged from 0 to 55% between five geographical populations collected in mainland Australia ($n = 45$), and from 0 to 2% between two populations collected in Tasmania ($n = 300$). For mainland sites at which parasitism was recorded, parasitism rates within sites differed significantly from either population in Tasmania. Reciprocal exposure experiments using one Tasmanian (Florentine Valley) and one parasitised mainland (Picadilly Circus, ACT) population were conducted in the laboratory to examine whether these different parasitism rates were attributable to egg or parasitoid origin. Parasitoids from the ACT parasitised *C. agricola* eggs of both origins more successfully than parasitoids from Tasmania, with up to 65% wasp emergence compared with 33% from Tasmania. Parasitoid origin significantly affected the number of wasps that emerged from exposed batches, but not the total loss from parasitism.

Key words eucalypt leaf beetle, geographical variation, parasitoid efficacy.

INTRODUCTION

Chrysophtharta agricola (Chapuis) is a pest of *Eucalyptus nitens* plantations in Tasmania (de Little 1989; Ramsden & Elek 1998) and occasionally of *E. globulus* and *E. viminalis* in Victoria (Elliott *et al.* 1998). It has been collected from around 20 species of eucalypts throughout its geographical range, which extends from the eastern New South Wales (NSW)–Queensland (Qld) border to southern Tasmania (Tas.). In Tasmania, *C. agricola* is usually univoltine (Ramsden & Elek 1998), while in Victoria (Vic.) it completes two generations per year (Neumann 1993). In Tasmania, adults emerge from overwintering in October–November and lay eggs from November to March in untidy batches of around 23 eggs on leaf tips (Ramsden & Elek 1998). Predators, including ladybirds, soldier beetles and mirids, consume the eggs and larvae of *C. agricola*, while at least two species of tachinid flies and one species of braconid wasp parasitise the larvae (G.R. Allen, A.D. Rice and V. Patel, pers. comms, 2000). However, there are no records of egg parasitism for *C. agricola*.

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Enoggera nassaui Girault (Hymenoptera: Pteromalidae) is an endemic Australian solitary egg parasitoid that has been recorded from five genera (*Paropsis* Olivier, *Chrysophtharta* Weise, *Trachymela* Weise, *Paropsisterna* Motschulsky and *Trocholodes* Weise) of eucalypt-feeding, paropsine chrysomelid beetles and from the psyllid genus *Creiis* Froggatt (Naumann 1991; Mo & Farrow 1993; Cox 1994; Tribe 2000). *Enoggera nassaui* has been recorded from Qld, NSW, the Australian Capital Territory (ACT) and Western Australia (WA) (Naumann 1991). It was successfully introduced to New Zealand from WA for the biological control of *Paropsis charybdis* Stål (Kay 1990). Its congener, *E. reticulata* Naumann, also introduced from WA, established in South Africa as a biological control agent for *Trachymela sinicollis* (Blackburn), with a parasitism rate of 96% (Tribe 2000).

Although *E. nassaui* has been recorded from the eggs of five species of *Chrysophtharta*, viz. *C. bimaculata* (Olivier), *C. decolorata* (Chapuis), *C. amoena* (Clark), *C. annularis* Blackburn and *C. variicollis* (Chapuis) (Naumann 1991; Mo & Farrow 1993; Cox 1994; Tribe 2000), it has not been previously recorded from *C. agricola*. Our study quantifies egg parasitism by *E. nassaui* of different populations of *C. agricola* in Tasmania and mainland Australia. As we found significant differences in the occurrence of egg parasitism between mainland and Tasmanian populations, we also tested whether host or parasitoid origin affected successful

egg parasitism of *C. agricola* eggs using a reciprocal exposure experiment.

MATERIALS AND METHODS

Egg batches of *C. agricola* were collected from roadside *E. viminalis* and *E. dalrympleana* by H.F.N. from Picadilly Circus, ACT (35°21′S 148°47′E); the Jindabyne–Thredbo roadside, NSW (36°27′S 148°27′E); Mt Buller, Vic. (37°06′S 146°25′E); and Marysville, Vic. (37°31′S 145°43′E) in early January 2001, and every 2 weeks from *E. nitens* plantations at Frankford, Tas. (41°20′S 146°46′E) and the Florentine Valley, Tas. (42°38′S 146°29′E), between November 2000 and March 2001. The number of eggs per batch was counted and compared between sites. Egg batches were held individually in petri dishes under ambient laboratory conditions (about 21°C, natural photoperiod) until emergence of beetle larvae or adult parasitoids. *Enoggera nassaui* were identified by B.D.M. using scanning electron microscopy, the key by Naumann (1991), and through comparison with Tasmanian specimens identified by Dr Stefan Schmidt (Australian National Insect Collection, Canberra). The number of egg batches from which *E. nassaui* emerged was recorded.

Reciprocal exposure experiments

Because parasitism of *C. agricola* eggs was too low at both Tasmanian sites to obtain sufficient specimens for experiments, *E. nassaui* was collected in egg batches of *Paropsis aegrota* Boisduval and *P. charybdis* from the Florentine Valley and reared through one generation on laboratory-reared *P. aegrota* eggs. All mainland *E. nassaui* used in testing originated from Picadilly Circus, ACT, but were first reared on laboratory-reared *P. aegrota* eggs to standardise testing. *Paropsis aegrota* was used as the rearing host because it is frequently found parasitised by *E. nassaui* in field surveys (Naumann 1991; B.D. Murphy, unpubl. data, 2000). Parasitoids were held in ventilated vials at ambient laboratory conditions and provided with a dilute (approximately 5%) honey solution, *P. aegrota* egg batches less than 24 h old, and water. Exposed egg batches were removed after 2–3 days and monitored so that hatching beetle larvae were removed before they damaged parasitised eggs. Parasitoids emerging from these batches were allowed to feed and mate under ambient laboratory conditions (about 21°C, natural photoperiod) for 2–3 days prior to use in the reciprocal exposure experiment.

The *C. agricola* eggs used in our experiments were obtained from adults collected from Picadilly Circus, ACT, and from adults collected from the Florentine Valley, Tasmania. Five male–female pairs of *C. agricola* from each source were placed in cylindrical plastic cages, provided with fresh *E. nitens* foliage and were held in a controlled temperature room at 21 ± 2°C, 16 L:8D photoperiod. Egg batches used in experiments were collected every 24 h.

To test the efficacy of *E. nassaui* from either geographical locality to parasitise *C. agricola* eggs from either locality, the four parasitoid origin/host origin combinations (i.e., ACT wasps with ACT eggs; ACT wasps with Tas. eggs; Tas. wasps with ACT eggs; Tas. wasps with Tas. eggs) were established as treatments, with 10 replicates of each. Because it is difficult to distinguish the sex of *E. nassaui* (Naumann 1991), each replicate commenced by introducing five host-inexperienced wasps into a Petri dish containing a *C. agricola* egg batch of approximately 30 eggs. We watched each replicate until a female inserted her ovipositor into a host egg and then the remaining four wasps were removed and returned to the rearing culture. The ovipositing female was then left with the egg batch for 2 h, after which she was removed and killed in 70% ethanol. Replicates where none of the five wasps oviposited with the *C. agricola* eggs within 1 h were then offered 30 *P. aegrota* eggs for 1 h. If oviposition subsequently occurred, the test with *C. agricola* was recorded as zero parasitism as at least one female physiologically capable of parasitism was present and chose not to parasitise the *C. agricola* eggs. If no oviposition occurred in the *P. aegrota* eggs, the replicate was discarded and repeated using different wasps.

All egg batches exposed to *E. nassaui* were held individually in petri dishes and monitored for beetle larvae or parasitoid emergence. As larvae and parasitoids emerged, they were removed from the petri dish and parasitoids were killed in 70% ethanol. Approximately 10 days after emergence ceased, egg batches were dissected under a microscope and the number of unhatched eggs was recorded. Ten unexposed *C. agricola* egg batches from each origin were placed in separate petri dishes and treated as controls for failure of eggs to hatch without exposure to *E. nassaui*. Data were square-root transformed and egg batch size was used as a covariate because egg batch size was not constant. The failure to hatch of eggs from control (unexposed) batches was not significantly different between the mainland and the Tasmanian populations (*t*-test; $t_{18} = 0.33$, $P = 0.75$): 6.1% of the mainland eggs and 7.4% of the Tasmanian eggs failed to hatch. These proportions were used to adjust exposed egg-batch numbers for each population, respectively, so that the total loss from parasitism was calculated as:

$$\%\ total\ loss\ from\ parasitism = [(\#\ wasps\ emerged + \#\ unhatched\ eggs) - (egg\ batch\ size \times proportion\ of\ unhatched\ eggs\ from\ unexposed\ batches)/egg\ batch\ size] \times 100 \quad \text{(Equation 1)}.$$

The head capsule width (± 0.02 mm) of a subsample of parental *E. nassaui* (reared from *P. aegrota* eggs), and their offspring (reared from *C. agricola* eggs of both origins) were measured under a dissecting microscope (45 times), but sexes were not considered separately because they are difficult to distinguish. Differences were detected using a one-way ANOVA and posthoc comparisons were made using Fisher's LSD test at $P < 0.05$.

Table 1 Occurrence of *Enoggera nassaui* in egg batches of *Chrysoptharta agricola* from seven geographical locations within Australia. Egg batch size did not differ significantly between sites (ANOVA, $F_{6,336} = 1.8$, $P = 0.1$)

Site	*n*	Egg batches parasitised (%)	Mean ± SE egg batch size (range)
Picadilly Circus, ACT	9	55	28.2 ± 5.9 (14–39)
Jindabyne, NSW	18	50	26.9 ± 2.1 (14–39)
Mt Buller, Vic.	7	0	29.1 ± 5.5 (18–57)
Lake Mountain, Vic.	5	0	34.2 ± 6.9 (19–54)
Marysville, Vic.	6	33	36.6 ± 3.9 (21–54)
Frankford, Tas.	138	2	32.9 ± 0.8 (14–67)
Florentine Valley, Tas.	160	0	34.1 ± 0.8 (11–64)

ACT, Australian Capital Territory; NSW, New South Wales; SE, standard error; Tas., Tasmania, Vic., Victoria.

RESULTS

Forty-five *C. agricola* egg batches were collected from five locations in mainland Australia (Table 1). *Enoggera nassaui* emerged from 0 to 55% (mean ± SE = 27.6 ± 13.2) of these egg batches, depending on site. *Neopolycystus* sp. emerged from one egg batch collected from Marysville. No egg batches collected from Lake Mountain and Mount Buller were parasitised.

Enoggera nassaui emerged from just three of the 300 egg batches collected from two sites in Tasmania. Only two egg batches collected on 27 December 2000 and one egg batch collected on 22 January 2001 from Frankford contained *E. nassaui*. No egg parasitism was recorded from the Florentine Valley. There was a significant difference in the proportion of parasitised egg batches between mainland and Tasmanian populations where parasitism occurred (e.g., Marysville compared with Frankford two-way contingency table, $\chi^2_1 = 16.9$, Fisher's exact test $P = 0.01$), but not between sites at which parasitism occurred on the mainland ($\chi^2_2 = 0.75$, $P = 0.69$).

Egg batch size was similar between mainland and Tasmanian sites (ANOVA, $F_{6,336} = 1.8$, $P = 0.1$) (Table 1).

Reciprocal exposure experiment

Parasitism occurred in each wasp/egg batch origin combination. There was no difference in the percentage of egg batches from which *E. nassaui* emerged according to wasp or host origin (two-way contingency table, $\chi^2_3 = 2.8$, $P = 0.09$) (Table 2). For parasitoids that originated in the ACT, parasitism within batches of either origin (i.e., the percentage of eggs from a single batch that bore adult wasps) ranged from 0 to 65% ($n = 10$), and for Tasmanian parasitoids the range was 0–33% ($n = 10$). For both parasitoid populations, the highest wasp emergence was recorded from eggs laid by beetles from the opposite origin to that of the wasp. The numbers of wasps that emerged from egg batches were variable, but higher for ACT-originated parasitoids (Fig. 1). Total loss from parasitism ranged from 3 to 93% for ACT wasps, and from 0 to 94% for Tasmanian wasps. Again, the highest loss from parasitism was recorded from eggs laid by beetles of the opposite origin to that of the wasp.

Table 2 Percentage of *Chrysophtharta agricola* egg batches from which *Enoggera nassaui* emerged from each parasitoid/host origin combination ($n = 10$ exposed batches per treatment). Female wasps were observed to oviposit in each egg batch

Source	Mainland *Enoggera*	Tasmanian *Enoggera*
Mainland eggs	100	50
Tasmanian eggs	80	60

The ACT population of parasitoids was significantly more successful at parasitising *C. agricola* eggs of either origin than the Tasmanian (Florentine Valley) population of parasitoids (two-way ANOVA, $F_1 = 11.29$, $P = 0.002$). That is, parasitoid origin had a significant effect on the number of F_1 adult parasitoids that emerged. However, neither host nor parasitoid origin affected the total loss from exposure to wasps (two-way ANOVA, $F_1 = 2.4$, $P = 0.13$).

Parasitoids from the ACT had significantly wider head capsules than parasitoids from southern Tasmania, and the rearing host also significantly affected head capsule width (Table 3) (ANOVA, $F_{5,78} = 151.1$, $P < 0.001$). The average head capsule width of ACT *E. nassaui* reared on *P. aegrota* eggs was 0.58 ± < 0.00 mm ($n = 40$), whereas the head capsule width of *E. nassaui* from the Florentine Valley, Tas., was 0.56 ± < 0.00 mm ($n = 15$) when reared on the same host.

DISCUSSION

The occurrence of *C. agricola* eggs parasitised by *E. nassaui* in the field was significantly lower in Tasmania compared with several populations in mainland Australia. Our field collection data for mainland populations were from native eucalypt bushland, whereas Tasmanian populations were collected from eucalypt plantations. Evidence suggests that natural enemies are less effective in monocultures than in mixed stands (e.g., Dazoj 2000) and this may partially account for the low field occurrence of *E. nassaui* in our survey. Furthermore, the role of plant volatiles in egg parasitoid searching behaviour is important (e.g., Honda & Walker

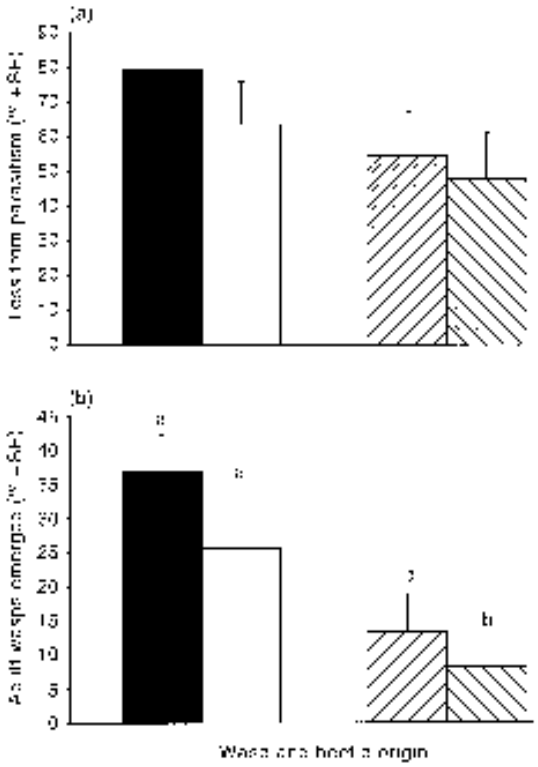

Fig. 1. (a) Percentage total loss from parasitism by *Enoggera nassaui* on eggs of *Chrysophtharta agricola*, and (b) the percentage of adult parasitoids that emerged. Total loss from parasitism includes the number of parasitoids emerging, plus the number of unhatched eggs. Different letters for each mean denote significant differences at $P < 0.05$. (■), Wasp and beetle from mainland; (□), wasp from mainland, beetle from Tasmania; (▨), wasp from Tasmania, beetle from mainland; (□), wasp and beetle from Tasmania.

Table 3 Head-capsule widths of *Enoggera nassaui* from different geographical and host origins, reared from *Chrysophtharta agricola* eggs. Different letters denote significant differences ($P < 0.001$)

Parasitoid origin	Egg origin	*n*	Mean head capsule width ± SE (mm)
Mainland	Tasmania	57	0.46 ± 0.01 a
Mainland	Mainland	77	0.46 ± 0.01 a
Tasmania	Tasmania	15	0.43 ± 0.01 b
Tasmania	Mainland	32	0.43 ± 0.01 b

1996); the low occurrence of *E. nassaui* in *C. agricola* eggs in Tasmania may be influenced by the relatively recent introduction of the host plant on which our collections were made. However, although *E. nitens* is an introduced tree species to Tasmania, a number of paropsine species occurring in sympatry with *C. agricola* are parasitised by *E. nassaui* (B.D. Murphy, unpubl. data, 2000), and parasitism of *P. charybdis* by *E. nassaui* in New Zealand on *E. nitens* can exceed 90% (Kay 1990).

Enoggera nassaui from the ACT were larger than their Tasmanian counterparts. Larger size is related to higher fecundity in a number of species of egg parasitoids (e.g., Kazmer & Luck 1995), and may account for the higher parasitism rate observed in the field at the ACT site, compared with Tasmanian sites. The results of our reciprocal exposure experiment showed that *E. nassaui* from the ACT were more effective at parasitising *C. agricola* eggs than *E. nassaui* from Tasmania under laboratory conditions.

Enoggera reticulata achieves up to 96% parasitism of *T. cincticollis* eggs in South Africa (Tribe 2000), and Tribe (2000) recorded an average of 80% parasitism by *E. nassaui* for the 12 paropsine species that he tested under laboratory conditions. Despite his wasps being left for 24 h with egg batches, only two host species recorded significant numbers of collapsed eggs. Tribe (2000) reported that collapsed eggs contained '. . . parasite larvae . . . but insufficient yolk . . . for any larvae to complete their development'. The proportion of collapsed eggs that we recorded from just 2 h of exposure was up to 94%, suggesting that *C. agricola* eggs are less suitable for *E. nassaui* development than the eggs of other paropsine species. Indeed, despite female wasps being observed to oviposit in almost all replicates, there were some egg batches from which no adult wasps emerged. Maximum parasitoid emergence from *C. agricola* eggs in our experiments was 65% for *E. nassaui* from the ACT and 33% for parasitoids originating from Tasmania, compared with maximum parasitoid emergence of >90% for eight paropsine species tested by Tribe (2000). Although this suggests that *C. agricola* may be a less suitable host for *E. nassaui* than other paropsine species, *E. nassaui* from the ACT nonetheless were more effective at using *C. agricola* as a host than *E. nassaui* from Tasmania.

Apart from the greater ability of ACT *E. nassaui* to successfully parasitise *C. agricola* eggs of either origin, other factors may have affected the differing parasitism rates that we recorded. The spatial arrangement of eggs within batches may affect their accessibility to parasitoids (Hilker 1994), and larger batches may protect more eggs from parasitism than smaller batches. However, mean egg batch size did not differ between Tasmanian and mainland Australian sites. The number of host generations each year may affect the abundance of *E. nassaui* and *C. agricola*, and differences in voltinism may influence the parasitoid's efficacy. In Tasmania, *C. agricola* is usually univoltine (Ramsden & Elek 1998), but it is reportedly bivoltine in Victoria (Neumann 1993). Further investigation of the phenology of *E. nassaui* in the field in mainland Australia and Tasmania may increase our understanding of this host–parasitoid interaction. Our experiments testing the efficacy of two geographical populations of *E. nassaui* were conducted under controlled laboratory conditions. It is possible that although *E. nassaui* performed better under these conditions, they may not parasitise *C. agricola*

as successfully at different temperatures. That *E. nassaui* of different origins may perform differently under different climatic conditions is being studied by Murphy and Kay (2000), introducing Tasmanian *E. nassaui* into New Zealand for biological control of *P. charybdis* in areas that the parasitoid is currently ineffective because of climatic intolerance.

Here, we have identified potential 'interstrain variation' between field populations of *E. nassaui* parasitising *C. agricola* eggs, and this difference persisted when wasps and hosts were reared under the same conditions in the laboratory. These are two of the five traits listed by Hopper *et al.* (1993) for identifying genetic variation in potential biological control agents. While the introduction of different 'strains' in classical biological control has rarely succeeded (Clarke & Walter 1995), it has seldom been tested using native systems such as the *E. nassaui–C. agricola* interaction. However, the possibility that the species identified here as *E. nassaui* from different geographical localities represent different sibling (or cryptic) species, rather than different 'strains' requires consideration (*sensu* Clarke & Walter 1995) before further investigation is undertaken.

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