Molecular Markers for *Lygus* Parasitoids to Assess Host Specificity of Candidate Entomophagous Biological Control Agents

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ABSTRACT

Lygus Hahn (Hemiptera: Miridae) are serious pests of economically important field, fruit, vegetable, and greenhouse crops in Canada. The release of European *Peristenus* Förster (Hymenoptera: Braconidae) in the USA has resulted in significant suppression of this pest (Day, 1996) and has renewed interest in the release of European *Peristenus* spp. in Canada. Prior to the release of exotic *Peristenus* spp., ecological host-range studies need to be conducted to define their habitat and host associations.

These associations can be difficult to study using conventional methods. Morphological similarity of related parasitoids prevents species-level identification by dissection. Host rearing is time-consuming and can result in high levels of host and parasitoid mortality. To facilitate identification of immature *Peristenus* spp. in their hosts, a multiplex PCR assay was developed. This assay provided a specific and sensitive tool to screen individual insects for three parasitoid species simultaneously.

To validate the utility of the multiplex PCR assay in ecological host-range studies, parasitism and parasitoid species composition obtained using conventional and molecular techniques were compared. Molecular methods compared favorably with conventional methods; however, more complete parasitoid species composition information was available with the multiplex assay. To improve the quality of risk-assessment studies and extract the most accurate ecological host-range data, molecular methods were used to evaluate host-parasitoid associations in mirid populations collected in two ecoregions. Several new host-parasitoid associations were recorded for *P. digoneutis* and *P. relictus*, but parasitism of non-target mirids was low.

Parasitism of the target host collected from different plant species was evaluated to help clarify *Peristenus* – host-plant associations. Despite the investigation of three different host plant species, no difference was observed in the parasitism level or parasitoid species composition in *L. rugulipennis*.

The post-release utility of the multiplex assay was investigated in Canada, where *Lygus* parasitoids may have dispersed following release in the USA. To confirm establishment, samples were analyzed using the multiplex PCR assay, and *P. digoneutis* was detected for the first time in southern Ontario.

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1. INTRODUCTION

1.1 Biological Control of Insect Pests

Biological control is defined as, "the actions of parasites, predators and pathogens in maintaining another organism's density at a lower average than would occur in their absence" (DeBach, 1964). There are three general approaches to biological control of pest organisms: classical biological control, inundative biological control, and conservation biological control. Classical biological control involves the importation and release of exotic natural enemies to control a native or invasive pest, and is the main focus of this thesis. The aim of inundative biological control is to increase the effectiveness of a natural enemy by the mass release of an agent prior to or during peak pest populations. Conservation biological control relies on the identification of ecological or environmental factors that affect the efficacy of beneficial organisms, and the optimization of conditions to favor the survival and retention of natural enemies in the agroecosystem.

The recognition of medical and ecological impacts of pesticides (e.g., non-target toxicity, residual effects, environmental contamination, pesticide resistance) has led to the adoption of pest control methods that are more environmentally sound. Such methods often involve the use of biological control. Biological control can provide an affordable and sustainable control method because biological organisms can reproduce and disperse throughout an agroecosystem. Chemical and cultural control methods do not offer this possibility; they require repeated treatments, labor, and expenditure to maintain control of a given pest. When responsible pest-management decisions are made, the use of natural enemies can provide an inexpensive, safe, non-toxic alternative to chemical control.

Despite the advantages over chemical control, biological control has not been widely adopted. This is likely related to the fact that biological control does not necessarily offer immediate results, nor does it provide complete eradication of the target pest (Gillott, 2005). Further, the threat of potential non-target effects (discussed in Section 1.2) has raised concerns about whether biological control is safe (Howarth, 1983; Simberloff & Stiling, 1996).

1.1.1 Classical Biological Control

Interest in classical biological control in North America began in the late 19th Century when accidentally introduced pests began to infest agricultural crops. Many of these pests arrived in North America without the natural enemies that were present in their area(s) of origin. This led to the idea of international transfer of beneficial insects for control of these pests (Doutt, 1964). The first international transfer occurred in 1883 when a European parasitoid, *Cotesia glomerata* L. (Hymenoptera: Braconidae) was introduced into the USA for control of the cabbageworm, *Pieris rapae* L. (Lepidoptera: Pieridae) (Coppel & Mertins, 1977).

There are numerous examples of successful classical biological control programmes for pestiferous insects and weeds, and perhaps the most well known is the programme that targeted prickly pear cactus, *Opuntia* Miller (Cactaceae) in Australia. This plant originated in the Western Hemisphere and was introduced into Australia in the late 1800's. By 1925, over 24 million hectares of land were overrun with exotic *Opuntia* spp. (Holloway, 1964). The search for host-specific insects for control of the plant led to the importation of 12 different phytophagous insect species in the early 1920's. The importation and release of *Cactoblastis cactorum* Berg (Lepidoptera: Pyralidae) from Argentina was responsible for the elimination of large areas of the prickly pear cactus, and by the 1960's *Opuntia* spp. were only occasionally found in Australia (Holloway, 1964). Biological control of *Opuntia* spp. with the same natural enemies used in the Australian programme was successful in an additional 16 countries (Julien, 1992; Greathead, 1995).

A well-known example of a successful classical biological control programme for an insect is the case of the cottonycushion scale, *Icerya purchasi* Maskell

(Homoptera: Margarodidae), in California citrus orchards. The threat of this pest to the citrus fruit industry fueled an international search for natural enemies. The establishment of the vedalia beetle, *Rodolia cardinalis* Mulsant (Coleoptera: Coccinellidae), from Australia was successful in the control of cottonycushion scale, and is thought to have saved the California citrus industry (Coppel & Mertins, 1977).

The International Institute of Biological Control reported that 4769 species of exotic insect parasitoids and predators were released world-wide between 1880 and 1992 (Greathead, 1995). Many of these programmes have seen success; however, some introductions of exotic natural enemies have also met with failure. It is estimated that less than 25% of introduced entomophagous biological control agents become established (DeBach, 1965). In addition, one study estimated that only 16% of over 600 introductions have reduced pest densities sufficiently to replace chemical control (Myers et al., 1989). Failure to establish and control the target pest may be attributed to climatic mismatch, competition with native species, hyperparasitism, and poor synchrony with the target host (Hawkins et al., 1993). The relatively poor success rate of biological control is due, in part, to a failure to consider these important factors in the initial stages of a biological control programme.

Environmental constraints, adaptability of the introduced agent, selection of suitable biotypes, and potential non-target effects are important considerations in the selection of candidate biological control agents. The importance of these factors is illustrated in the biological control programme for *Sitona discoideus* Gyllenhal (Coleoptera: Curculionidae) on lucerne in Australia and New Zealand. Three biotypes of *Microctonus aethiopoides* Loan (Hymenoptera: Braconidae) were released in an effort to reduce populations of this pest (Aeschlimann, 1995). Parasitoids were obtained from France, Morocco and Greece; however, it was realized after importation that *M. aethiopoides* represented a complex of biotypes, each associated with a particular host (Aeschlimann, 1995). The Greek and French biotypes failed to establish because one preferred a different host species (*S. bicolor*), and the other was unable to adapt to the climatic conditions in the area of introduction (Aeschlimann, 1995). The Moroccan biotype adapted to the host and climatic conditions and resulted in high levels of parasitism in *S. discoideus*; however, it has since been shown to attack several native,

non-target species in laboratory tests and in the field (Barratt et al., 1997; Barratt & Johnstone, 2001; Barratt, 2004).

1.1.2 Classical Biological Control in Canada

Biological control in Canada dates back to 1882, when *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) was imported from the USA to Canada (Doutt, 1964). The first intercontinental transfer took place when *Encarsia formosa* Gahan (Hymenoptera: Encyrtidae) was imported from England in 1935, and was mass-reared and released in Ontario for whitefly (*Trialeurodes vaporariorum* Westwood; Hemiptera: Aleyrodidae) control in greenhouses (Elliott, 2005).

Between 1981 and 2000, biological control programmes targeted 98 pest species in Canada; 55 of these programmes were against insect pests and mites, 24 were aimed at weed control, and 19 focused on the suppression of plant pathogens (Mason & Huber, 2002).

A recent and on-going biological control effort in Canada involves the classical biological control programme for the orange wheat blossom midge, *Sitodiplosis mosellana* Géhin (Diptera: Cecidomyiidae), a pest that was accidentally introduced in North America in the 1800's (Doane et al., 2002). In Europe, *S. mosellana* is not a serious pest due to the presence of effective natural enemies. Surveys in Saskatchewan revealed that a European parasitoid, *Macroglenes penetrans* Kirby (Hymenoptera: Pteromalidae), is already present in the Canadian prairies and was likely introduced with the pest. *Macroglenes penetrans* has significantly suppressed *S. mosellana* infestations in many areas of western Canada, with parasitism levels between 30% and 80% (Doane et al., 2002). Nonetheless, outbreaks occasionally occur. Thus, an additional species, *Platygaster tuberosula* Kieffer (Hymenoptera: Platygastridae), was released in an effort to increase the suppression of *S. mosellana*. Establishment in Saskatchewan has been confirmed, parasitism levels are increasing from year to year, and the parasitoids have begun to migrate from release sites to the surrounding area (Olfert et al., 2003).

Another potential classical biological control programme being considered in Canada is aimed at the control of *Lygus* (Hemiptera: Miridae) plant bugs. This

programme was initiated in 1998 to address the potential of European parasitoids to suppress *Lygus* spp. populations in Canada (see Section 1.3).

1.2 Non-Target Risk Assessment in Biological Control

There are risks and benefits involved in any biological control programme, and it is prudent to investigate the host range of candidate biological control agents prior to their release to ensure minimal non-target impact. Host range refers to the group of species in or on which a control organism can develop (McEvoy, 1996). Evaluation of the host range of a candidate biological control agent can predict whether this agent is able to complete its life cycle in or on target organisms and non-target organisms (Nechols et al., 1992; McEvoy, 1996).

Target organisms can be native or invasive pest species that are of concern due to the economic damage they cause in agroecosystems. In contrast, non-target organisms are species other than the intended target that may be parasitized by introduced natural enemies. The displacement of native species with similar niches, attacks on alternate hosts, host-preference shifts, and dispersal of introduced agents into other habitats are of concern because biological control introductions are irreversible (Howarth, 1983, 1991; Simberloff & Stiling, 1996; Follett et al., 2000; Howarth, 2000; Kuris, 2003).

Louda et al. (2003) provided a retrospective analysis of several biological control programmes where risk assessment was not adequately considered. One of these case studies involved the release of a generalist parasitoid, *Compsilura concinnata* Meigen (Diptera: Tachinidae), in the USA in 1906 for the control of gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae). Limited research was done prior to importation, and the only information available was that the parasitoid was polyphagous, gregarious, and multivoltine (Louda et al., 2003). However, the gypsy moth is univoltine, which suggests that *C. concinnata* would require alternate hosts on which to survive in subsequent generations. Originally, it was thought that a parasitoid with a broad host range would be advantageous, as it would allow populations of *C. concinnata* to complete multiple generations (Elkinton & Boettner, 2004). In North America, *C. concinnata* has now been reared from over 150 native butterfly and moth species, and 8

native sawfly species (Arnaud, 1978; Strazanac et al., 2001; Kellogg et al., 2003; Elkinton & Boettner, 2004). In parts of the eastern USA, this parasitoid may be responsible for the placement of several Saturniidae on the endangered-species list, the extirpation of two species of *Citheronia* Hübner (Lepidoptera: Saturniidae), and the decline of native silk moth, *Hyalophora cecropia* L. (Lepidoptera: Saturniidae) populations (Boettner et al., 2000). To add insult to injury, this parasitoid has not been effective in the effort to control or contain the spread of gypsy moth in the USA.

Despite the focus on potential negative effects of biological control agents, Onstad and McManus (1996) and Duan and Messing (1997) stated that the debate over non-target effects is often philosophical in nature, as thorough, large-scale case studies are lacking. Further, of the exotic natural enemy introductions reported worldwide, only 1.7% have been linked to non-target effects, most of which were minor or negligible (Lynch et al., 2001; Louda et al., 2003). Onstad and McManus (1996) suggested that habitat loss and destruction are more likely to be the cause of native species extinctions.

It is important to make the distinction between polyphagous biological control agents, which have broad host ranges, and oligophagous agents, which are more host-specific (Onstad & McManus, 1996; Simberloff & Stiling, 1996). Currently, generalist parasitoids and predators are rarely considered for use as biological control agents due to the high potential for non-target effects. Further, the recent requirement for host-specificity testing prevents authorization of the release of non-specific agents in most countries (Miller & Aplet, 1992; Greathead, 1997; Sands, 1998; Van Driesche, 2004; Barratt & Moeed, 2005; Bigler et al., 2005; Simberloff, 2005; Babendreier et al., 2006). In the early 20th Century, non-target effects were rarely considered and regulations were not in place to ensure that only host-specific agents were released. Today, polyphagous parasitoids, such as *C. concinnata*, would surely not be released, as they would fail to meet host-specificity standards.

Protocols for the evaluation of host specificity often focus on field studies in the area of origin to determine the ecological host range of a parasitoid and the biotic and abiotic factors that restrict the natural enemy to a particular niche (Greathead, 1997). The ecological host range is defined as the current and evolving set of host species used by a natural enemy to support its reproduction under natural conditions (Onstad &

McManus, 1996). Ecological host-range studies on parasitoids involve the collection of potential hosts in the field to identify the parasitoid community associated with target and non-target hosts.

Pre-release laboratory experiments to assess the fundamental host range of a parasitoid are also used as a measure of host-specificity (Sands & Van Driesche, 1999). The fundamental host range refers to the set of species that can support the development of a parasitoid under laboratory conditions (Onstad & McManus, 1996; Strand & Obrycki, 1996). However, fundamental host-range studies may overestimate the host range of a natural enemy because they do not take into account some of the biological and environmental constraints that may be present in the field (Sands, 1993; Strand & Obrycki, 1996; Van Driesche & Hoddle, 1997).

For example, Duan and Messing (1997) studied the ecological host range of two braconids introduced for the control of fruit flies in Hawaii, with particular attention to potential non-target effects on native flies (Diptera: Tephritidae). Although the braconid parasitoids attacked non-target tephritids in the laboratory, field studies showed that the imported parasitoids failed to recognize the ovipositional cues associated with non-target hosts. This is likely because native tephritids are associated with flower heads, whereas the imported parasitoids are adapted to search for hosts on fruit or galls. Thus, ecological host-range studies are often used to complement fundamental host-range studies, as they allow a more accurate interpretation of laboratory results (Clement & Cristofaro, 1995; McEvoy, 1996).

1.3 Biological Control of *Lygus* Bugs

1.3.1 Biology and Pest Status of Lygus *in North America*

Lygus Hahn plant bugs are Hemiptera of the family Miridae. Adult *Lygus* overwinter in plant litter along field margins and become active in the spring, when they move to budding or flowering plants and begin to oviposit (Craig & Loan, 1987; Jones, 1999; Broadbent et al., 2002; Carcamo et al., 2002). By late spring, first-instar nymphs are present in the field. There are five nymphal instars, and the first generation of adults generally appears in June. Adults disperse to colonize new plants, and one to five generations occur (Broadbent et al., 2002).

Worldwide there are 43 species of *Lygus*; 31 are native to North America, 10 to Europe, and 2 to China (Kelton, 1975; Schwartz & Foottit, 1998). *Lygus* bugs feed on meristematic tissue and developing reproductive parts of the plant and cause fruit abscission or deformation, necrosis, reduction of seed viability, and decreased vegetative growth (Strong, 1970). Of the native species present in North America, *L. lineolaris* Palisot de Beauvois, *L. hesperus* Knight, *L. elisus* van Duzee, and *L. borealis* Kelton, are among the most damaging and prevalent (Schwartz & Foottit, 1992). The most widespread of these pests is *L. lineolaris*, the tarnished plant bug, which occurs throughout North America. *Lygus lineolaris* is associated with 130 economically important host plants, including 21 of the 30 most important crops produced in the USA (Young, 1986).

In Canada, *Lygus* is a common pest on celery, lettuce, spinach, cabbage, broccoli, cauliflower, pepper, eggplant, tomato, potato, cucumber, and bean (Chaput & Uyenaka, 1998). *Lygus* outbreaks in Ontario result in losses of $12 million annually for the orchard and horticultural industries (Broadbent et al., 2002). Of particular concern in western Canada is the impact of *Lygus* on seed alfalfa and canola. In Saskatchewan, the seed alfalfa industry loses approximately $50 million per year due to *Lygus* infestations (Broadbent et al., 2002). Similarly, *Lygus* outbreaks in canola in southern Alberta in 1997 resulted in the loss of over $70 million despite the application of chemical insecticides to 200 000 hectares of canola (Broadbent et al., 2002). Other industries in Canada that are affected by *Lygus* plant bugs include the greenhouse industry (Gillespie & Foottit, 1997) and the nursery seedling (forestry) industry (Shrimpton, 1985; Schowalter, 1990). Cereal crops, such as wheat, buckwheat, oat, rye, and barley are also commonly used as host plants by *Lygus* spp. in Canada (Wise et al., 2005).

1.3.2 Control Measures for Lygus *spp.*

Chemical Control

Lygus control often relies on chemical pesticides, primarily carbamates, pyrethroids and organophosphates. There are no selective pesticides for the control of *Lygus*, and therefore broad-spectrum insecticides are the only option available. In the USA, *Lygus* populations in alfalfa and cotton have started to develop resistance to

insecticides, likely due to repeated application of broad-spectrum insecticides (Grafton-Cardwell et al., 2000; Godfrey et al., 2001). In addition, the use of pesticides for *Lygus* control in alfalfa may disrupt biological control programmes for three other alfalfa pests: the alfalfa weevil (*Hypera postica* Gyllenhal; Coleoptera: Curculionidae), pea aphid (*Acyrthosiphon pisum* Harris; Homoptera: Aphididae), and alfalfa blotch leafminer (*Agromyza frontella* Rondani; Diptera: Agromyzidae) (Day, 1987).

Cultural Control

Removal of overwintering habitats (e.g., weeds, crop debris, leaf litter), selection of resistant or tolerant plant varieties, and management of weeds that are potential hosts for *Lygus* are recommended. Although these crop management practices may help reduce crop damage from insect feeding and disease, they generally do not prevent *Lygus* damage from exceeding the economic threshold (Chaput & Uyenaka, 1998).

For *Lygus* management in cotton, Stern et al. (1969) recommended interplanting 6 m strips of alfalfa for every 90 – 150 m of cotton. *Lygus* would be more likely to stay in the alfalfa, and therefore cause less damage to the cotton crop.

Other studies investigated the possibility of strip-cutting alfalfa, rather than completely harvesting the crop, to limit the mass migration of *Lygus* from forage alfalfa to more susceptible, higher value crops (Mueller et al., 2005). Similarly, the use of alfalfa as a trap crop has been investigated to help manage *L. rugulipennis* populations. However, effective management was only possible when localized chemical treatments were applied to the trap crop (Ferrari et al., 2003; Accinelli et al., 2005).

Ferrari et al. (2004) and Accinelli et al. (2004) used trap crops, colored sticky traps, and netting around the entrance of horticultural tunnels where cucumbers and aubergines were grown. The use of nets as a physical barrier appeared to be the more effective of these methods, but was still unable to completely exclude *Lygus*.

Biological Control

Several organisms attack *Lygus* bugs, including microbial pathogens, parasitoids and predators. Among the microbial pathogens is the entomopathogenic fungus *Beauveria bassiana*, which has shown some potential in early-season management of

Lygus populations (Ruberson & Williams, 2000). Two commercial mycoinsecticides derived from isolates of *B. bassiana* have been evaluated for their efficacy against *Lygus*; however, they function poorly under field conditions because of UV instability (Leland et al., 2005).

Another microbial insecticide being investigated for *Lygus* control is *Bacillus thuringiensis* (*Bt*). Wellman-Desbiens and Côté (2004) screened 94 strains of *Bt* and found that 5 demonstrated significant insecticidal activity against *Lygus hesperus* Knight. However, all 5 strains produced ß-exotoxin, which interferes with RNA biosynthesis and results in a broad spectrum of activity against invertebrates and vertebrates (Wellman-Desbiens & Côté, 2004). Most countries will not register *Bt* strains that produce ß-exotoxin because of the potential non-target effects (Glare & O'Callanghan, 2000). Thus, the strains investigated by Wellman-Desbiens and Côté (2004) are unlikely to be registered for use. Nonetheless, further screening of *Bt* isolates may lead to the discovery of a novel microbial insecticide for *Lygus* plant bugs.

Several arthropod natural enemies have also shown promise as biological control agents for *Lygus* plant bugs. *Anaphes iole* Girault (Hymenoptera: Mymaridae) is a native parasitoid that attacks *Lygus* eggs in western North America. Although parasitism levels are variable, they can reach 100% (Graham et al., 1986). Thus, *A. iole* has potential as an inundative biological control agent for *Lygus*, and techniques for mass rearing are being developed (Norton et al., 1992; Williams et al., 2003; Manrique et al., 2005; Riddick, 2005).

Leiophron Nees (Hymenoptera: Braconidae) are nymphal parasitoids of mirids in Europe and North America. Two multivoltine species are associated with *Lygus* in North America, *L. uniformis* Gahan and *L. lygivorus* Loan. Although parasitism of *Lygus* by *Leiophron* is not very high in alfalfa (maximum 10% parasitism), it is significantly higher in *Chenopodium* spp. (maximum 30%) (Clancy & Pierce, 1966; Graham et al., 1986). Further, preliminary field-cage trials suggest that *L. uniformis* may be useful in an inundative biological control programme for *Lygus* in strawberries (Norton et al., 1992).

Members of the genus *Peristenus* Förster (Hymenoptera: Braconidae) are also nymphal parasitoids associated with *Lygus*. Species of *Peristenus* occur in North

America and Europe. However, most North American species are univoltine and thus, their ability to suppress multivoltine *Lygus* is limited. In contrast, multivoltine *Peristenus* species attack *Lygus* plant bugs in Europe and are likely better synchronized with their hosts (Kuhlmann et al., 1998). See Section 1.3.3 for further details.

Parasitoids that attack *Lygus* adults are also known from Europe and North America. *Phasia obesa* Fabricius (Diptera: Tachinidae) emerge from overwintered *Lygus* adults, and parasitism levels can reach up to 38% in northern Europe (Hellqvist et al., 2001; Rämert et al., 2005). Clancy and Pierce (1966) also listed this species as a parasitoid of *Lygus* in Canada and the USA, along with several other *Phasia* species (*P. opaca* Coquillett, *P. aeneoventris* Williston, *P. fumosa* Coquillett, *P. pulverea* Coquillett, and an unidentified species of *Phasia*). However, these tachinids occur in low numbers and are likely of little or no importance in the suppression of *Lygus* plant bugs in North America (Clancy & Pierce, 1966).

Several generalist predators are thought to reduce *Lygus* populations (Arnoldi et al., 1991). Clancy and Pierce (1966) noted the importance of *Geocoris* Fallén (Hemiptera: Lygaeidae) and *Nabis* Latreille (Hemiptera: Nabidae) species as predators of *Lygus* nymphs. Although the polyphagous nature of these predators discourages their use in most inundative biological control programmes, the retention of naturally occurring predators in a crop (e.g., by not applying pesticides) can help reduce pest densities.

1.3.3 Parasitoids in the Genus Peristenus

Adult *Peristenus* females inject a single egg in the haemocoel of a mirid nymph. After egg hatching, the parasitoid goes through three larval instars before it exits the host (killing it in the process) and drops into the soil where it spins a cocoon and pupates. Parasitoid adults emerge from the cocoon during the same season (if multivoltine) or the pupae go into diapause, overwinter, and emerge in the spring of the following season (if univoltine).

Boundaries between species, subspecies and geographic strains of *Peristenus* are poorly understood. A holarctic species complex, the *P. pallipes* Curtis complex, is currently undergoing taxonomic revision. Members of this complex appear to fill diverse

ecological niches in terms of distribution and host range, and yet most species are indistinguishable based on morphological characters; thus detailed biological data from their hosts are required to separate the species within this complex (Goulet & Mason, 2006). Nine species within the *P. pallipes* complex in North America have been described, six of which use *Lygus* spp. as their main hosts in Canada (Goulet & Mason, 2006). Two species in the *P. pallipes* complex, *P. mellipes* Cresson and *P. pseudopallipes* Loan, are found in eastern Canada (Loan & Craig, 1976; Craig & Loan, 1987; Goulet & Mason, 2006), and four species (*P. carcamoi* n. sp., *P. otaniae* n. sp., *P. broadbenti* n. sp., and *P. gillespiei* n. sp.) are recognized in western Canada (Goulet & Mason, 2006). All native *Peristenus* species in Canada are univoltine.

Currently, species designations within the European *P. pallipes* complex are not available. Henceforth, the use of the name '*P. pallipes*' broadly refers to the holarctic members of this complex, as the majority of the members of this complex have yet to be individually described.

Despite the abundance of species in the native *P. pallipes* complex, parasitism is not sufficient to reduce *Lygus* spp. populations. In Ontario, surveys were conducted to determine parasitism levels in various regions from 1998 to 2000; parasitism by native parasitoids was low, with mean values below 11% (Broadbent et al., 2006). Studies conducted in Saskatchewan in 1998 and 1999 on parasitism of *Lygus* by *Peristenus pallipes* showed moderate parasitism levels (9 to 28%) in alfalfa, but very low parasitism (0 to 1%) in canola and mustard (Braun et al., 2001). Native parasitoids are ineffective in the control of *Lygus* plant bugs, as the first generation of *Lygus* nymphs are primarily attacked while the second generation remains virtually unparasitized (Clancy & Pierce, 1966; Day, 1987). The introduction of exotic parasitoids with life cycles that are better synchronized with the pest may enhance control of *Lygus* plant bugs in North America.

In Europe, two multivoltine species, *P. digoneutis* Loan and *P. relictus* Ruthe (syn. *P. stygicus* Loan) are associated with *L. rugulipennis* and may significantly reduce populations of this species (Broadbent et al., 2002).

In an attempt to increase the overall parasitism of *Lygus* plant bugs, several European *Peristenus* species were released in Canada between 1978 and 1981.

Peristenus digoneutis, *P. relictus*, *P. rubricollis* Thomson and *P. adelphocoridis* Loan were liberated near Saskatoon, SK. However, specimens were never recovered from subsequent field collections, and it is assumed that they failed to establish (Craig & Loan, 1987). The same European species were released in several states in the USA between 1963 and 1983. Despite the fact that these initial parasitoid introductions were not successful, further releases resulted in the establishment of *P. digoneutis* in New Jersey and *P. relictus* in California (Day et al., 1990; Pickett et al., 2001; Pickett et al., 2005). The success of the *Lygus* biological control programme in the USA has renewed interest in the introduction of European parasitoids in Canada (Broadbent et al., 2002), and at least one species (*P. digoneutis*) is being considered for release (Haye, 2004).

1.4 Molecular Diagnostics in Biological Control of Arthropods

In entomology, DNA-based techniques have proven useful in many areas of research, particularly in the study of taxonomic and phylogenetic relationships (reviewed by Caterino et al., 2000) and population genetics (see Behura, 2006). Molecular techniques have also been used to clarify phylogenetic relationships among parasitic Hymenoptera (Dowton & Austin, 1994) and to facilitate ecological studies on parasitoids and predators used in biological control programmes (Symondson, 2002; Greenstone, 2006). The need to accurately identify natural enemies, understand their population dynamics, and ensure minimal non-target impacts has spurred the development of molecular diagnostic tools, particularly for species that are difficult to identify based on morphological traits.

Beyond agent identification, molecular phylogeny may be able to predict natural enemy host range, climatic adaptability, and other important biological traits that can be used in the selection of efficient candidate biological control agents (Unruh & Woolley, 1999). Menalled et al. (2004) suggested that new molecular technologies could lead to increased adoption and success of biological control agents, as they provide taxonomic accuracy, precise identification, and a thorough understanding of population genetics and gene flow.

Traditional methods for identification of parasitoids and quantification of parasitism in a host population generally rely on rearing and dissection of host material. However, there has been increasing interest in the use of molecular methods to identify parasitoids and assess parasitism in host populations. These methods may provide data that are not easily obtained by rearing and dissection. In systems where host dissection provides no information on parasitoid species composition, molecular diagnostics using species-specific PCR primers for parasitoid species of interest could be helpful. Like dissection, they provide a rapid estimate of parasitism level, but also give the species composition data lacking in host dissection. Molecular markers for parasitoids are capable of detecting minute quantities of DNA. This enables the detection of parasitoid eggs within a host, which are often missed by dissection. Additionally, molecular techniques can provide information more rapidly than rearing, which is often delayed by lengthy diapause periods prior to parasitoid emergence. Furthermore, in comparison to rearing, molecular analysis is not affected by host and parasitoid mortality; thus, a more accurate estimate of parasitism level may be possible.

However, detection of parasitoid DNA in a host does not necessarily indicate parasitoid survival, as host immune response may kill immature stages of the natural enemy. Thus, overestimation of the parasitism level may occur when molecular methods are used. In addition, molecular analysis can only detect the presence or absence of parasitoid DNA. It is unable to identify the parasitoid stage or the presence of multiple parasitoids of the same species within a single host. For this reason, data obtained by traditional dissection and rearing methods should be used to supplement molecular data.

Several gene regions have been used in insect molecular studies (Simon et al., 1994; Caterino et al., 2000; Greenstone, 2006; Stouthamer, 2006). The two regions most often targeted for sequencing in insect systematics are mitochondrial DNA (mtDNA) and nuclear ribosomal DNA (rDNA). For mtDNA, the genes most commonly used include cytochrome oxidase I and II (COI, COII), and the 16S and 12S subunits of rDNA. In nuclear rDNA, the 18S and 28S subunits of rRNA and the first and second internal transcribed spacer regions (ITS 1 and ITS 2) are commonly sequenced. All these genetic elements have multiple copies per cell, which increases the target density for DNA analysis.

For a review of the use of molecular diagnostics for *Peristenus* spp. identification and in biological control programmes for arthropods, see Chapter 2 and Appendix A.

1.5 Project Rationale – Molecular Markers for *Peristenus*

One of the difficulties encountered in studies on parasitism of *Lygus* bugs is that *Peristenus* species are virtually indistinguishable in the larval stage. Thus, dissection of mirid hosts to quantify parasitism does not yield information on species identity. Identification requires host rearing until parasitoid larvae emerge from the host, pupate, and eclose. However, the lengthy diapause period prior to eclosion can result in high levels of pupal mortality. In addition, the adult stage offers few morphological characteristics that can be used to separate species within this genus, and taxonomic expertise is often required.

The use of molecular marker systems may preclude the need for tedious and time-consuming dissection and rearing procedures. PCR-based methods would provide an efficient and accurate technique to facilitate the identification of parasitoid species and conduct ecological studies, including pre- and post-release studies in the area of origin and in the area of introduction.

1.6 Objectives

Prior to the release of an exotic natural enemy for pest control, detailed studies on the biology, ecology, and host range of the organism are necessary. These details are essential in any biological control programme to ensure establishment and predict potential risks associated with the introduction of an exotic agent.

The main goal of this research is to clarify some of the host-parasitoid associations of European *Peristenus* spp. to determine whether *P. digoneutis* and *P. relictus* attack non-target mirid species in the area of origin. Hence, the specific objectives of this research are:

(1) To develop species-specific molecular markers for *P. digoneutis*, *P. relictus*, and *P. pallipes*. The development of a molecular diagnostic tool for these

species is expected to facilitate non-target risk assessment studies and improve current techniques for the identification of *Peristenus* species;

(2) To estimate parasitism levels and parasitoid species composition in field-collected mirid populations by rearing, dissection and molecular analysis to provide a comparative assessment of the three techniques;

(3) To use molecular analysis to evaluate the ecological host range of *P. digoneutis* and *P. relictus* in two European ecoregions;

(4) To determine if host plant influences the parasitism level and parasitoid species composition in *Lygus rugulipennis*; and

(5) To use the molecular diagnostic tool in preliminary post-release studies to assess the establishment of *P. digoneutis* and *P. relictus* in mirid populations in southern Ontario.

2. DEVELOPMENT OF A MOLECULAR DIAGNOSTIC TOOL FOR DETECTION AND IDENTIFICATION OF *PERISTENUS* SPP.[1]

2.1 Introduction

Accurate identification of natural enemies is the cornerstone of biological control, and methods that can definitively identify biological control agents are essential, especially when morphological variation among species is slight. Members of the genus *Peristenus* Förster (Hymenoptera: Braconidae) are difficult to distinguish because species differences are small (Loan & Bilewicz-Pawinska, 1973; Bilewicz-Pawinska & Pankanin, 1974). Thus, PCR-based methods to distinguish between species would be advantageous in the identification of adult *Peristenus* species and in the detection of immature stages of *Peristenus* in parasitized *Lygus* Hahn (Hemiptera: Miridae).

Recently, molecular techniques have been investigated for their utility in identification of some species of *Peristenus* (Tilmon et al., 2000; Erlandson et al., 2003; Ashfaq et al., 2004; Zhu et al., 2004). These molecular marker systems include PCR-based methods that potentially preclude the need for tedious and time-consuming dissection and rearing procedures. This would be advantageous as dissection provides no information as to which parasitoid species is present (Carignan et al., 1995), and rearing methods require several months because of diapause prior to adult emergence and identification (Loan & Bilewicz-Pawinska, 1973).

Tilmon et al. (2000) developed molecular markers based on the cytochrome oxidase I (COI) gene for North American *P. pallipes* Curtis, and two European species introduced in the USA (*P. digoneutis* Loan and *P. conradi* Marsh) to assess parasitism

[1] Contents of this Chapter are published in *Biocontrol Science and Technology* **15**, 481-495.

levels in *Lygus lineolaris* Palisot de Beauvois. Similarly, Erlandson et al. (2003) developed molecular markers based on sequences from the internal transcribed spacer (ITS) regions for two European species, *P. digoneutis* and *P. relictus* Ruthe, and North American *P. pallipes*. However, the molecular markers developed in both these studies amplify fragment sizes that are identical for all *Peristenus* species investigated, and therefore require the additional step of restriction enzyme digestion or additional PCRs to distinguish between species. Zhu et al. (2004) screened a collection of single, short oligonucleotide primers and selected several which produced PCR product patterns that distinguish between three species in the *P. pallipes* complex (*P. mellipes* Cresson, *P. pseudopallipes* Loan and *P. howardi* Shaw). Although no additional restriction digests or PCRs were required, the fragment patterns were difficult to interpret. Thus, a single-step PCR assay that allows clear identification of multiple *Peristenus* species is not available.

Multiplex PCR uses multiple species-specific primer pairs in a single reaction to amplify unique fragment sizes for different DNA targets, which allows samples to be screened for several organisms simultaneously (Bej et al., 1991; Henson & French, 1993). Although this type of system has only recently been used in host-parasitoid studies to identify multiple parasitoid species (Gariepy et al., 2005; Traugott et al., 2006), multiplex PCR is routinely used in the diagnosis of medical and veterinary diseases (Rossiter & Caskey, 1993; Favia et al., 2000; Zarlenga & Higgins, 2001) as well as in the detection of plant pathogens (Hamelin et al., 1996; Cullen et al., 2000; Taylor et al., 2001; Bertolini et al., 2003; Gariepy et al., 2003; Lopez et al., 2003).

The purpose of this study was to design PCR primers based on the ITS region of rDNA for *P. digoneutis*, *P. relictus*, and *P. pallipes* that produce unique fragment sizes, and that could be used in multiplex to improve their diagnostic utility. The ITS region is useful in the identification of organisms at the genus, species, and population level because there is high degree of sequence variation in comparison to other genomic regions (Stouthamer, 2006). Hence, DNA sequences from this region are commonly used to develop molecular markers for the identification of parasitoids (Erlandson & Gariepy, 2005). In addition, a large amount of sequence information can be obtained from this region, approximately 1600-bp (Erlandson et al., 2003), which should provide

several potential target areas for primer design. This is particularly important in a multiplex system, as the development of primers that amplify different fragment sizes relies on the availability of several regions of sequence variation.

Peristenus digoneutis and *P. relictus* have been released as biological control agents for *Lygus* plant bugs in the USA (Coulson, 1987; Day, 1999), and are being considered for release in Canada. As previously discussed (Chapter 1), *P. pallipes* is a species complex associated with different mirid species (Craig, 1963; Loan, 1965; Bilewicz-Pawinska, 1977b, 1982). Thus, for *P. pallipes*, a primer set that universally amplifies members of this complex is desirable. A multiplex PCR protocol for *P. digoneutis*, *P. relictus* and *P. pallipes* would be useful to detect immature stages of *Peristenus* in *Lygus* nymphs. This would help evaluate host-parasitoid associations, which is an important step in non-target risk assessment studies.

2.2 Materials and Methods

2.2.1 Acquisition of Voucher Specimens

Cocoons of *P. digoneutis* and *P. relictus* were reared from *Lygus rugulipennis* Poppius nymphs collected from geographically dispersed red clover (*Trifolium pratense* L.; Fabaceae) and chamomile (*Matricaria recutita* L.; Asteraceae) fields in Schleswig-Holstein, northern Germany. *Peristenus pallipes* cocoons were obtained from *Liocoris tripustulatus* Fabricius (Hemiptera: Miridae) and *Closterotomus norwegicus* Gmelin (Hemiptera: Miridae) collected from stinging nettles (*Urtica dioica* L.; Urticaceae) in the same region. Emerged adults were put in 95% ethanol and identified based on morphological characteristics by H. Goulet (Taxonomist, Agriculture and Agri-Food Canada, Ottawa, ON). Twenty identified specimens for each of the *Peristenus* species investigated were randomly selected from collections and were used as voucher samples for molecular analysis.

2.2.2 DNA Extraction

Peristenus adults preserved in 95% ethanol were rinsed in sterile, distilled water, and placed individually in 1.5-mL centrifuge tubes. Individual *Peristenus* adults were homogenized using disposable pestles (Kimble Kontes, Vineland, NJ, USA) in 250 μL

of Lifton buffer [0.2 M sucrose, 50 mM ethylene-diaminetetraacetic acid, 100 mM Tris-HCl (pH 7.5), and 0.5% sodium dodecyl sulfate] and incubated for 2 h at room temperature. Proteinase K (1.25 μL of 20 μg/mL) was added, and samples were incubated for 4 h at 65°C. Samples were put on ice for 30 min following the addition of 35 μL of 5 M potassium acetate, and centrifuged for 20 min at 20,000 xg. The supernatant was extracted once with 200 μL of phenol-chloroform-isoamyl alcohol (25:24:1), and once with 200 μL chloroform-isoamyl alcohol (24:1). The aqueous phase was precipitated with two volumes of cold 95% ethanol by overnight incubation at -20°C. Precipitated DNA was pelleted by centrifugation (30 min at 20,000 xg), washed with cold 70% ethanol, and air-dried for 15 min. DNA samples were resuspended in 50 μL Tris-EDTA (pH 7) with 0.5 μL of 10 mg/mL RNase A.

2.2.3 DNA Sequencing and Primer Design

The oligonucleotide primers inDNA44 (5'-TCCTCCGCTTATTGATATGC-3') and inDNA45 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (Nucleic Acid – Protein Service, University of British Columbia, Vancouver, BC, Canada) were used in PCR to amplify conserved rRNA gene sequences and ITS regions of nuclear DNA from voucher specimens. PCR conditions were as follows: 25 μL reactions contained 2.5 μL of 10X *Taq* reaction buffer, 2.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 μM of each primer, 1 U of *Taq* DNA polymerase (Invitrogen, Burlington, ON, Canada), and approximately 20 ng of DNA template. PCR reactions were carried out in a Stratagene Robocycler thermocycler (LaJolla, CA, USA) with an initial denaturation at 94°C for 120 sec, followed by 30 cycles at 94°C for 60 sec, 55°C for 60 sec, and 72°C for 90 sec. A final extension period at 72°C for 5 min followed the 30 cycles. PCR products were electrophoresed at 75 V for 45 – 60 min on 1% agarose gels that contained 0.5 μg/mL ethidium bromide, and products were visualized by UV transillumination. PCR products were directly cloned into pGem® - T Easy vectors (Promega, Madison, WI, USA) using the manufacturer's protocol. Cloned PCR products were sequenced at the DNA Services Laboratory, Plant Biotechnology Institute (NRCC, Saskatoon, SK, Canada) using universal primers to sequence from both ends of the cloned insert. DNA sequence data were assembled and aligned using SeqMan (DNAStar Inc., Madison, WI, USA) and

Vector NTI (InfoMax, Fredrick, MD, USA). Areas of sequence variation determined by visual inspection of the sequence data were used to design primers for *P. digoneutis, P. relictus* and *P. pallipes*.

2.2.4 Specificity of Peristenus *Species-Specific Primers*

The specificity of the primer pairs designed for *P. digoneutis*, *P. relictus* and *P. pallipes* was evaluated by the amplification of DNA from 20 voucher specimens of each species with each of the primer pairs. Adult *Lygus* DNA was used as a negative control to ensure that the primer sets did not amplify *Lygus* sequences. Amplification of DNA was performed in a Stratagene Robocycler thermocycler using the same reagents as above, except the primer pairs used were Per R1 (a presumed universal *Peristenus* primer) in combination with either dig F1096 for *P. digoneutis*, sty F1230 for *P. relictus*, or pal F517 for *P. pallipes*. Amplification conditions were: initial denaturation at 94°C for 120 sec, followed by 35 cycles of 94°C for 45 sec, 54°C for 45 sec, and 72°C for 60 sec. A final extension period of 5 min at 72°C followed.

2.2.5 Multiplex PCR Assay and Detection Sensitivity

To enhance the diagnostic utility of *Peristenus* spp. primers, a multiplex PCR protocol was tested. For this approach, the reagents mentioned above were used with the following modifications: 0.2 μM dig F1096, 0.2 μM sty F1230, 0.2 μM pal F517 and 0.4 μM Per R1 were combined in the reaction mixture. Amplification conditions were as described above for specificity testing of the *Peristenus* species-specific primers.

DNA from adult *P. digoneutis, P. relictus,* and *P. pallipes* voucher specimens was serially diluted from 20 – 0.02 ng, and used in a multiplex PCR assay to determine the sensitivity of the primers.

2.2.6 Detection of Immature Stages of Peristenus *in Parasitized Lygus Nymphs*

To determine the ability of the molecular markers to detect immature stages of *P. digoneutis* and *P. relictus* inside their host, *L. rugulipennis* nymphs were parasitized in the laboratory. Between 20 and 25 experienced, mated *P. digoneutis* and *P. relictus* females (5 to 10 days old) were provided with second- or third-instar *Lygus* nymphs in a

5-cm diameter Petri dish. Each wasp was transferred into a Petri dish containing five nymphs, and attacks were observed. When an attack occurred, the nymph was immediately removed to prevent superparasitism. In total, 100 – 125 nymphs attacked by *P. digoneutis* and 100 – 125 nymphs attacked by *P. relictus* were obtained. Attacked nymphs were either placed immediately in 95% ethanol (0 h) or reared for fixed periods at approximately 22 ± 2°C, 16L:8D photoperiod, and 50% RH in rearing cylinders with sprouting potatoes (*Solanum tuberosum* L.), lettuce (*Lactuca sativa* L.), and green beans (*Phaseolus vulgaris* L.). Rearing periods were chosen to cover the documented range of developmental stages of *P. digoneutis* and *P. relictus* within *Lygus* spp. nymphs (Drea et al., 1973; Carignan et al., 1995). Rearing periods included: 3 days (egg), 6 days (first instar), 9 days (second instar), and 12 days (third instar). After the specified rearing period, attacked nymphs were placed in vials containing 95% ethanol. DNA was extracted from the attacked nymphs (20 nymphs per rearing period for each *Peristenus* species), and amplified using the multiplex PCR assay.

To determine the lower limit of detection for parasitoid eggs within parasitized *Lygus* spp. nymphs, additional intervals were later included. Following the same protocol as above, nymphs parasitized by *P. digoneutis* and *P. relictus* were reared for 6, 12, and 24 h post-parasitism before being killed and preserved in 95% ethanol. DNA was extracted and analyzed using the multiplex PCR assay.

2.3 Results

2.3.1 DNA Sequencing and Primer Design

DNA sequences were obtained for cloned PCR products generated using inDNA44 and inDNA45 from at least 4 individuals (1 to 3 clones per individual) for each of the *Peristenus* species investigated. Sequences were obtained for the ITS1 regions of *P. digoneutis* (GenBank accession no. **AY605234**) and *P. relictus* (GenBank accession no. **AY605233**). ITS1 and ITS2 sequences were obtained for *P. pallipes* (GenBank accession no. **AY608602**). Sequences were aligned to analyse sequence variation among species. Several areas of variation were identified, which allowed the design of species-specific primers for each species (Table 2.1). Primer sets consisted of a

Table 2.1 *Peristenus* species-specific primers, their respective sequences, and fragment sizes when used in combination with a genus-specific forward primer (Per R1).

Species	**Primer**	**Primer Sequence**	**Size**
Peristenus spp.	Per R1	5' ACAAGGTTTCCGTAGGTG 3'	-
P. digoneutis	dig F1096	5' GAACATAAAAACTTCTTCTACGC 3'	515
P. relictus	sty F1230	5' CAGGTAGAGATACATGGCTGT 3'	330
P. pallipes	pal F517	5' TAAACTTTGGCCAGATAAATG 3'	1060

species-specific reverse primer (dig F1096, sty F1230, or pal F517) used in combination with a genus-specific forward primer (Per R1) common to all three species investigated. The combinations of Per R1 with dig F1096 for *P. digoneutis*, with sty F1230 for *P. relictus*, and with pal F517 for *P. pallipes* produced PCR products of 515-, 330-, and 1060-bp, respectively (Figure 2.1).

2.3.2 Specificity of Peristenus *Species-Specific Primers*

Each primer set selectively amplified the DNA of the species for which it was designed, and yielded the expected product sizes when using the PCR conditions described above. Species-specific amplification was consistent for all voucher specimens tested (20 specimens per species), and a representative PCR reaction for each species is shown (Figure 2.2). None of the primer sets amplified *Lygus* DNA.

2.3.3 Multiplex PCR Assay and Detection Sensitivity

The combination of all three species-specific reverse primers (dig F1096, sty F1230, pal F517) and the genus-specific forward primer (Per R1) in a multiplex PCR protocol distinguished each species, and amplified the predicted species-specific fragment (Figure 2.3). These results were consistent with those obtained using the species-specific primer sets individually (Figure 2.2). When used in multiplex, the lower limit of detection appears to be 0.2 ng for *P. digoneutis* primers, and 0.02 ng for *P. relictus* and *P. pallipes* primers (Figure 2.4).

2.3.4 Detection of Immature Stages of Peristenus *in Parasitized* Lygus *Nymphs*

Using the multiplex PCR protocol, primers successfully detected *Peristenus* eggs and larvae in *L. rugulipennis* nymphs 3, 6, 9, and 12 days post-parasitism. For these periods, detection of both parasitoid species was consistent among *Lygus* nymphs (20 attacked nymphs per rearing period for *P. digoneutis* and *P. relictus*), and 90 – 100% of observed attacks resulted in parasitism. Detection of *Peristenus* within nymphs placed in 95% ethanol immediately after an attack (0 h) was inconsistent. Faint bands of the

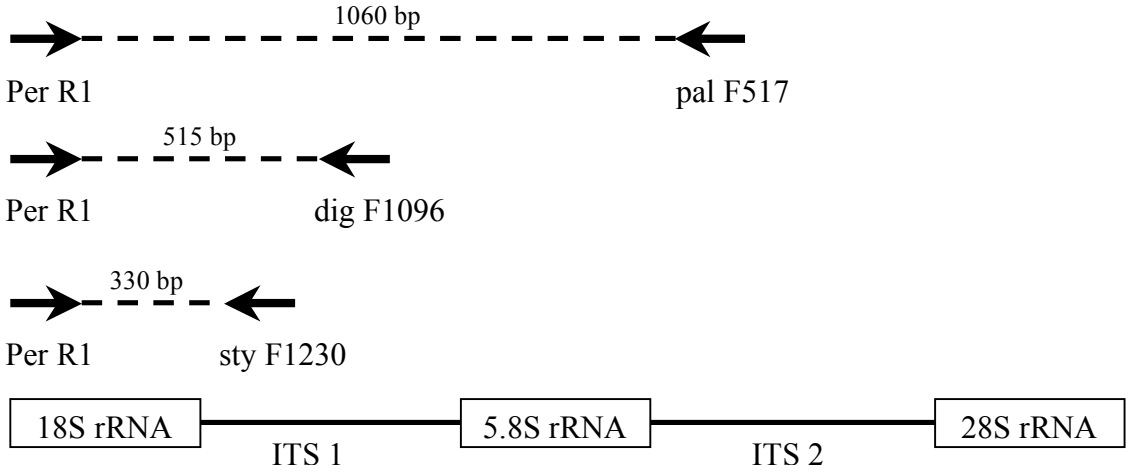

Figure 2.1 Diagram of the ITS region of rDNA, with locations of species-specific primers for *P. digoneutis* (Per R1 and dig F1096), *P. relictus* (Per R1 and sty F1230), and *P. pallipes* (Per R1 and pal F517).

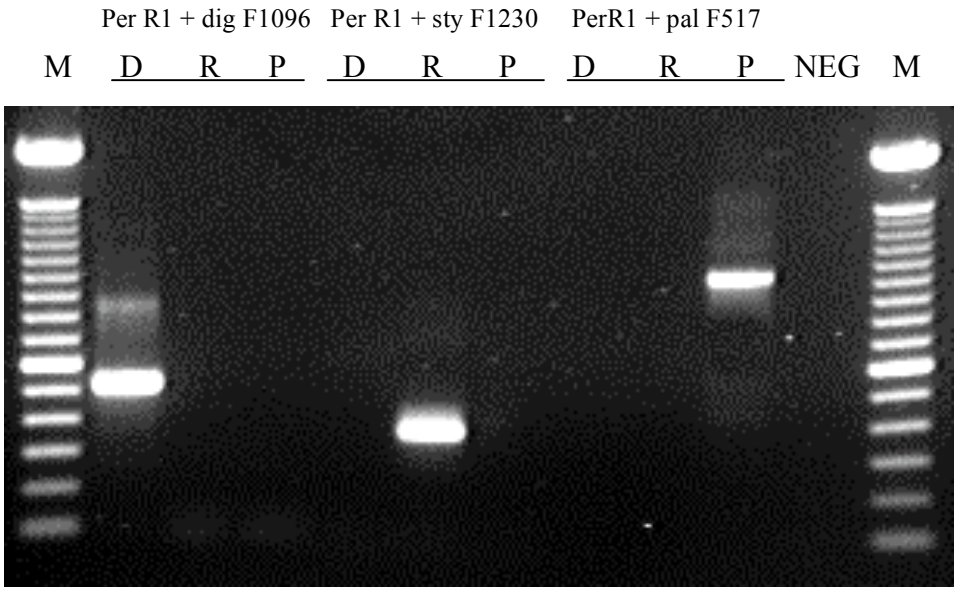

Figure 2.2 Diagnostic PCR using species-specific primers for *P. digoneutis* (Per R1 and dig F1096), *P. relictus* (Per R1 and sty F1230), and *P. pallipes* (Per R1 and pal F517). D, R, and P designate *P. digoneutis*, *P. relictus*, and *P. pallipes*, respectively. M, 100 bp DNA marker; NEG, negative control (no DNA).

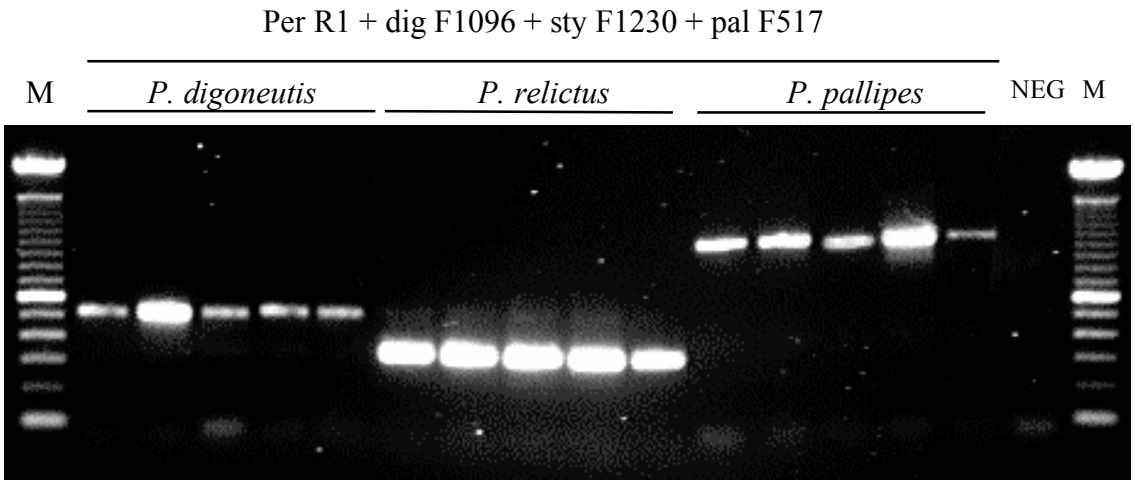

Figure 2.3 Diagnostic multiplex PCR of *P. digoneutis*, *P. relictus*, and *P. pallipes* DNA. M, 100 bp DNA marker; NEG, negative control (*Lygus* DNA).

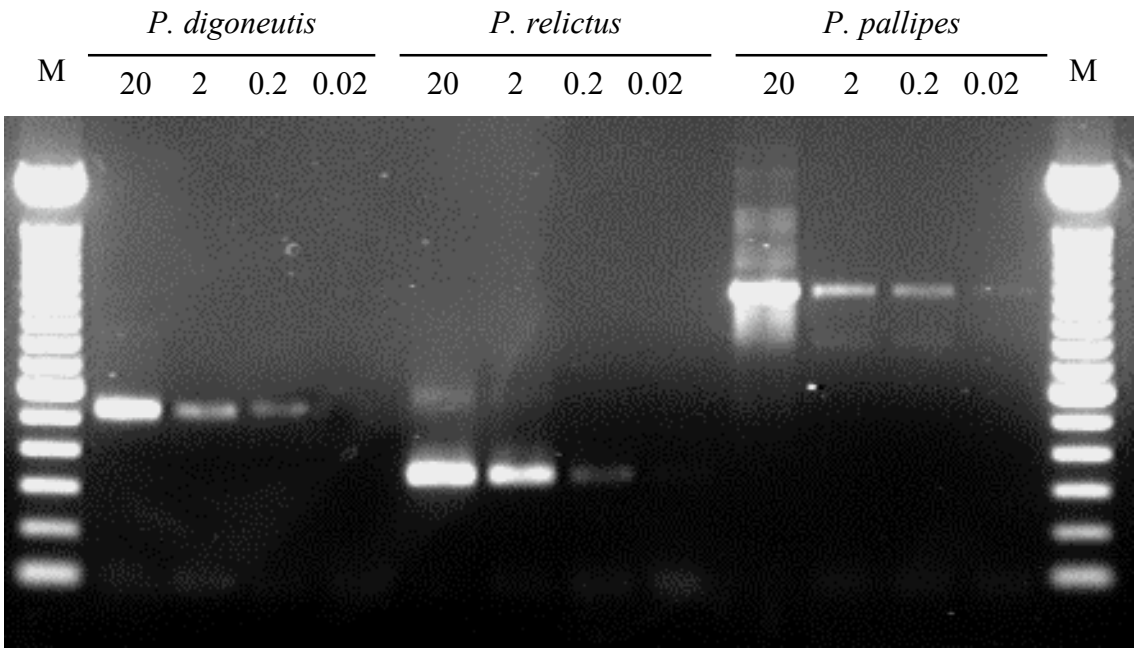

Figure 2.4 Diagnostic multiplex PCR using 20, 2, 0.2, and 0.02 ng of DNA template from *P. digoneutis*, *P. relictus*, and *P. pallipes* adults (serial dilutions). M, 100 bp marker.

expected size were occasionally present, but required 4 – 5 times more DNA template than was required for samples from the other post-parasitism periods. The primer sets did not amplify *Lygus* DNA.

Analysis of DNA from nymphs reared for 6, 12, and 24 h post-parasitism showed that *P. relictus* DNA was detected as early as 6 h post-parasitism, but only in 8 of the 20 nymphs processed. *Peristenus digoneutis* DNA was never detected in the 6-h interval, but was detected within 12 h post-parasitism in 10 of the 20 nymphs processed. Consistent detection (100% detection) of parasitoid DNA was possible at 12 h post-parasitism for *P. relictus* and 24 h post-parasitism for *P. digoneutis.*

2.4 Discussion

The PCR primers developed for *P. digoneutis*, *P. relictus*, and *P. pallipes* are specific and sensitive when used individually or in multiplex with DNA extracted from *Peristenus* adults or from parasitized *Lygus* nymphs. When used individually or in multiplex, the primers yield unique fragment sizes for each species and identified *Peristenus* eggs, larvae, and adults based on the size of the PCR fragment generated.

The inability of molecular markers to detect newly laid parasitoid eggs in their host is not uncommon. Primers developed by Amornsak et al. (1998) for detection of *Trichogramma australicum* Girault (Hymenoptera: Trichogrammatidae) in *Helicoverpa armigera* Hubner and *H. punctigera* Wallengren (Lepidoptera: Noctuidae) were unable to detect the parasitoid immediately after oviposition; positive PCR products were only generated 12 h after parasitism (and onwards). Within the first 24 h after oviposition, *Peristenus* eggs swell and further embryonic development occurs (Carignan et al., 1995). It is within this time period that *P. digoneutis* and *P. relictus* eggs were detected by PCR, and this increased development explains why consistent detection was possible between 12 and 24 h after parasitism. The earliest time-point for detection of parasitoid eggs within *Lygus* spp. nymphs was approximately 6 and 12 h after oviposition for *P. relictus* and *P. digoneutis*, respectively. However, parasitoid DNA was only detectable in 40% - 50% of the parasitized nymphs from these intervals.

Lopez et al. (2003) list multiplex PCR methods among the innovative tools currently used in pathogen diagnosis based on the sensitivity of the technique and the ability to simultaneously detect different DNA targets in a single reaction. In insect parasitology, molecular diagnostic tools based on multiplex PCR have been used to identify different mosquito species and strains that carry malaria (Kengne et al., 2001; Phuc et al., 2003), to detect animal viruses and plant pathogens in insect vectors (Wilson & Chase, 1993; Rodrigues et al., 2003), to identify microsporidian infection in ants (Valles et al., 2002) and bacterial infection in insect cadavers (Bourque et al., 1993).

The use of a multiplex PCR assay for *Peristenus* species improves upon the conventional and molecular techniques previously used to identify *Peristenus* spp. It allows rapid and accurate identification of multiple *Peristenus* species and eliminates mortality issues encountered in the rearing process. Further, unlike dissection, the multiplex assay allows the determination of species composition, and unlike rearing, it does so without the time-delay due to the lengthy diapause period of the parasitoid prior to adult emergence. This is a novel approach that could be valuable in insect biological control programmes, particularly for pre-release and post-release studies on potential biological control agents.

The single-step multiplex PCR assay described here would be useful in pre-release studies in the area of origin, as DNA from mirid nymphs can be screened to determine host-parasitoid associations and quantify parasitism levels in target and non-target mirid populations in Europe (see Chapters 3 and 4).

In addition, after the introduction of an exotic parasitoid, molecular tools can facilitate post-release monitoring studies. Recent post-introduction studies in New York State used molecular markers for the COI gene to map the distribution of *P. digoneutis* in *Lygus* populations in alfalfa and strawberry fields (Tilmon et al., 2000; Tilmon & Hoffmann, 2003). However, as previously mentioned, the markers used in these studies produce identical fragment sizes for each species and require additional steps to obtain species-specific identifications. The use of the single-step multiplex PCR protocol described here could facilitate similar post-introduction studies, as shown in Chapter 6.

It should be noted that the *P. pallipes* primers (Per R1 and palF517), developed based on DNA sequences from European *P. pallipes*, also amplify North American *P.*

pallipes (Gariepy et al., 2005). The availability of universal primers for the *P. pallipes* complex is advantageous, as samples can be screened for introduced *Peristenus* species and indigenous *P. pallipes* simultaneously to assess establishment, distribution, displacement of native parasitoids, and non-target parasitism.

However, prior to the use of the multiplex PCR assay in ecological host-range studies, a comparative assessment of conventional and molecular methods should be done to validate the utility of the multiplex assay in the estimation of parasitism levels and parasitoid species composition, as described in the following chapter.

3. ESTIMATING PARASITISM LEVELS AND PARASITOID SPECIES COMPOSITION IN A HOST POPULATION BY DISSECTION, REARING, AND MULTIPLEX PCR: A COMPARATIVE ASSESSMENT OF TECHNIQUES

3.1 Introduction

In biological control, the level of parasitism in a host population is generally used as a measure of parasitoid efficacy and provides a quantitative evaluation of the impact of a parasitoid on a pest population. According to Hawkins et al. (1993), maximum parasitism levels provide a highly significant estimate of the probability that the parasitoid will suppress the host population. To evaluate the effectiveness of a natural enemy, methods must be available that can accurately quantify parasitism levels (Van Driesche, 1983). This is necessary not only in the evaluation of parasitoids as biological control agents, but also in the construction of life tables (Van Driesche et al., 1991).

Conventional methods for the identification of parasitoids and quantification of parasitism levels in a host population rely on rearing and dissection of field-collected insects. However, these two methods often provide different results, and failure to acknowledge that differences exist can result in data that are inaccurate (Day, 1994). Furthermore, it has been suggested that accurate estimation of parasitism levels is one of the main methodological problems encountered in the study of parasitoid efficiency (Höller & Braune, 1988). Nonetheless, few studies mention the accuracy of parasitism level estimates derived from rearing and/or dissection data, and it appears to be a topic that is largely neglected in the literature (Day, 1994).

Several studies have shown that dissection (or other forms of microscopic examination) provides higher parasitism estimates than rearing (Clancy & Pierce, 1966; Cartwright et al., 1982; Day, 1994; Berberet & Bisges, 1998; Heng-Moss et al., 1999; Moretti & Calvitti, 2003). Other studies have shown that there is no significant difference between rearing and dissection (Kenis et al., 2001), or that rearing provides higher estimates of parasitism than dissection (Burrell, 1966). Clearly, the outcome of these comparisons depends on the host-parasitoid association being investigated, the rearing and dissection protocols used, and the impact of host and parasitoid mortality in rearing.

Molecular methods have recently been used to estimate parasitism levels and identify parasitoid species in host populations (Greenstone, 2006). Like dissection, molecular analysis provides a rapid estimate of parasitism level. However, using species-specific PCR primers, molecular analysis can identify parasitoids to species, which is often impossible by dissection alone. Molecular markers for parasitoids are able to detect minute quantities of DNA and allow the detection of parasitoid eggs within a host, which are often missed by dissection. Additionally, information is obtained more rapidly than rearing, which can be delayed by lengthy diapause periods prior to parasitoid emergence. Most importantly, host and parasitoid mortality (commonly encountered in rearing) do not affect results obtained by molecular analysis.

Studies that compare parasitism levels obtained using molecular methods and dissection show that there is no statistical difference between these methods (Tilmon et al., 2000; Gariepy et al., 2005; Jones et al., 2005). Most studies that compare rearing and molecular analysis have also yielded similar results (i.e., no significant difference) with respect to parasitism level (Ratcliffe et al., 2002; Agustí et al., 2005; Gariepy et al., 2005; Jones et al., 2005). However, there are studies that indicate that discrepancies exist between parasitism estimates by rearing and molecular analysis (Tilmon et al., 2000) or between rearing, dissection, and molecular analysis (Ashfaq et al., 2004).

Studies that compare molecular and conventional methods are often based on small sample sizes that are disproportionate between methods; this may affect the statistical significance of such data. Nonetheless, large-scale assessments of the accuracy

of rearing, dissection, and molecular analysis in the estimation of parasitism levels in a host population have rarely been done.

Although some qualitative comparisons have been made, rigorous statistical comparison of parasitoid species composition data obtained from rearing and molecular analysis has never been done. The accuracy of parasitoid species composition data is equally important as the accuracy of parasitism level data. Therefore, it is necessary to apply methods that provide more complete species composition information. Pupal mortality in rearing prevents the identification of individuals that successfully killed their host, but failed to complete development to the (identifiable) adult stage. The inability to identify a parasitoid species causing host mortality can lead to an underestimation of the impact the parasitoid has on the host population.

Species of *Lygus* Hahn (Hemiptera: Miridae) are serious pests of a wide variety of economically important crops in North America. Two European *Peristenus* Förster species (Hymenoptera: Braconidae), *P. digoneutis* Loan and *P. relictus* Ruthe, have been introduced in the USA as part of a biological control program for *Lygus*. Other members of the genus *Peristenus* include holarctic *P. pallipes* Curtis, a species complex (currently undergoing taxonomic revision) associated with a variety of Miridae in Europe and North America.

Dissection and rearing are used to study host-parasitoid associations in Miridae. Although dissection provides a rapid estimate of parasitism level, it does not permit distinction between *Peristenus* species (Bilewicz-Pawinska & Pankanin, 1974). Rearing is therefore necessary to determine which parasitoid species are present in a mirid host population (Day, 1994). Recently, molecular tools have been developed for the identification of *Peristenus* species and estimation of parasitism levels in *Lygus* (Tilmon et al., 2000; Erlandson et al., 2003; Zhu et al., 2004; Gariepy et al., 2005). These molecular tools provide a promising alternative to conventional methods to identify *Peristenus* and estimate their impact on mirid populations.

The objective of the present study was to conduct a large-scale comparative assessment of methods used to estimate the parasitism level and parasitoid species composition in mirid host populations. Four mirid species were used as case studies to

compare the accuracy of rearing, dissection, and molecular methods in the estimation of parasitism by three *Peristenus* species in Europe. The potential utility of these methods and their accuracy are discussed in the context of non-target risk assessment.

3.2 Materials and Methods

3.2.1 Field Collections

Four mirid species were collected: *Lygus rugulipennis* Poppius (the target host for *P. digoneutis* and *P. relictus*), and *Closterotomus norwegicus* Gmelin, *Liocoris tripustulatus* F., and *Leptopterna dolobrata* L. (potential non-target hosts for *P. digoneutis* and *P. relictus*). The potential non-target species were selected based on the criteria outlined by Kuhlmann et al. (2006): (1) availability in the field; (2) phylogenetic relatedness to the target host; (3) overlap in time of occurrence with the target host; (4) habitat overlap with the target host; and (5) literature reports suggesting potential alternative hosts for the parasitoid species of interest.

Mirids were collected from various locations in northern Germany, the southern Rhine Valley of Germany, Switzerland, and eastern France (GPS coordinates are listed in Appendix B; see Chapter 4 for maps of these regions).

Collections were made using a standard sweep net (38 cm in diameter) and insects were sorted in the field in white trays coated with Fluon® (polytetrafluoro-ethylene, Dyneon Werk Gendorf, Burgkirchen, Germany) to prevent insects from climbing out of the trays. Mirid nymphs were separated from other insects and spiders using a mouth aspirator, and were placed in ventilated plastic containers during transport to the laboratory. *Lygus rugulipennis* was collected in red clover (*Trifolium pratense* L.; Fabaceae), alfalfa (*Medicago sativa* L.; Fabaceae), and chamomile (*Matricaria recutita* L.; Asteraceae). *Liocoris tripustulatus* was collected in stinging nettles (*Urtica doica* L.; Urticaceae). *Leptopterna dolobrata* was collected in grassland habitats, particularly on orchard grass, *Dactylus glomerata* L (Poaceae). *Closterotomus norwegicus* was collected in chamomile and in fallow fields with mixed grasses and flowers.

Lygus rugulipennis was collected from 30 field sites. Collections took place during the last 2 weeks of August in 2003 and 2004. Nineteen field collections were made for *C. norwegicus*. These collections took place throughout the month of June in

2003 and 2005. *Liocoris tripustulatus* was collected from 20 field sites during the first 3 weeks of July in 2003 and 2004. *Leptopterna dolobrata* was collected from 18 field sites, with collections taking place in the last 3 weeks of June in 2003 and 2004.

At each site, 300 nymphs of a given species were collected, and sub-samples of 100 nymphs were designated for rearing, dissection, and molecular analysis, respectively. Mirids were collected in the third to the fifth nymphal instar, when maximum parasitism levels in the field are reached (Haye, 2004).

3.2.2 Parasitism and Parasitoid Species Composition

Rearing

Nymphs were reared in plastic cylinders (1.2 L) fitted with removable Petri-dish bottoms. Moist vermiculite (Opticulit, Optima-Werke, Oberwil, Switzerland) was added to Petri dishes to serve as a pupation medium for parasitoid larvae. The vermiculite was separated from the rest of the cylinder by a mesh screen that allowed parasitoid larvae to pass through, but which excluded nymphs from the vermiculite. Organic beans (*Phaseolus vulgaris* L.) were used as a food source for *L. rugulipennis* and *C. norwegicus* nymphs. Beans were changed two or three times a week, depending on their condition. Nymphs of *L. tripustulatus* were provided with *Urtica* leaves and flowers as a food source. Freshly cut stems were placed in plastic vials with moist florist foam to prevent wilting, and were replaced with new stems every second day. Nymphs of *L. dolobrata* were provided with *D. glomerata* blades and flowers. Freshly cut flowers and blades were placed in plastic vials with moist florist foam to prolong their freshness. Grass flowers and blades were changed every second day.

A maximum of 50 nymphs were reared in each cylinder, and mirid adults were counted and removed daily. When there were no nymphs left, Petri dishes were removed and the parasitoid cocoons were counted. Parasitism was recorded as the number of cocoons obtained from 100 nymphs reared from a given site. Unexplained mortality of mirid nymphs was recorded for each site. This was calculated by adding the number of mirid adults and the number of cocoons, and then subtracting this number from the total number of nymphs collected from that site.

Parasitoid cocoons were overwintered in Petri dishes filled with vermiculite in a subterranean insectary. Temperatures inside the insectary tend to fluctuate in accordance with outside temperatures, and range from 2–10ºC. In April of the following year, cocoons were brought indoors, incubated at 20–22˚C, and emergence was monitored daily. Parasitoids that emerged from cocoons were killed, preserved in 95% ethanol, and sent to H. Goulet (Taxonomist, Agriculture and Agri-Food Canada, Ottawa, ON) for identification.

Parasitoid species composition (based on the identification of parasitoid adults) was calculated as the proportion of each species that emerged from cocoons obtained at each site. Parasitoid pupal or overwintering mortality was calculated for each site based on the proportion of cocoons from which no parasitoid adults emerged.

Dissections

Sub-samples of 100 nymphs per site were dissected under a compound microscope in 70% ethanol. The abdomen of each nymph was opened carefully using fine forceps, and the contents were visually examined for the presence of eggs, first-, second-, and third-instar parasitoid larvae. At each site, parasitism was recorded as the number of nymphs in which immature parasitoids were found upon dissection. On the very rare occasion when two parasitoid larvae were found in a single host, this was still counted as a single event in the estimation of parasitism level.

Species composition information was not obtained from dissected host material, as immature *Peristenus* species are morphologically indistinguishable (Bilewicz-Pawinska & Pankanin, 1974).

Molecular analysis

Sub-samples of 100 nymphs per site were preserved in 95% ethanol for molecular analysis. DNA was extracted and amplified using the multiplex PCR protocol described in Chapter 2. Parasitism level was recorded as the number of nymphs that produced a positive PCR result, which indicated the presence of parasitoid DNA. On the rare occasion when multiparasitism was detected, this was still counted as a single event in the estimation of parasitism level.

Parasitoid species composition was estimated based on the proportion of species-specific PCR reactions that were positive for *P. digoneutis*, *P. relictus*, and *P. pallipes* (respectively) from among the total number of parasitoids detected.

3.2.3 Statistical Analysis

To test the null hypothesis that there was no significant difference in the overall parasitism level estimated by rearing, dissection and molecular analysis, a one-way ANOVA ($P = 0.05$) was used. When a significant difference was detected, a Tukey HSD test ($P = 0.10$) was used to determine which means were significantly different.

To test the null hypothesis that there was no significant difference between rearing and molecular analysis in the estimation of the proportion of each parasitoid species, a Wilcoxon paired samples test ($P = 0.05$) for non-parametric data was used.

3.3 Results

Mean parasitism levels based on dissection, rearing, and molecular analysis of the four host species are shown in Figure 3.1.

Peristenus species composition in each of the hosts is presented in Table 3.1. The mean proportion of each parasitoid species obtained in rearing and molecular analysis is shown in Figure 3.2. This figure also presents information regarding the proportion of hyperparasitoids and pupal mortality in rearing, and the proportion of multiparasitism detected by molecular analysis.

Lygus rugulipennis

Parasitism levels ranged from 4.0% to 66.0% by dissection, 8.0% to 63.0% by rearing, and 7.0% to 70.0% by molecular analysis. There was no significant difference between the mean parasitism levels determined using the three methods ($F_{2, 87} = 1.00$; $p = 0.37$).

During the rearing process, some nymphs presumably died, and were not accounted for as adults or parasitoid cocoons. The mean proportion of unexplained nymphal mortality was 15.2±1.8(SE)%.

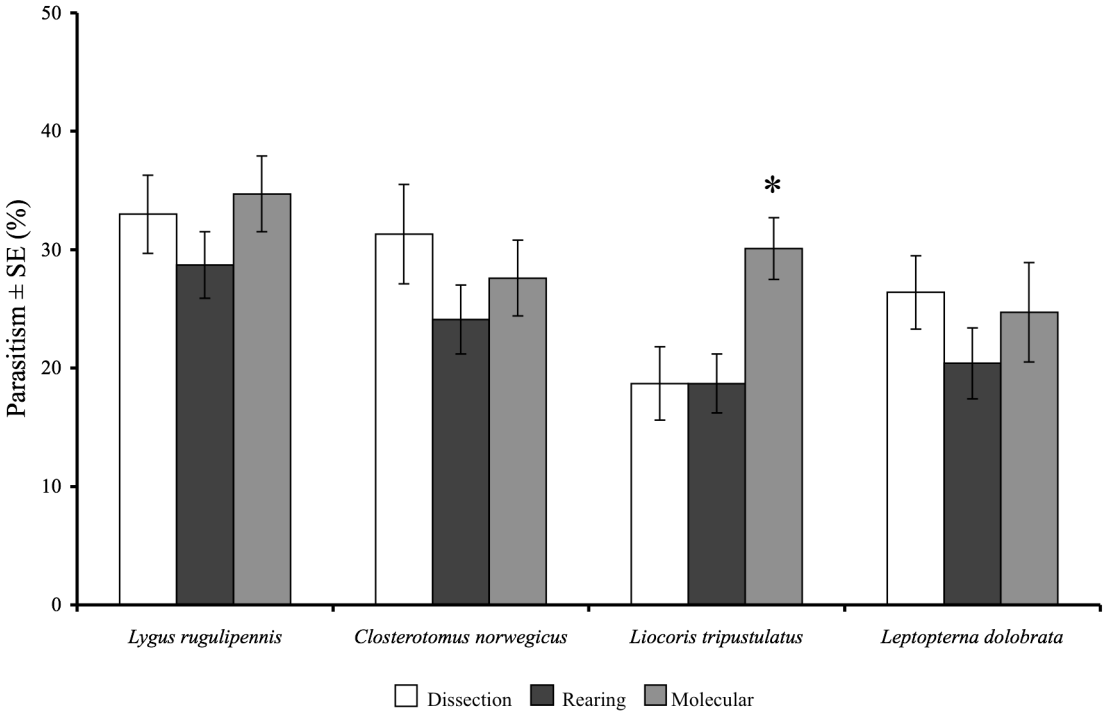

Figure 3.1 Mean parasitism levels estimated by dissection, rearing, and molecular analysis of four mirid species collected in Europe. For a given host species, significant differences between methods are indicated by an asterisk (ANOVA, $P < 0.05$; Tukey HSD, $P < 0.10$).

Table 3.1 Comparison of the mean proportion of *P. digoneutis*, *P. relictus*, and *P. pallipes* in four mirid host species, estimated by rearing and molecular analysis. Significant differences in the proportion estimated by rearing and molecular analysis are indicated by an asterisk (Wilcoxon matched pairs sign test, $P < 0.05$).[1]

	P. digoneutis **Mean ± SE**		***P. relictus*** **Mean ± SE**		***P. pallipes*** **Mean ± SE**	
Mirid Species	**Rearing**	**Molecular**	**Rearing**	**Molecular**	**Rearing**	**Molecular**
Lygus rugulipennis	77.4±3.4	82.1±3.3	8.0±2.2	15.3±3.2*	0.0±0.0	0.5±0.3
Closterotomus norwegicus	3.9±2.9	3.6±1.4	0.7±0.5	7.2±3.1*	58.3±6.3	88.6±4.7*
Liocoris tripustulatus	0.2±0.2	0.1±0.1	0.0±0.0	0.4±0.2	32.9±5.0	99.5±0.2*
Leptopterna dolobrata	0.0±0.0	0.4±0.3	0.2±0.2	3.2±1.4*	42.3±4.9	96.0±1.4*

[1]As this table presents partial parasitoid species composition information, the mean values do not add up to 100. Additional contributions from hyperparasitoids, multiparasitism, and pupal mortality are not shown in this table but are used in the calculation of the proportion of each parasitoid species.

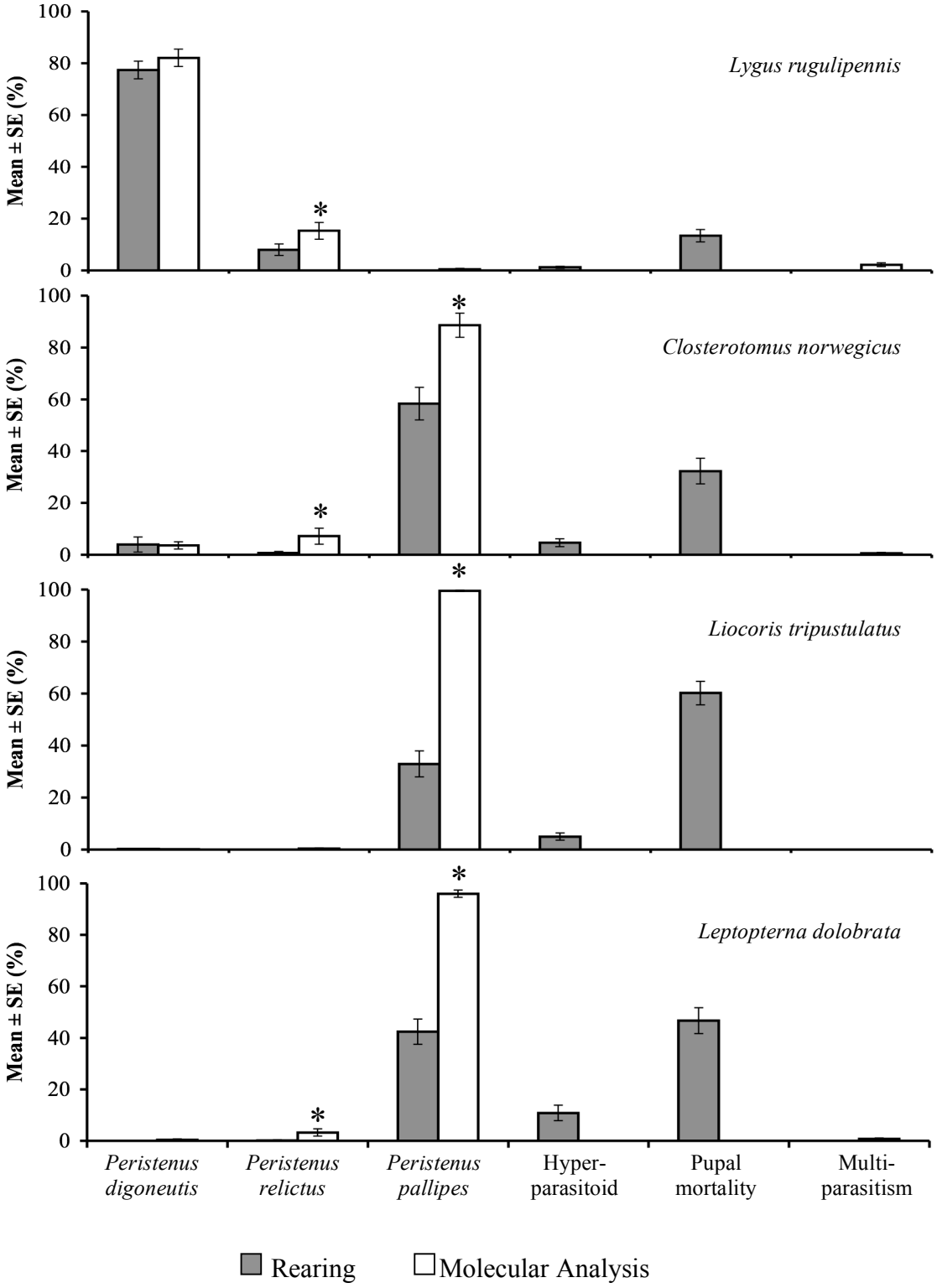

Figure 3.2 Mean proportion of parasitoids, hyperparasitoids, pupal mortality, and multiparasitism from rearing and molecular analysis of mirid host species. Significant differences in rearing and molecular analysis are indicated by an asterisk (Wilcoxon matched pairs sign test, $P < 0.05$).

Of the 860 cocoons obtained from *L. rugulipennis* during the rearing process, approximately 76.3% were *P. digoneutis*, and 9.9% were *P. relictus*. *Peristenus pallipes* was not reared from *L. rugulipennis*. Hyperparasitoids (*Mesochorus* spp.) emerged from 1.4% of the cocoons, and were present at 7 of the 30 sites. Relatively few parasitoids failed to emerge from their cocoons (pupal mortality), but nonetheless accounted for 12.4% of the total number of cocoons collected.

Molecular analysis of *L. rugulipennis* nymphs resulted in 1040 positive PCR reactions. *Peristenus digoneutis*- and *P. relictus*-specific PCR products accounted for 79.4% and 16.7% of the positive PCR reactions, respectively. *Peristenus pallipes*-specific PCR products were rarely generated. Of the five parasitized nymphs (accounting for 0.5% of the positive PCR reactions) that generated *P. pallipes*-specific PCR fragments, four were from a single site. Of the 1040 PCR positives, 3.4% were positive for *P. digoneutis* and *P. relictus* simultaneously, an indication of multiparasitism.

The mean proportion of *P. digoneutis* estimated by rearing and molecular analysis was not significantly different ($z = -1.59$; $p = 0.11$). However, the mean proportion of *P. relictus* was significantly higher by molecular analysis when compared to similar results obtained in rearing of *L. rugulipennis* ($z = -3.25$; $p = 0.001$). No significant difference in the mean proportion of *P. pallipes* estimated by rearing and molecular analysis was observed ($z = -1.34$; $p = 0.18$).

Closterotomus norwegicus

By dissection, the minimum and maximum parasitism levels were 4.0% and 78.0%, respectively. Rearing provided parasitism levels that ranged from 4.0% to 43.0%, and parasitism estimated by molecular analysis ranged from 7.0% to 57.0%. There was no significant difference in the mean parasitism level estimated using the three methods ($F_{2, 54} = 1.07$; $p = 0.35$).

In field-collected *C. norwegicus* reared in the laboratory for parasitoids, nymphal mortality was 14.1±2.1(SE)%. The cause of this mortality remains unexplained.

Laboratory-reared *C. norwegicus* yielded 458 cocoons. *Peristenus digoneutis* was reared from nymphs collected at 3 of 19 field sites, and emerged from 2.2% of the

cocoons. *Peristenus relictus* was reared from nymphs collected from 3 of the 19 sites, and 0.6% of the cocoons produced adults of this species. *Peristenus pallipes* was the most common parasitoid reared from *C. norwegicus*, and emerged from 58.1% of the cocoons. Hyperparasitoids belonging to the genus *Mesochorus* emerged from cocoons collected at 8 of the 19 sites, and represented 5.5% of the cocoons. Some of the parasitoids reared from *C. norwegicus* failed to emerge from their cocoons. This was evident in all collections, and represented a total of 33.6% of the cocoons.

Molecular analysis of *C. norwegicus* nymphs yielded 522 positive PCR reactions. *Peristenus digoneutis* was detected from 7 of the 19 sites, and represented 2.5% of the positive PCRs. Nymphs collected from 8 of the 19 sites contained *P. relictus*, and 5.0% of parasitized nymphs produced positive PCR results for this species. *Peristenus pallipes* was detected in 92.1% of parasitized nymphs, and was found at all sites. Multiparasitism was detected only twice, and represented 0.4% of the positive reactions.

Despite the similarity of the results for species composition based on rearing and molecular analysis, only the proportion of *P. digoneutis* was not significantly different when estimated by rearing and molecular analysis ($z = 0.42$; $p = 0.68$). The proportion of *P. relictus* detected by molecular analysis was significantly higher than in rearing ($z = -2.55$; $p = 0.01$). Similarly, the proportion of *P. pallipes* identified by molecular analysis was significantly higher than the proportion obtained in rearing ($z = -3.51$; $p < 0.001$).

Liocoris tripustulatus

Parasitism ranged from 0% to 56.0% in dissected samples, 6.0% to 53.0% in reared samples, and 9.0% to 76.0% in samples analyzed using PCR. There was a significant difference in the mean parasitism level estimated by the three methods ($F_{2, 57} = 3.58$, $p = 0.03$). A post-hoc analysis using a Tukey HSD test ($P = 0.10$) showed that the mean parasitism level estimated by molecular analysis was significantly higher than by rearing and dissection.

Unexplained nymphal mortality in the rearing process accounted for 16.4±1.9(SE)% of the nymphs collected.

Rearing of *L. tripustulatus* nymphs provided 374 parasitoid cocoons. *Peristenus digoneutis* was reared from nymphs collected from only 1 of 20 *L. tripustulatus* sites, and represented 0.3% of the cocoons reared from this host. *Peristenus relictus* was not reared from *L. tripustulatus*. *Peristenus pallipes* was the most commonly reared parasitoid, and emerged from 33.7% of the cocoons. Hyperparasitoids were reared from 10 of the 20 *L. tripustulatus* collections, and emerged from 5.9% of the cocoons. A high proportion of pupal mortality was evident, and 60.1% of the cocoons failed to produce a parasitoid adult.

A total of 602 parasitoid-specific PCR fragments were obtained from molecular analysis of *L. tripustulatus* nymphs. Two *P. digoneutis* individuals were detected from a single site, and represented 0.3% of the total number of positive PCR results. *Peristenus relictus* was detected at 3 of the 20 sites, and represented 0.5% of the positive PCR reactions. *Peristenus pallipes* was detected at all sites, and represented 99.1% of the positive PCR reactions.

Rearing and molecular analysis did not differ significantly in the estimation of the mean proportion of *P. digoneutis* ($z = 0.45$; $p = 0.66$) and *P. relictus* ($z = -1.60$; $p = 0.11$). However, the proportion of *P. pallipes* estimated by molecular analysis was significantly higher than by rearing ($z = -3.92$; $p < 0.001$).

Leptopterna dolobrata

Dissection, rearing, and molecular analysis of field-collected *L. dolobrata* nymphs provided parasitism levels that ranged from 4.0% to 48.0%, 5.0% to 37.0%, and 8.0% to 47.0%, respectively. Comparison of the mean parasitism levels showed that there was no significant difference between the mean values estimated using the three methods (ANOVA, $F_{2, 51} = 1.29$; $p = 0.28$). Unexplained nymphal mortality in rearing was 17.3±2.5(SE)%.

Rearing of *L. dolobrata* nymphs yielded 368 parasitoid cocoons. *Peristenus digoneutis* was not reared from this host, and only one *P. relictus* emerged from the collections. *Peristenus pallipes* was the most frequently reared parasitoid, and emerged from 42.1% of the cocoons. The remainder of the cocoons contained either

hyperparasitoids (13.8%), or parasitoids that failed to emerge (43.8%). Hyperparasitism was evident at 10 of the 18 sites, and pupal mortality was evident in all collections.

Molecular analysis of *L. dolobrata* nymphs produced 443 parasitoid-specific PCR reactions. Three of these generated *P. digoneutis*-specific PCR fragments, and 13 generated *P. relictus*-specific PCR fragments. These two parasitoid species accounted for 0.7% and 2.9% of the positive PCR reactions for *L. dolobrata*. The majority of the positive PCR reactions (95.7%) generated *P. pallipes*-specific PCR products. Three *L. dolobrata* nymphs (0.7% of the positive PCR reactions) showed strong positive results for *P. pallipes* and *P. relictus* simultaneously, an indication of multiparasitism.

The mean proportion of *P. digoneutis* was not significantly different when estimated by rearing and molecular analysis ($z = -1.34$; $p = 0.18$). However, there was a significant difference between the mean proportion of *P. relictus* reared and the mean proportion detected by molecular analysis ($z = -2.38$; $p = 0.02$). Similarly, the mean proportion of *P. pallipes* was significantly higher by molecular analysis than by rearing ($z = -3.73$; $p < 0.001$).

3.4 Discussion

In the current study, a multiplex PCR assay for *Peristenus* was compared to conventional methods in the estimation of parasitism level and parasitoid species composition in four mirid species. The intent was to conduct a large-scale comparison of the three methods to assess their accuracy. Over 26,000 mirid nymphs were collected, with 8,700 nymphs analyzed using each of the methods.

Two earlier studies on plant bug parasitoids suggested that discrepancies between molecular and traditional methods may occur (Tilmon et al., 2000; Ashfaq et al., 2004). Both of these studies used rearing, dissection, and molecular methods to estimate parasitism levels in *Lygus* plant bugs. Tilmon et al. (2000) indicated that molecular analysis and/or dissection provided higher estimates of parasitism than rearing; however, no statistical comparisons were made between rearing and molecular analysis, or between rearing and dissection. Furthermore, collections were made at a single site, and the sample size was small and involved unequal sample sizes for the

methods tested. Ashfaq et al. (2004) showed that PCR provided significantly higher estimates of parasitism level when compared to dissection and rearing. However, sample sizes were again small, and differed between methods.

In contrast with the previously cited studies, but consistent with several other studies comparing molecular and conventional methods (Ratcliffe et al., 2002; Agustí et al., 2005; Gariepy et al., 2005; Jones et al., 2005), the current study shows that in most cases, all three methods provide statistically equivalent estimates of parasitism level in a variety of host populations. However, in *L. tripustulatus* rearing and dissection provided significantly lower mean parasitism values than molecular analysis. One possible explanation for this result is that *L. tripustulatus* nymphs were collected too early in the season. If this were the case, some nymphs would still contain the egg stage of the parasitoid. Although easily detected by molecular analysis, parasitoid eggs are often overlooked in dissection because of their small size, which would result in an underestimation of the parasitism level. For rearing, it is likely the effect of host mortality during the rearing phase that led to a slightly lower parasitism level. Therefore, the slight discrepancy between the data obtained using the two methods could have resulted from the fact that molecular analysis was able to detect parasitism at an earlier stage than dissection, and because molecular estimates were not confounded by mortality factors.

Numerous studies have examined host-parasitoid associations in Miridae in Europe (Bilewicz-Pawinska, 1976, 1977a, 1977b, 1982; Bilewicz-Pawinska & Varis, 1985; Haye et al., 2005b; Haye et al., 2006a). Results from rearing and molecular analysis in the present study were consistent with results from previous studies, and showed that the dominant parasitoid associated with *L. rugulipennis* was *P. digoneutis*, followed by *P. relictus*. *Peristenus pallipes* was virtually absent in *Lygus* collections. Members of the *P. pallipes* complex dominated the parasitoid community associated with *C. norwegicus*, *L. tripustulatus*, and *L. dolobrata*. Relatively few *P. digoneutis* and *P. relictus* were detected in the selected non-target hosts.

For each mirid species, the mean proportion of *P. digoneutis* estimated by rearing and molecular methods was not significantly different. This indicates that both

methods provide equivalent estimates of the occurrence of this parasitoid species in the target and non-target hosts investigated.

However, in *L. rugulipennis*, *C. norwegicus*, and *L. dolobrata*, the mean proportion of *P. relictus* was significantly higher when estimated using molecular methods. Studies on the reproductive biology of *P. relictus* have shown that this species readily superparasitizes *Lygus* nymphs, even when unparasitized individuals are available (Haye, 2004). Multiparasitism was detected by molecular analysis, and indicates that occasionally parasitoids oviposited in an already parasitized host. Superparasitism and multiparasitism often increase mortality, with reared specimens failing to produce either a host or parasitoid adult (Fisher, 1961; King et al., 1976; Reader & Jones, 1990; Agboka et al., 2002; De Moraes & Mescher, 2005). Therefore, it is possible that *P. relictus*-multiparasitized or -superparasitized nymphs were subject to higher mortality in rearing. As DNA extracted from *P. relictus*-superparasitized or -multiparasitized nymphs would produce a *P. relictus* species-specific PCR product, these individuals would still be detected by molecular analysis. The proportion of *P. relictus* would therefore be lower in reared samples than in samples analyzed with molecular methods. Although plausible, further research would be necessary to support this suggestion.

The methods used to estimate the proportion of *P. pallipes* in *C. norwegicus*, *L. tripustulatus*, and *L. dolobrata* provided different results. The mean proportion estimated by molecular analysis was significantly higher than the mean proportion estimated by rearing. This is because of the large proportion of pupal mortality that occurred in reared samples. Pupal mortality in these hosts was often extreme, with approximately 30% to 60% of the cocoons failing to produce a parasitoid adult. In contrast, pupal mortality in *L. rugulipennis* collections was considerably lower (approximately 13%). This suggests that *P. pallipes* is subject to a higher degree of overwintering mortality than *P. digoneutis* and *P. relictus*. This is consistent with previous research (Lachance et al., 2001) that demonstrated *P. digoneutis* and *P. relictus* suffer less pupal mortality than members of the *P. pallipes* complex.

In biological control programmes, hyperparasitism is believed to have a critical impact on the suppression of a host population (Rosenheim et al., 1995; Batchelor et al.,

2006) and may contribute to the failure of parasitoid establishment (Gaines & Kok, 1999; Perez-Lachaud et al., 2004). Furthermore, hyperparasitoid host preference may change the relative abundance of co-occurring parasitoid species, thereby influencing host suppression (Bogran & Heinz, 2002). In the current study, hyperparasitoids were obtained from reared samples, but generally in fairly low proportions (usually less than 10%). Day (2002) suggested that in North America, the polyphagous hyperparasitoid *Mesochorus curvulus* Thomson prefers *P. pallipes*, and that low hyperparasitism levels are unlikely to have a strong negative effect on primary parasitoids of *Lygus*.

Because a hyperparasitoid consumes the *Peristenus* larva within the cocoon, only the hyperparasitoid adult emerges in rearing, and the identity of the *Peristenus* host is lost. This can have a large impact if one parasitoid species in a community of natural enemies is hyperparasitized more frequently than the others, as it can profoundly affect the abundance of that species. In addition, it would render rearing data inaccurate, as the proportion of the preferred parasitoid species would be underestimated when only hyperparasitoids emerge from these individuals. Molecular analysis was unable to provide information regarding hyperparasitism because PCR primers for *Mesochorus* were not used in the multiplex assay. Theoretically, molecular analysis of mirid nymphs would allow the detection and identification of *Peristenus* individuals even when hyperparasitized because parasitoid DNA would still be present. The development of a system in which parasitoids and hyperparasitoids could be detected simultaneously would be ideal. Such an assay would facilitate investigations on host – parasitoid – hyperparasitoid associations, and allow questions regarding the impact and host preferences of *Mesochorus* to be addressed further.

Accurate measurement of levels of multiparasitism is important as it could provide information on competition between parasitoids that utilize the same host species. Interspecific competition for host resources can be detrimental to achieving biological control of a pest species (Levesque et al., 1993; Heinz & Nelson, 1996; Urbaneja et al., 2003; De Moraes & Mescher, 2005; Batchelor et al., 2006; Muli et al., 2006). Very little data is available on multiparasitism in field-collected Miridae. Euphorine wasps are solitary endoparasitoids, and even when parasitized by multiple individuals, only one parasitoid (if any) will be reared from the host (Lachance et al.,

2001). Although host dissection may detect the presence of multiple parasitoid larvae, the inability to distinguish species prevents further information from being recorded. The use of the multiplex PCR assay allowed the detection of multiparasitism, as well as the identification of the species responsible. However, it cannot provide information on which parasitoid (if any) would develop successfully.

Concern has been raised regarding the competitive displacement of native parasitoids by exotic parasitoids (Bennett, 1993; Simberloff & Stiling, 1996; Waage, 2001; Messing et al., 2006). As *P. digoneutis* and *P. relictus* are established in North America, the potential displacement of native braconid species is a possibility. However, post-release studies by Day (1996; 2005) suggested that displacement of native species by *P. digoneutis* in the USA has not occurred. If multiparasitism can be used to indicate competition between native and exotic natural enemies, then molecular methods that can detect multiple parasitoid species could be used to assess these competitive interactions. Although rarely used in the characterization of guild interactions in natural enemy communities, molecular methods would be useful in demonstrating the occurrence of indirect non-target effects resulting from competitive interactions and displacement (Messing et al., 2006).

Direct non-target effects are often evaluated by rearing field-collected non-target hosts to determine if the biological control agent of interest is present in the population. In this context, the loss of species composition information due to host and/or parasitoid mortality may allow non-target parasitism to go undocumented. This is conceivable, as there could be fitness consequences for a parasitoid that develops in a non-target host. If this is the case, it may lead to an increased level of parasitoid mortality. These parasitoids may still result in the death of the host, and thereby in non-target effects, but the identity of the responsible parasitoid would not be obtained in the rearing process. It has been acknowledged that so-called 'duds' (i.e., hosts which fail to produce a parasitoid adult) may bias parasitism estimates obtained in rearing (Ratcliffe et al., 2002; Greenstone, 2006). However, there appears to be no reference to the potential impact of 'duds' on the accuracy of measurements for parasitoid species composition and/or host-rangedata in non-target risk assessment studies.

In the present study, a high proportion of cocoons reared from *C. norwegicus*, *L. tripustulatus*, and *L. dolobrata* failed to produce parasitoid adults. It is clear that the parasitoid pupal mortality observed in these reared samples is attributable to *P. pallipes*, as opposed to *P. digoneutis* and *P. relictus*, because the molecular data show that *P. pallipes* made up over 90% of the parasitoid species composition in these hosts. Furthermore, if the proportion of pupal mortality is added to the proportion of *P. pallipes* in reared samples, this value is approximately the same as the proportion of *P. pallipes* detected by molecular analysis. In most cases, rearing alone would not allow the conclusive statement that unemerged parasitoids belong to a certain species because there is no way to identify such specimens. However, the identity of unemerged parasitoids can be quite accurately inferred using paired molecular data. Without this information, the possibility that some or all of the unemerged parasitoids were *P. digoneutis* or *P. relictus* could not be excluded. Correct estimation of the impact of candidate biological control agents in non-target populations is essential in risk assessment studies. Knowledge of parasitoid species and their specificity will enable more rigorous selection of candidate agents for the biological control of economically important plant bugs (Loan, 1980).

Although molecular methods are valuable tools in the study of parasitism and parasitoid species composition, they cannot replace rearing. Agustí et al. (2005) stated that rearing is essential to detect non-expected species, particularly in previously unexplored geographic regions. In the current context, extensive field surveys in Europe have already defined the parasitoid community associated with the Miridae (Bilewicz-Pawinska, 1982; Haye et al., 2005b; Haye et al., 2006a). Availability of these records allowed the development of PCR primers for the primary parasitoid species that attack the mirids surveyed in this study.

According to Shaw (1994), methods that allow quantitative expression of the realized host range to be related to the performance of the parasitoid within a host population are essential. When sufficient rearing data are available on the parasitoid community associated with a given host species, molecular assays can be developed that encompass all members of this community. Molecular methods for the estimation of parasitoid species composition have the potential to provide more accurate information

than rearing. Although rearing provides important qualitative data on the parasitoid community associated with a given host, molecular methods are likely to provide better quantitative data for these associations.

4. MOLECULAR DIAGNOSTICS AND RISK ASSESSMENT: PARASITISM OF MIRIDAE BY *P. DIGONEUTIS* LOAN AND *P. RELICTUS* RUTHE IN TWO ECOREGIONS

4.1 Introduction

There are risks and benefits in any biological control programme, and it can be difficult to predict how an agent will behave when introduced into a new environment. Because biological control introductions are generally irreversible, there are some concerns about the release of exotic natural enemies; these include the displacement of native species with similar niches, attack on alternate hosts, host-preference shifts, and dispersal into other habitats (Howarth, 1983, 1991; Simberloff & Stiling, 1996; Follett et al., 2000; Howarth, 2000; Kuris, 2003).

Risk-assessment studies often attempt to define the ecological host range of a candidate biological control agent in order to gauge the specificity of the agent under natural conditions. These studies rely on extensive field collection of potential target and non-target host species, followed by laboratory rearing to obtain parasitoids and identify the parasitoid community associated with each host species. However, the use of molecular methods to identify parasitoids within their hosts may be more appropriate for risk assessment studies, as they can provide more complete parasitoid species-composition information than traditional rearing methods (see Chapter 3).

Peristenus digoneutis Loan and *P. relictus* Ruthe (Hymenoptera: Braconidae) are under consideration as biological control agents for *Lygus* Hahn (Hemiptera: Miridae) plant bugs in Canada. These species are widely distributed geographically in Europe (Coutinot & Hoelmer, 1999). However, studies have focused primarily on the occurrence of *P. digoneutis* and *P. relictus* in *Lygus* spp. Few studies have investigated parasitism of additional mirid species by these two candidate biological control agents.

The fundamental and ecological host ranges of *P. digoneutis* and *P. relictus* were investigated in northern Germany (Haye et al., 2005b; Haye et al., 2006a), and limited host-parasitoid association studies were conducted in cultivated habitats in Poland (Bilewicz-Pawinska, 1977b, 1982). However, extensive host-range data from different geographic regions or climatic zones are lacking, despite the possibility that different biotypes of these parasitoid species may exist.

It is not uncommon for a parasitoid species to consist of biotypes that differ in climatic adaptation or host-population suitability, and non-target effects may be associated with a particular biotype of a species rather than the species as a whole (Etzel & Legner, 1999; Unruh & Woolley, 1999). Thus, host-range surveys that include different geographic regions are more likely to predict parasitoid impact on target and non-target hosts. Coutinot and Hoelmer (1999) suggested that characterization of climatic strains of *P. digoneutis* and *P. relictus* may help evaluate host-parasitoid associations within and between geographic regions. In addition, strains or biotypes may be responsible for differences in parasitism by *P. digoneutis* and *P. relictus* from different hosts, climates and/or geographic locations in Europe (Coutinot & Hoelmer, 1999). However, before such comparisons can be made, a suitable scale on which to compare them needs to be identified. In the assessment of risks associated with exotic biological control agents, Cock et al. (2006) suggested that the ecoregion concept be used, as ecological boundaries are more relevant than national boundaries in this context.

Ecoregions are relatively large units of land that contain a distinct assemblage of natural communities and species. They are delineated based on biogeographic, taxonomic, conservational, and ecological information (Bailey, 1983, 1989; Olson et al., 2001). Olson et al. (2001) presented a detailed global ecoregion map composed of 867 ecoregions nested within 14 biomes (see www.worldwildlife.org/wildworld for an interactive digital map).

Ecoregions show regional and global distributions of a wide variety of flora and fauna, and provide a framework that allows comparison between units (Olson et al., 2001). The existence of a given ecological feature in one ecoregion can be inferred from similar ecoregions on the same continent, or across analogous ecoregions on different

continents (Bailey, 2005). Thus, the ecoregion concept is useful for a broad range of ecological studies. In fact, the use of an ecoregion approach in biological control can help interpret and manage risks associated with the movement of natural enemies (Cock et al., 2006).

To improve the quality of information generated in risk-assessment studies, and to extract the most precise ecological host-range data, molecular methods were used to evaluate host-parasitoid associations in mirid populations from two ecoregions, the Northern Temperate Atlantic (NTA) Ecoregion and the Western European Broadleaf Forest (WEBF) Ecoregion. The NTA Ecoregion is characterized by a wet, temperate, coastal climate, typical of areas in northern continental Europe including parts of France, Germany, Belgium, the Netherlands, and Denmark (DMEER, 2000). The WEBF Ecoregion consists of lowland mixed forests that dominate western Europe, and includes most of western France, central and northern Switzerland, central and southern Germany and Austria (DMEER, 2000). Both ecoregions are within the Temperate Broadleaf and Mixed Forests Biome (Olson et al., 2001). Analogous ecoregions in the same biome occur in Canada, where *P. digoneutis* may be released.

Previous ecological host-range studies on *Peristenus* relied on rearing methods to obtain and identify parasitoids from non-target hosts. However, overwintering mortality of parasitoid cocoons often prevents identification of some of the specimens obtained. In contrast, the multiplex PCR assay for *Peristenus* spp. provides more complete information regarding *Peristenus* species composition (see Chapter 3). Although molecular methods have been used to identify parasitoid species and quantify parasitism (Greenstone, 2006), they have not been applied to ecological host-range studies.

The objectives of this study were to determine if parasitism by *P. digoneutis* and *P. relictus* in target and non-target mirids differs between ecoregions, and whether *P. digoneutis* and *P. relictus* parasitize non-target and target mirids equally. The non-target impacts of the two candidate biological control agents are discussed in context with previous studies on their ecological and fundamental host ranges in Europe and North America.

4.2 Materials and Methods

4.2.1 Selection of Target and Non-target Hosts

Lygus rugulipennis Poppius was selected as the target host, as it is considered the European equivalent of pestiferous *Lygus* species in North American. Criteria outlined by Kuhlmann et al. (2006) were used to select non-target species for risk-assessment studies. These selection criteria include phylogenetic affinity and ecological similarity with the target host, as well as safeguard considerations (i.e., potential effects on beneficial insects, and rare or endangered species). To reduce the test list to a manageable size, only those species with similar spatial, temporal and morphological attributes as the target pest, and which are readily available and accessible in the field should be included (Kuhlmann et al., 2006). Results from previous studies on the ecological and fundamental host ranges of *P. digoneutis* and *P. relictus* (Haye et al., 2005b; Haye et al., 2006a) were used to further restrict the test-list of non-target species.

Four non-target Miridae in the subfamily Mirinae were selected: *Adelphocoris lineolatus* Goeze, *Closterotomus norwegicus* Gmelin, *Liocoris tripustulatus* F., and *Leptopterna dolobrata* L. These species were abundant in the field and share similar spatial, temporal and morphological attributes with the target species. *Adelphocoris lineolatus*, *C. norwegicus* and *L. tripustulatus* belong to the same tribe as *Lygus* (Mirini) whereas *L. dolobrata* belongs to the tribe Stenodemini. A maximum fit cladogram for *Lygus* and its outgroup taxa (Schwartz & Foottit, 1998) placed *L. tripustulatus* among the species most closely related to *Lygus* species. *Adelphocoris lineolatus* and *C. norwegicus* share ecological similarities with *L. rugulipennis*. In contrast, *L. tripustulatus* and *L. dolobrata* do not occur on the same host plants as *Lygus*, although some overlap may occur when neighboring agricultural fields contain *Lygus*. It should be noted that in the current context, 'host plant' can refer to the assemblage of plant species that occur in the same field (e.g., mixed grasses).

Several other non-target species were collected when they were found in high numbers, simultaneously with *L. rugulipennis*. These were: *Adelphocoris seticornis* F. (Mirinae: Mirini), *Notostira elongata* Geoffroy, *Stenodema calcerata* Fallén, and *Trigonotylus caelestialum* Kirkaldy (all Mirinae: Stenodemini). None of these species was collected at more than two sites.

4.2.2 Field Collections in the NTA and WEBF Ecoregions

Mirid nymphs were collected from 2003 to 2005 in two distinct ecoregions in the temperate broadleaf and mixed forests Biome. Ecoregions were defined from the Digital Map of European Ecological Regions (DMEER, 2000). In 2003, field collections took place in northern Germany (Schleswig-Holstein), part of the NTA Ecoregion (Figure 4.1). In 2004 and 2005 collections took place in Switzerland, the Baden-Württemburg area of Germany, and the Alsace and Franche-Compté areas of France (Figure 4.2 and 4.3). These areas are part of the WEBF Ecoregion. See Appendix B for GPS coordinates.

The selected target and non-target species were collected from 9 to 10 field sites in each of the ecoregions examined (Figures 4.1 – 4.3). Host plants for each species – ecoregion combination are listed in Table 4.1. At each field site, 100 third- to fifth-instar nymphs were collected for molecular analysis. For *A. lineolatus*, collections were conducted primarily in the WEBF Ecoregion. This species was not common in the NTA Ecoregion, likely because the main host plant (alfalfa, *Medicago sativa* L.) is not grown in the Schleswig-Holstein area. However, a single collection of *A. lineolatus* was possible from black medic (*Medicago lupulina* L.) in the NTA Ecoregion.

A standard sweep net (38 cm in diameter) was used to collect insects from host plants. Insects were sorted in white trays coated with Fluon® (polytetrafluoroethylene, Dyneon Werk Gendorf, Burgkirchen, Germany), and species of interest were separated from other insects and spiders using a mouth aspirator. Specimens were transported to the laboratory in 1.2 L plastic containers with plant material as a food source. Samples were then re-counted, separated by species, and preserved in 95% ethanol for molecular analysis. During long field trips, mirids were directly sorted and preserved in ethanol in the field to prevent loss of specimens due to mortality and cannibalism during transport.

4.2.3 Parasitism by P. digoneutis *and* P. relictus *in Target and Non-target Miridae*

Nymphs preserved in 95% ethanol were stored at -20ºC until processing. DNA was extracted and amplified using the multiplex PCR assay (see Chapter 2) to screen for the presence of three *Peristenus* species simultaneously. This allowed detection of *P.*

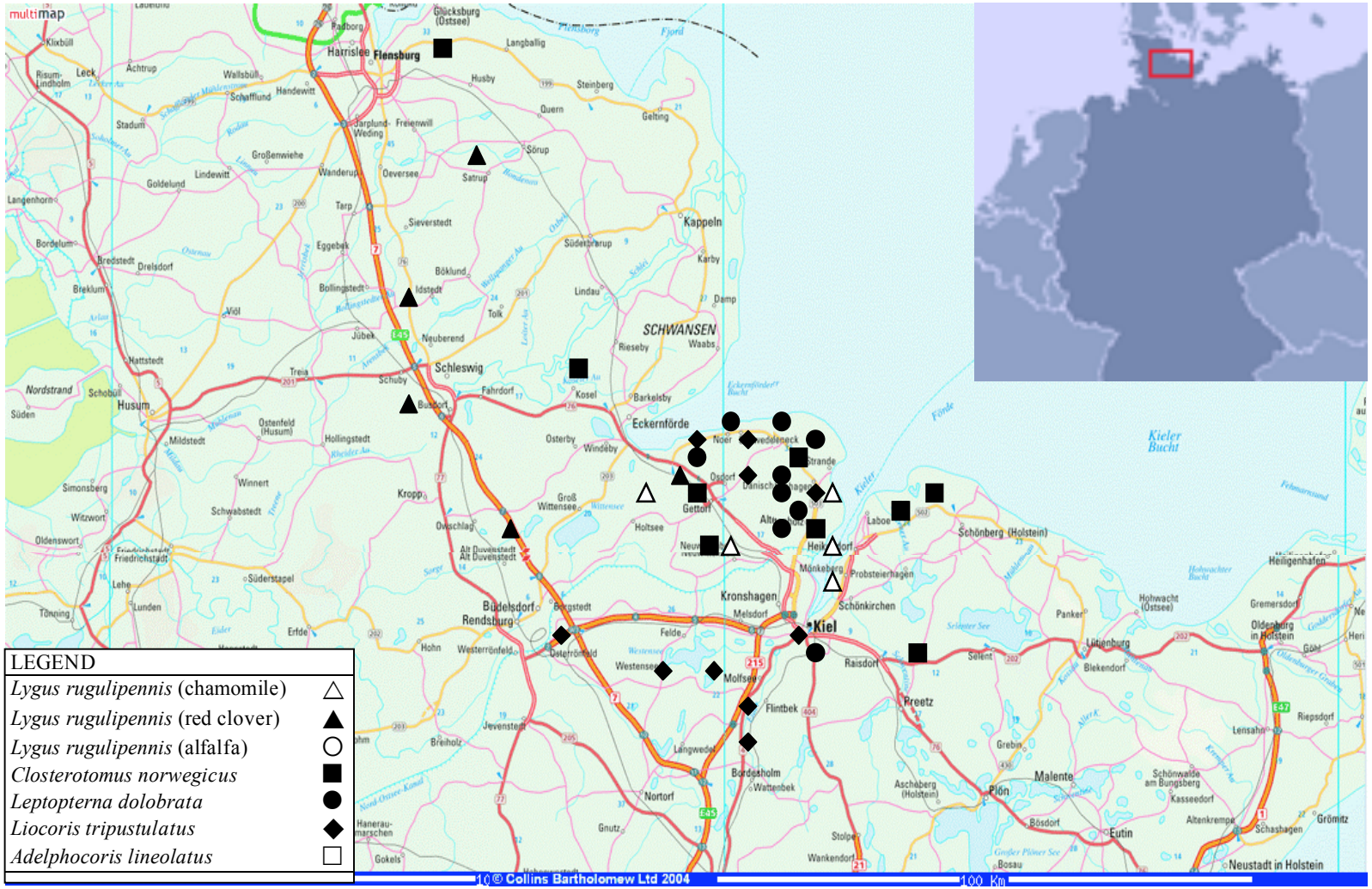

Figure 4.1 Field sites in the Northern Temperate Atlantic Ecoregion: Schleswig-Holstein, Germany (maps from multimap.com)

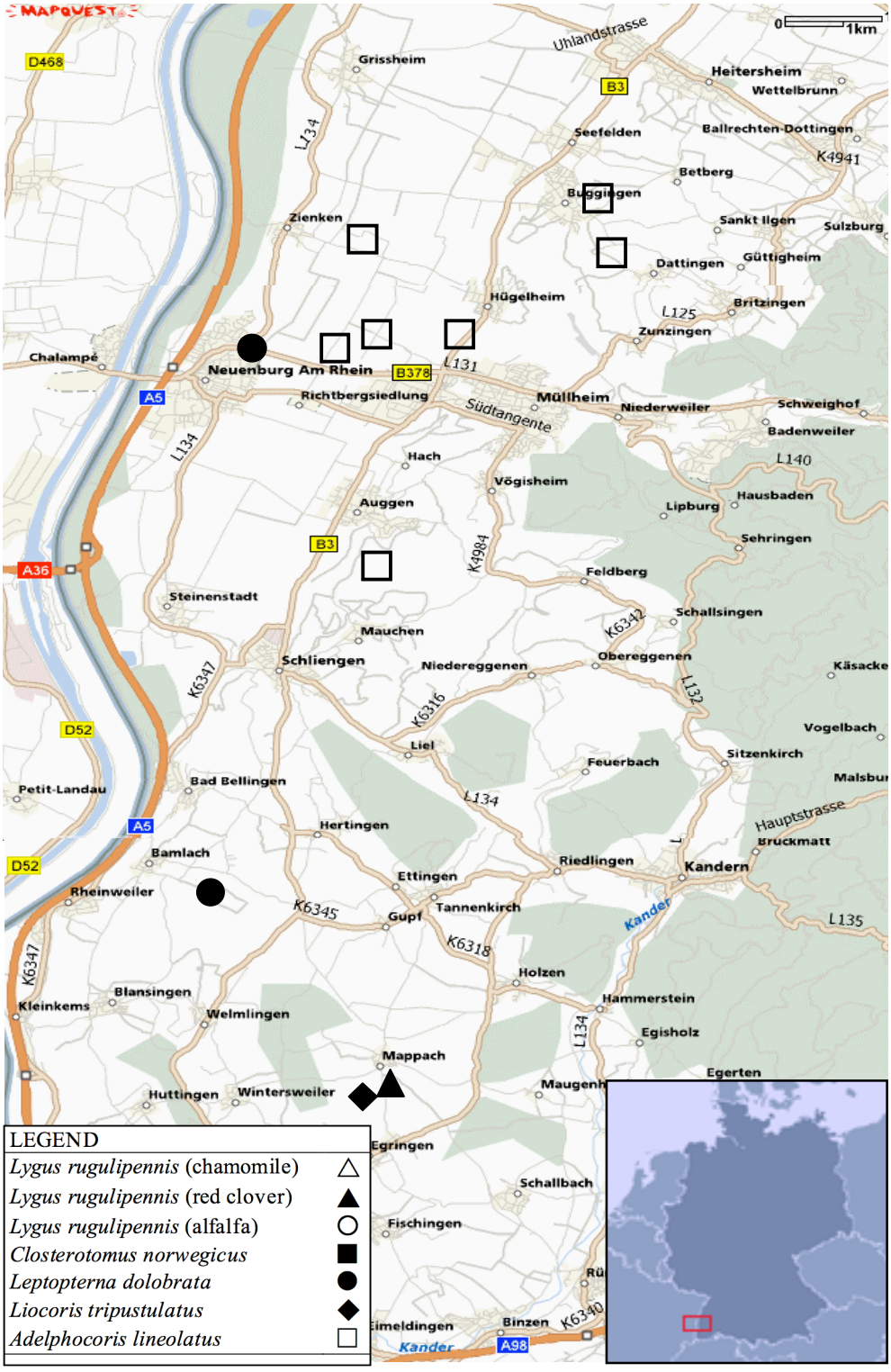

Figure 4.2 Field sites in the Western European Broadleaf Forest Ecoregion: Baden-Württemburg, Germany (maps from mapquest.com).

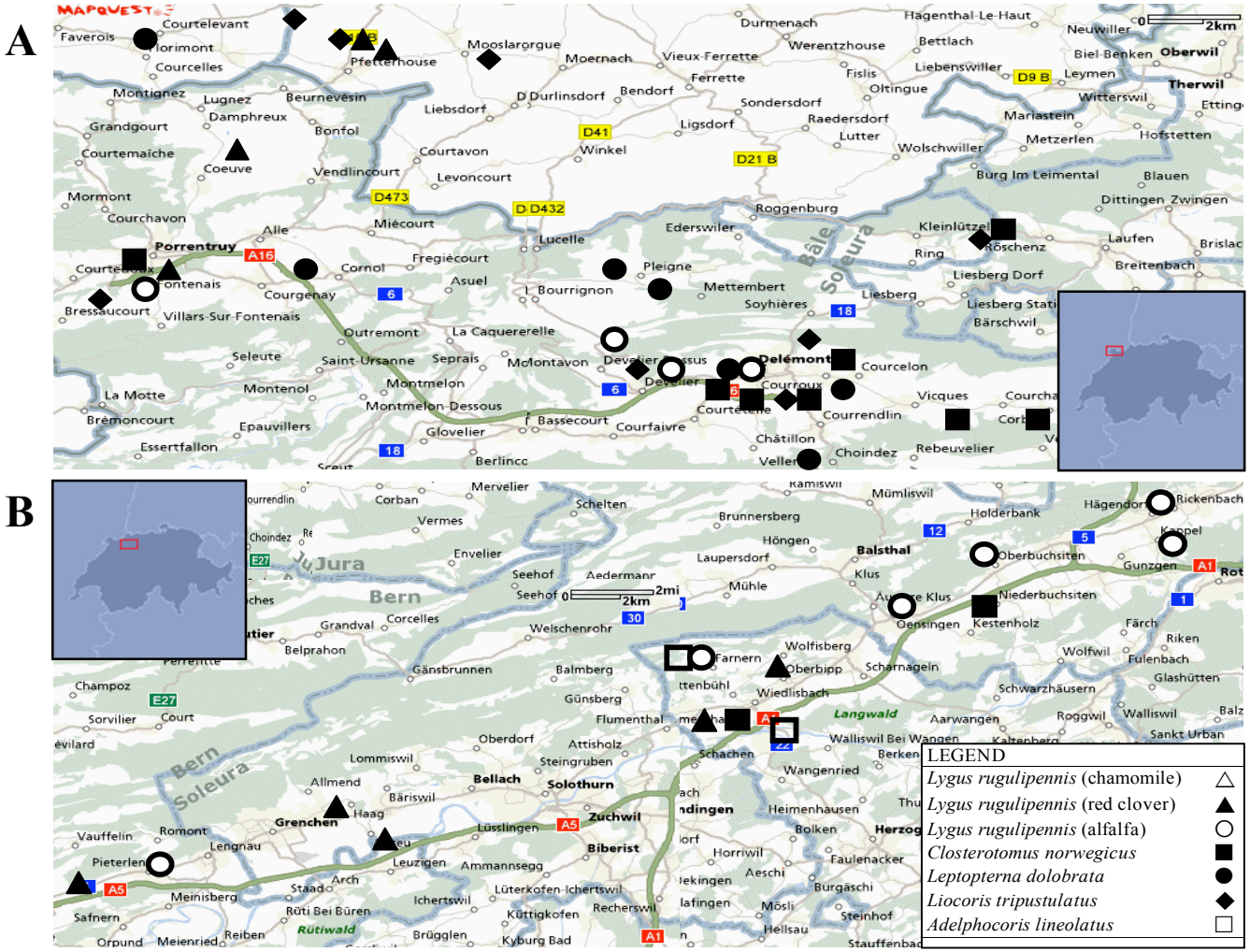

Figure 4.3 Field sites in the Western European Broadleaf Forest: (A) Jura, Switzerland, Franche-Compté and Alsace, France; (B) Solothurn and Bern, Switzerland (maps from mapquest.com).

Table 4.1. Host plants or habitats of target and non-target Miridae collected in Northern Temperate Altantic (NTA) and Western European Broadleaf Forest (WEBF) Ecoregions.

Mirid Species	**Ecoregion**	**Host Plant(s)**
Selected Species:		
Lygus rugulipennis	NTA	Chamomile (*Matricaria recutita* L.), red clover (*Trifolium pratense* L.)
	WEBF	Red clover (*T. pratense*)
Adelphocoris lineolatus	NTA	Black medic (*Medicago lupulina* L.)
	WEBF	Alfalfa (*Medicago sativa* L.)
Closterotomus norwegicus	NTA	Chamomile (*M. recutita*), mixed weeds and grasses
	WEBF	Mixed weeds and grasses
Leptopterna dolobrata	NTA	Orchard grass (*Dactylus glomerata* L.)
	WEBF	Orchard grass (*D. glomerata*)
Liocoris tripustulatus	NTA	Stinging nettles (*Urtica dioica* L.)
	WEBF	Stinging nettles (*U. dioica*)
'Additional' Species		
Adelphocoris seticornis	WEBF	Alfalfa (*M. sativa*)
Notostira elongata	NTA	Mixed grasses
Stenodema calcerata	NTA	Mixed grasses
Trigonotylus caelestialium	WEBF	Red clover (*T. pratense*) with mixed grasses

digoneutis and *P. relictus* (the two candidate biological control agents for *Lygus*), as well as *P. pallipes* Curtis (a complex of species associated with Miridae in Europe and North America). A positive PCR reaction indicated the presence of parasitoid DNA in a mirid nymph and provided information regarding the *Peristenus* species present. This was possible because PCR primers for *P. digoneutis*, *P. relictus*, and *P. pallipes* produced distinct PCR fragments (515, 330, and 1060-bp, respectively), which allows each species to be identified based on the fragment size generated.

Percent parasitism by *P. digoneutis* and *P. relictus* was calculated by dividing the number of each parasitoid species detected by the total number of nymphs collected for each mirid species, and multiplying by 100. In this way, the impact of the biological control agents on target and non-target mirid populations could be estimated. Estimates of parasitism by *P. pallipes* were used in the calculation of total parasitism. However, as this species is not intended for use as a biological control agent, the influence of this species on mirid host populations is not presented.

4.2.4 Statistical Analysis

To test the null hypothesis that total parasitism of each mirid species does not differ between the NTA and WEBF Ecoregions, an independent t-test (two-tailed; $P = 0.05$) was used. Parasitism of *A. lineolatus* was not included in the comparison, as representative collections were not possible in the NTA Ecoregion.

To test the null hypothesis that parasitism of Miridae by *P. digoneutis* and *P. relictus* does not differ between the two ecoregions, Mann-Whitney U-tests (two-tailed; $P = 0.05$) were used for non-parametric data (following Shapiro-Wilks tests that demonstrated the data were not normally distributed).

Similarly, a Mann-Whitney U-test (one-tailed; $P = 0.05$) was used to test the null hypothesis that parasitism by *P. digoneutis* and *P. relictus* is the same between target and non-target hosts. The alternate hypothesis is that parasitism by *P. digoneutis* and *P. relictus* is higher in the target host than in the non-target hosts.

Mirid species that were only occasionally collected (i.e., collected from one or two sites) were not included in statistical comparisons, as the number of collections was too low.

4.3 Results

4.3.1 Influence of Ecoregion on Parasitism by P. digoneutis *and* P. relictus

Parasitism of *L. rugulipennis* was 47.4±5.2(SE) in the NTA Ecoregion; this was significantly higher than in the WEBF Ecoregion, where parasitism was 31.8±4.2(SE) ($t = 2.32$, $p = 0.03$). In *C. norwegicus*, *L. dolobrata*, and *L. tripustulatus*, parasitism levels did not differ significantly between ecoregions ($t = -0.45$, $p = 0.66$; $t = 1.31$, $p = 0.21$; $t = 0.02$, $p = 0.98$). Parasitism levels were 25.9±4.9(SE) (NTA Ecoregion) and 28.9±4.6(SE) (WEBF) in *C. norwegicus*, 27.9±3.8(SE) (NTA Ecoregion) and 21.3±3.2(SE) (WEBF Ecoregion) in *L. dolobrata*, and 30.2±5.7(SE) (NTA Ecoregion) and 30.0±6.6(SE) (WEBF Ecoregion) in *L. tripustulatus*.

Parasitism by *P. digoneutis* and *P. relictus* in the two ecoregions are shown in Figures 4.4 and 4.5 for target and non-target hosts, respectively.

Parasitism by *P. digoneutis* in each host investigated did not differ significantly by ecoregion (*L. rugulipennis*: $z = -0.23$, $p = 0.82$; *C. norwegicus*: $z = -0.83$, $p = 0.41$; *L. tripustulatus*: $z = -1.00$, $p = 0.32$; *L. dolobrata*: $z = -0.08$, $p = 0.94$).

Similarly, ecoregion did not significantly influence parasitism by *P. relictus* in the non-target mirids investigated [*C. norwegicus* ($z = -1.96$, $p = 0.08$), *L. tripustulatus* ($z = -0.61$, $p = 0.54$), or *L. dolobrata* ($z = -1.21$, $p = 0.23$)]. However, parasitism of *L. rugulipennis* by *P. relictus* was significantly higher in the NTA Ecoregion than in the WEBF Ecoregion ($z = 3.09$, $p < 0.01$).

4.3.2 Parasitism by P. digoneutis *and* P. relictus *in Target and Non-target Mirid Species*

Peristenus digoneutis was responsible for 31.0% and 28.7% parasitism in *L. rugulipennis* in the NTA and WEBF Ecoregions (Figure 4.4, Table 4.2). In contrast, non-target parasitism ranged from 0 to 6.0%, depending on mirid species (Figure 4.5, Table 4.2). The highest non-target parasitism by *P. digoneutis* was in *A. seticornis* (6.0%). Although *A. seticornis* was collected from only two sites, *P. digoneutis* was the only parasitoid detected from this host. Of the non-target species originally intended for collection (i.e. species for which the number of sites was ≥ 9), the highest parasitism level by *P. digoneutis* was in *A. lineolatus* (2.2%).

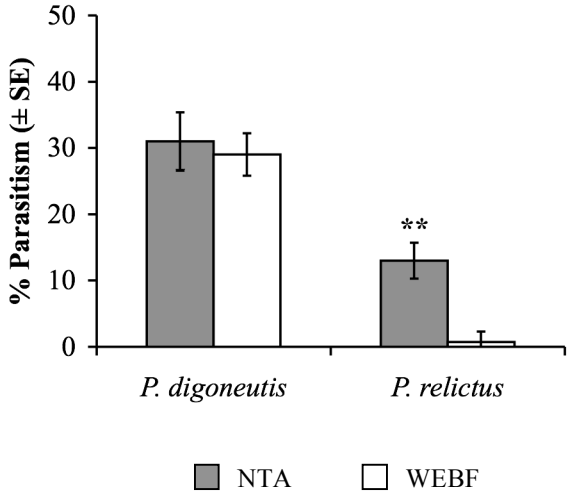

Figure 4.4 Parasitism of the target host, *L. rugulipennis*, by *P. digoneutis* and *P. relictus* in the Northern Temperate Atlantic (NTA) and Western European Broadleaf Forest (WEBF) Ecoregions of Europe. A significant difference in parasitism between ecoregions is indicated by an asterisk (Mann-Whitney U-test, ** $P < 0.01$).

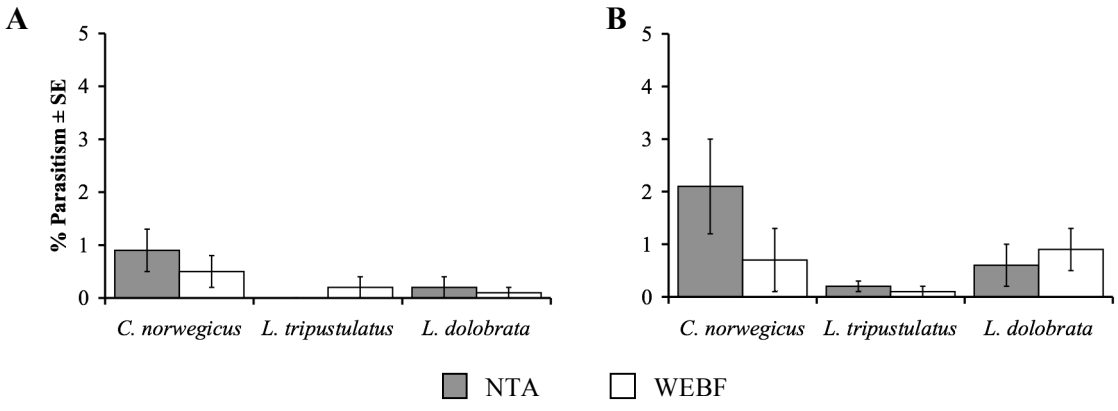

Figure 4.5 Parasitism of non-target hosts by (A) *P. digoneutis* and (B) *P. relictus* in the Northern Temperate Atlantic (NTA) and Western European Broadleaf Forest (WEBF) Ecoregions of Europe.

Table 4.2 Total parasitism and parasitism by *P. digoneutis* and *P. relictus* in Miridae collected from the Northern Temperate Atlantic (NTA) and Western European Broadleaf Forest (WEBF) Ecoregions in Europe.

Mirid Species	Ecoregion	No. Sites	No. Nymphs	No. PCR Positives			% Parasitism		
				Total	*P. digoneutis*	*P. relictus*	Total	*P. digoneutis*	*P. relictus*
Mirinae: Mirini									
Adelphocoris lineolatus	NTA	1	100	62	2 (1)	4 (1)	62.0	2.0	4.0
	WEBF	9	900	21	20 (7)	1 (1)	2.3	2.2	0.1
Adelphocoris seticornis	NTA	0	0	-	-	-	-	-	-
	WEBF	2	200	12	12 (2)	0	6.0	6.0	0
Closterotomus norwegicus	NTA	9	900	233	8 (5)	19 (6)	25.9	0.9	2.1
	WEBF	10	1000	289	5 (3)	7 (2)	28.9	0.5	0.7
Liocoris tripustulatus	NTA	10	1000	302	0	2 (2)	30.2	0.0	0.2
	WEBF	10	1000	300	2 (1)	1 (1)	30.0	0.2	0.1
Lygus rugulipennis	NTA	10	1000	474	310 (10)	132 (10)	47.4	31.0	13.2
	WEBF	10	1000	318	287 (10)	26 (5)	31.8	28.7	2.6
Miriniae: Stenodemini									
Leptopterna dolobrata	NTA	9	900	251	2 (1)	5 (2)	27.9	0.2	0.6
	WEBF	9	900	192	1 (1)	8 (5)	21.4	0.1	0.9
Notostira elongata	NTA	3	300	97	3 (1)	0	32.3	1.0	0
	WEBF	0	0	-	-	-	-	-	-
Stenodema calcerata	NTA	1	100	7	0	0	7	0.0	0
	WEBF	0	0	-	-	-	-	-	-
Trigonotylus caelestialium	NTA	0	0	-	-	-	-	-	-
	WEBF	2	90	45	0	39 (2)	50.0	0.0	43.3

Values in parentheses represent the number of sites from which the parasitoid was detected; '-' indicates that no information was collected.

Percent parasitism in the target host compared to each non-target host confirmed that parasitism by *P. digoneutis* was significantly higher in *L. rugulipennis* than in *A. lineolatus* (WEBF: $z = -3.68$, $p < 0.001$), *C. norwegicus* (NTA: $z = -3.71$, $p < 0.001$; WEBF: $z = -3.86$, $p < 0.001$), *L. tripustulatus* (NTA: $z = -3.96$, $p < 0.001$; WEBF: $z = -4.04$, $p < 0.001$), and *L. dolobrata* (NTA: $z = -3.82$, $p < 0.001$; WEBF: $z = -3.82$, $p < 0.001$). In fact, parasitism levels by *P. digoneutis* were relatively high in the target host, yet almost zero in the non-target species.

Parasitism by *P. relictus* was 13.2% and 2.6% in *L. rugulipennis* nymphs in the NTA and WEBF Ecoregions, respectively (Figure 4.4, Table 4.2). In non-target hosts, parasitism by *P. relictus* ranged from 0% to 43.0%, depending on mirid species. Maximum parasitism (43.0%) occurred in *T. caelestialium*, where *P. relictus* was the dominant parasitoid species detected. In the non-target species collected from ≥ 9 field sites, the highest parasitism level by *P. relictus* was in *C. norwegicus* collected from the NTA Ecoregion (2.1%) (Figure 4.5B).

In the NTA Ecoregion parasitism by *P. relictus* was significantly higher in *L. rugulipennis* than in *C. norwegicus* ($z = -3.16$, $p = 0.002$), *L. tripustulatus* ($z = -3.91$, $p < 0.001$), and *L. dolobrata* ($z = -3.64$, $p < 0.001$).

In contrast, parasitism by *P. relictus* in the WEBF Ecoregion was extremely low in the target host (significantly lower than parasitism of the target host in the NTA Ecoregion, as noted in Section 4.3.1), with levels similar to those observed in non-target hosts. Parasitism attributed to *P. relictus* in the WEBF Ecoregion was not significantly different between *L. rugulipennis* and *C. norwegicus* ($z = -1.38$, $p = 0.09$), or between *L. rugulipennis*, and *L. dolobrata* ($z = -0.35$, $p = 0.36$). However, parasitism by *P. relictus* in *L. rugulipennis* was significantly higher than in *L. tripustulatus* ($z = -2.05$, $p = 0.02$) and *A. lineolatus* ($z = -1.93$, $p = 0.03$).

4.4 Discussion

Parasitoid species from different ecoregions may differ in host specificity, and non-target effects may be attributable to a particular biotype collected from a certain habitat, climate, or host, rather than a particular species (Sands & Van Driesche, 1999;

Unruh & Woolley, 1999; Vink et al., 2003; Vazquez et al., 2004; Goldson et al., 2005). To determine the impact of a parasitoid on target and non-target species at the population level, studies should be done in several ecoclimatic zones, with replicated populations in each zone (Hopper, 2001). Expanding ecological host-range studies to include sympatric populations from different regions decreases the chance of underestimating the ecological host range of a candidate biological control agent (Haye et al., 2005b). In the present study, replicated populations of five mirid species from two ecoregions were sampled and used to describe the ecological host range of *Peristenus* species associated with *Lygus* plant bugs in Europe.

Parasitism of the Target Host in different Ecoregions

Total parasitism levels in *L. rugulipennis* were significantly higher in the NTA Ecoregion than in the WEBF Ecoregion. This likely due to the fact that two parasitoid species (*P. digoneutis* and *P. relictus*) regularly parasitize this host in the NTA Ecoregion. In contrast, *P. digoneutis* is the dominant parasitoid species associated with *L. rugulipennis* in the WEBF Ecoregion, and *P. relictus* occurs only sporadically in the target host population (see below). Thus, the presence of two parasitoid species appears to have an additive effect on parasitism of *L. rugulipennis* in the NTA Ecoregion.

Peristenus digoneutis is widely distributed throughout Europe and, consistent with the data presented here, is known to comprise 70–90% of the parasitoid community of *L. rugulipennis* in continental and oceanic climates (including the WEBF and NTA Ecoregions) (Coutinot & Hoelmer, 1999).

Parasitism of *L. rugulipennis* by *P. relictus* was significantly higher in the NTA Ecoregion than in the WEBF Ecoregion. Differences in parasitism levels by *P. relictus* in different areas of Europe may suggest the existence of biotypes that differ in climatic suitability and host preference. This is supported by the fact that, in Turkey, *P. relictus* was collected primarily from another mirid host species (*Polymerus unifasciatus* F.) (Coutinot & Hoelmer, 1999).

In the process of selecting parasitoid biotypes for release in North America, information on regional differences in parasitism levels should be considered. This would enhance the likelihood that the released parasitoids attack the target host at levels

that can result in a significant reduction of the pest population. Hypothetically, biotypes of *P. relictus* from the NTA Ecoregion would be more suitable for release efforts in North America than those from the WEBF Ecoregion, where parasitism levels in the target host were very low.

In terms of the release of European *Peristenus* in Canada for *Lygus* control, it may be useful to select *Lygus* parasitoids from European ecoregions that are similar to those in the receiving area. This would maximize parasitoid establishment, as the area of origin and introduction would be ecologically similar with respect to climate, natural communities, and species assemblages. For example, the Palearctic ecoregions surveyed here are within the Temperate Broadleaf and Mixed Forests Biome, and analogous Nearctic ecoregions also exist within the same biome. Although the ecoregion designations are somewhat different, the Nearctic equivalent of the NTA and WEBF Ecoregions would likely include the Eastern Great Lakes Lowland Forest and the Southern Great Lakes Lowland Forest Ecoregions. These ecoregions occupy most of southern Ontario, southern Quebec, and the northeastern United States (Olson et al., 2001), and would be best suited for releases of European *Peristenus* from the NTA and WEBF Ecoregions.

Parasitism of Non-target Hosts in Different Ecoregions

Peristenus digoneutis was detected in six of eight non-target species collected, but parasitism levels by this species were generally below 1%. This is consistent with research by Haye et al. (2005b) on parasitism levels in some of the same mirid species investigated here.

Adelphocoris seticornis is a new host record for *P. digoneutis*; however, potential non-target effects on *A. seticornis* at the population level are difficult to evaluate, as this species was collected from only two field sites in the WEBF Ecoregion. This mirid occurs throughout Europe (Wagner & Weber, 1964), but is absent or rare in northern Germany (Wagner, 1952). It is likely that parasitism of this host by *P. digoneutis* was not previously reported because limited geographic regions were surveyed in host-range studies. *Peristenus digoneutis* was also the main parasitoid associated with *A. lineolatus* in the WEBF Ecoregion. However, in the NTA Ecoregion limited collections suggested

that *A. lineolatus* was parasitized almost exclusively by *P. pallipes*. This is consistent with more extensive studies in the same ecoregion by Haye et al. (2005b). The difference in parasitoid species composition in the two ecoregions may result from differences in host phenology, as *A. lineolatus* is bivoltine in the WEBF Ecoregion and univoltine in the NTA Ecoregion (Wagner, 1952). This shows that the parasitoid community associated with a given host species can vary from one ecoregion to the next because host phenology may also vary.

Peristenus digoneutis was detected in field-collected *N. elongata* and *L. dolobrata* using molecular analysis. This parasitoid was never documented from these species in ecological host-range surveys in Europe that used rearing techniques (Haye et al., 2005b). However, both hosts are known from fundamental host-range studies on *P. digoneutis* (Haye et al., 2005b), and this parasitoid has been reared from *L. dolobrata* in North America (Day, 1999). This demonstrates the utility of molecular diagnostic tools in the detection of rare events, such as non-target parasitism.

Although *P. relictus* was detected in five of eight non-target species collected, parasitism levels were generally below 1.0%. This is consistent with previous research in northern Germany and suggests that *P. relictus* is likely of minor importance in the regulation of non-target populations (Haye et al., 2006a).

Adelphocoris lineolatus is a new host record for *P. relictus*. Although detected in both ecoregions, it is difficult to interpret the significance of this host-parasitoid association in the NTA Ecoregion as *A. lineolatus* was only found at one site. However, *P. relictus* was detected only once in the nine *A. lineolatus* populations sampled in the WEBF Ecoregion, and thus the evidence suggests that the impact is minor.

Trigonotylus caelestialium was highly parasitized by *P. relictus* (43.0%). This parasitoid was the dominant species in *T. caelestialium* at the two sites where this mirid was collected and accounted for 87.0% of the parasitoid community detected in this host. Bilewicz-Pawinska (1982) noted that *P. relictus* comprised 60.0% of the parasitoid community in *T. caelestialium* in Poland (the Central European Broadleaf Forest, CEBF, Ecoregion), with parasitism levels of up to 62.0%. Haye et al. (2006a) also reported parasitism of *T. caelestialium* by *P. relictus* in northern Germany, where it accounted for 42.0% of the parasitoid community in this host. This implies that *P. relictus* frequently

uses *T. caelestialium* as a host species, and may have the potential to regulate populations of this species.

In the present study, four new host records were reported in the ecological host range of *P. digoneutis*, and one new host record was reported in the ecological host range of *P. relictus*. It is unlikely that this represents a recent host range expansion of these parasitoid species; rather, it is likely due to the limited geographic scale of previous host-range studies. When studies are restricted to a single ecoregion, only a fraction of the host range may be documented. This is of particular concern when a parasitoid occurs across a broad range of ecoregions, and when the phenology and distribution of potential host species differ between ecoregions. The expansion of ecological host-range studies to include additional ecoregions provides a more thorough account of host records for candidate biological control agents.

Despite the new non-target host records in the ecological host ranges of *P. digoneutis* and *P. relictus*, parasitism levels in these mirids were consistently low, and do not necessarily translate into an increased likelihood of non-target effects. However, in sensitive or threatened ecosystems, even low non-target parasitism may be unacceptable. In the current context, the use of *Peristenus* spp. host records from European ecoregions may identify related native species that are at risk in similar ecoregions in North America.

Non-target Impact of P. digoneutis *and* P. relictus

Sands (1997) noted that effective biological control agents are rarely monophagous in their area of origin, but are typically adapted to a narrow range of closely related arthropod hosts. This suggests that some non-target parasitism will occur, but it does not necessarily result in severe non-target effects. The severity of the non-target impact needs to be addressed to ensure that effective, oligophagous parasitoids are not immediately discarded as candidate agents.

In classical biological control, severe non-target effects are unlikely when parasitoid-induced mortality is lower than 30% (Hawkins et al., 1993; Hawkins & Cornell, 1994; Lynch et al., 2001). Based on limited data from *T. caelestialium* from the NTA, WEBF, and CEBF Ecoregions (Bilewicz-Pawinska, 1982; Haye et al., 2006;

present study), this species may be at risk of severe non-target effects from *P. relictus*. Further research on larger populations of this species is needed to confirm this conclusion. The release of *P. relictus* in North America as part of a biological control program for *Lygus* spp. could raise concern over non-target effects, as 13 *Trigonotylus* species are native to Canada (Maw et al., 2000). Many of these species occur across Canada and potentially overlap with habitats and locations where European *Peristenus* would be released.

Lynch et al. (2001) developed severity indices, ranging from 0 (no records of parasitism) to 9 (extinction of non-target species over large areas), for non-target effects in biological control. A severity index of 1 indicates less than 5% mortality, with no significant effect at the population level (Lynch et al., 2001). If this approach is applied to estimate the impact of *P. digoneutis* and *P. relictus* on the majority of non-target species, a severity index of 1 is assignable. In contrast, a severity index of 3 to 5 indicates parasitism levels over 40% and/or significant suppression of non-target populations on the short or long term (Lynch et al., 2001). When applied to the current study, parasitism of *T. caelestialium* by *P. relictus* falls into this category.

The Utility of Molecular Diagnostics in Non-target Risk Assessment

The current study provides the first example of the use of molecular methods for ecological host-range studies. Although the test list of non-target mirids was not extensive, the data obtained by molecular analysis compare favorably with data on the host ranges of *P. digoneutis* and *P. relictus* based on rearing records from different regions (Bilewicz-Pawinska, 1982; Haye et al., 2005b; Haye et al., 2006a). From this comparison, it can be confidently stated that molecular analysis does not overestimate the host range of *P. digoneutis* and *P. relictus*. In fact, molecular methods may provide a more precise estimate of the host range as they are not biased by host and parasitoid mortality. This is particularly advantageous when evaluating non-target effects in hosts that are rare, difficult to rear, and/or subject to high mortality.

The use of molecular methods may facilitate ecological host-range studies conducted over large geographic areas, as rearing of numerous non-target species can be complicated, expensive, and time-consuming. Further, surveys over large geographic

areas can be difficult because live samples must be transported to the laboratory shortly after collection. In contrast, a molecular approach would allow samples to be collected, preserved, and processed when convenient. By precluding the need to transport and rear live specimens, collecting or receiving non-target insects from diverse and distant ecoregions is more practical. Further, molecular diagnostic techniques, such as those based on PCR, would provide a novel approach to more thoroughly assess the ecological host range of a parasitoid over a broad geographic area where different biotypes may occur.

5. DOES HOST PLANT INFLUENCE PARASITISM AND PARASITOID SPECIES COMPOSITION IN *LYGUS RUGULIPENNIS*?

5.1 Introduction

Parasitism levels on the same insect species have been known to vary from one host plant to another (Vinson, 1976). *Aphidius smithi* Sharma (Hymentoptera: Braconidae) readily parasitized the green peach aphid, *Myzus persicae* Sulzer (Homoptera: Aphididae) when it was reared on broad bean, but not on tobacco (Fox et al., 1967). Le Corff et al. (2000) found that parasitism levels on leaf-feeding Lepidoptera were predictably higher on black oak *Quercus velutina* L. (Fagaceae) than on white oak (*Q. alba* L.). Similarly, in a study on leafminers and their parasitoids, Rott and Godfray (2000) demonstrated that the composition of the parasitoid community was largely influenced by host plant.

Plants often provide the first cue in the sequence of events that lead to host location by a parasitoid (Vinson, 1976), and characteristics of a given host plant may affect the susceptibility of an insect to parasitoid attack (Price et al., 1980; Le Corff et al., 2000).

Price et al. (1980) discussed some of the host plant characteristics that may influence parasitism level and parasitoid species composition in a host population. These include: plant-secreted attractants, differences in parasitoid search behavior on different host plants, structural refuges that conceal the host, and plant toxins sequestered by the host insect that adversely affect parasitoid survival.

North American studies showed that plants influence the search and parasitization behavior of *Peristenus pseudopallipes* Loan (Hymenoptera: Braconidae), a native parasitoid of *Lygus* Hahn (Hemiptera: Miridae) (Streams et al., 1968). Parasitism

of *Lygus lineolaris* Palisot de Beauvois by *P. pseudopallipes* was high on *Erigeron* (Asteraceae) and negligible on other plants growing in the same field (Streams et al., 1968; Shahjahan & Streams, 1973). Olfactometer assays and experiments on *P. pseudopallipes* feeding preference showed that *Erigeron* spp. were more attractive than several other host plants of *Lygus* bugs, and that parasitoid longevity was significantly higher when female parasitoids were provided with *Erigeron* flowers (Shahjahan, 1974). This evidence suggests that plant attractants may be an important cue for location of food and host resources by *Peristenus* species.

Studies on European *P. relictus* Ruthe showed that parasitoid females responded to volatiles from *L. lineolaris* nymphs on green bean (*Phaseolus vulgaris* L.), but not from nymphs or green bean alone (Condit & Cate, 1982). This supports the statement by Vinson (1976) that plant injury, or a mixture of plant and host factors may guide parasitoids to plants with potential hosts. Thus, odors from *Lygus*-infested plants may serve as attractants that allow *P. relictus* females to localize their search for hosts (Condit & Cate, 1982).

As discussed in Chapter 1, native univoltine *Peristenus* species do not provide adequate suppression of *Lygus* plant bugs in North America. Thus, bivoltine European parasitoids are being considered for release as part of a classical biological control programme for *Lygus*. However, the attractiveness of different host plants to European *Peristenus* spp. has not been addressed. In Europe, *P. digoneutis* Loan and *P. relictus* are associated with *L. rugulipennis* Poppius, and a third species or species complex (*P. pallipes* Curtis) is known to attack several other species of Miridae. The impact of host plant on parasitism level and parasitoid species composition in *Lygus* in a multiparasitoid system is unknown, and may be an important consideration prior to parasitoid release.

Interspecific competition may result when multiple parasitoid species share a common host (Heinz & Nelson, 1996; Batchelor et al., 2006). However, parasitoid species in the same guild can coexist if some degree of spatial or niche separation occurs (Tscharnke, 1992; Muli et al., 2006). Closely related parasitoids that attack the same insect may adapt to the same host on a different plant to avoid competitive interactions. Because *L. rugulipennis* is known to occur on a wide variety of plants in Europe

(Holopainen & Varis, 1991), there is the potential that parasitism levels and parasitoid species composition may differ in *Lygus* populations on different host plants. This would provide niche separation for the different *Peristenus* species and would allow coexistence on the same host resource.

To address this, *L. rugulipennis* populations were studied in crop habitats and/or natural habitats in two ecoregions. In the Northern Temperate Atlantic (NTA) Ecoregion parasitism and parasitoid species composition were compared in red clover and chamomile. In the Western European Broadleaf Forest (WEBF) Ecoregion, parasitism and parasitoid species composition were compared in red clover and alfalfa.

5.2 Materials and Methods

5.2.1 Field Collections

Lygus rugulipennis nymphs were collected from 5 chamomile (*Matricaria recutita* L.; Asteraceae) and 5 red clover (*Trifolium pratense* L.; Fabaceae) fields in the NTA Ecoregion, and in 10 red clover and 10 alfalfa (*Medicago sativa* L.; Fabaceae) fields in the WEBF Ecoregion (Appendix B). At each site, 100 nymphs were collected using a standard sweep net (38 cm in diameter), sorted in white trays coated with Fluon® (polytetrafluoroethylene, Dyneon Werk Gendorf, Burgkirchen, Germany) and preserved in 95% ethanol, as described in Chapter 4.

Chamomile sites were generally fallow fields colonized almost exclusively by this plant species (>90% chamomile relative to other plant species). Red clover and alfalfa fields were generally monocultures, with few other plant species present. When other plant species were present at a given site, only the host plants of interest were swept for *Lygus* nymphs.

5.2.2 Parasitism Level and Parasitoid Species Composition in Lygus rugulipennis *Collected From Different Host Plants*

Nymphs preserved in 95% ethanol were stored at -20°C until processing. DNA was extracted and amplified using the multiplex PCR protocol described in Chapter 2. As parasitized nymphs provide a positive PCR result and unparasitized nymphs provide a negative result, amplification of DNA from individual nymphs was used to calculate

the parasitism level at each site. Parasitoid species composition at each site was estimated based on the number of PCR reactions that generated species-specific products for *P. digoneutis*, *P. relictus*, and *P. pallipes*. This was expressed as the proportion of each parasitoid species in the total number of parasitoids detected. The proportion of multiparasitism (evidenced by PCR reactions that showed positive results for two species simultaneously) in parasitized nymphs was also estimated.

5.2.3 Statistical Analysis

To test the null hypothesis that parasitism levels in *L. rugulipennis* do not differ between red clover and chamomile or between red clover and alfalfa, independent t-tests (two-tailed; $P = 0.05$) were used.

Similarly, to test the null hypothesis that parasitoid species composition does not differ between red clover and chamomile or between red clover and alfalfa, a Mann-Whitney U-test (two-tailed; $P = 0.05$) was used to compare the proportion of a given parasitoid species from each host plant investigated (for example, the proportion of *P. digoneutis* detected in *L. rugulipennis* collected in red clover was compared to the proportion of this species detected in *L. rugulipennis* collected in chamomile).

Parasitism level and parasitoid species composition were not compared between the NTA and WEBF ecoregions, as they are discussed in Chapter 4.

5.3 Results

The mean parasitism level in *L. rugulipennis* collected in red clover and chamomile was 49.6±7.5(SE) and 45.2±8.1(SE) in the NTA Ecoregion. In *L. rugulipennis* collected in red clover and alfalfa in the WEBF Ecoregion, the mean parasitism level was 31.8±4.2(SE) and 24.8±5.0(SE), respectively. Parasitism levels in *L. rugulipennis* did not differ significantly in the red clover and chamomile sites surveyed in the NTA Ecoregion ($t = -0.40$; $p = 0.70$), nor did they differ significantly in the red clover and alfalfa habitats surveyed in the WEBF Ecoregion ($t = 1.08$; $p = 0.30$).

In the samples collected and analyzed from the NTA Ecoregion, the species composition and the proportion of multiparasitism was not significantly different in the red clover and chamomile fields investigated (*P. digoneutis*: $z = 0.10$, $p = 0.91$; *P.*

relictus: z = 0.31, p = 0.75; *P. pallipes*: z = -0.52, p = 0.60; multiparasitism: z = -0.31, p = 0.75). In the host plants surveyed, *P. digoneutis* and *P. relictus* were the dominant parasitoid species associated with *L. rugulipennis* (approximately 65% and 30%, respectively), whereas *P. pallipes* was virtually absent (Figure 5.1). Multiparasitism was low (4% – 5%) in both habitats.

Similarly, there was no significant difference in parasitoid species composition or multiparasitism of *L. rugulipennis* collected from red clover and alfalfa in the WEBF Ecoregion (Figure 5.2). This was true for *P. digoneutis* (z = 0.57, p = 0.57), *P. relictus* (z = -0.26, p = 0.79), *P. pallipes* (z = 0.38, p = 0.71), and multiparasitized individuals (z = -1.09, p = 0.27). In the WEBF Ecoregion *P. digoneutis* was the dominant parasitoid species associated with *L. rugulipennis* (90.0% and 91.7% of the parasitoid community in red clover and alfalfa, respectively). Although *P. relictus* was present, only 9.0% and 6.7% of the parasitoid species composition in red clover and alfalfa were attributed to this species. In the *Lygus* populations sampled, *P. pallipes* was virtually absent (0.7% and 0% of the species composition in red clover and alfalfa), as was multiparasitism (0.4% and 1.6% of parasitized individuals in red clover and alfalfa, respectively).

5.4 Discussion

Trophic interactions between parasitoids, herbivorous insects and their host plants have become a key focus of studies in insect community ecology (Hawkins & Sheehan, 1994). Price et al. (1980) suggested that several host plant characteristics can influence parasitism level and species richness of a parasitoid community. One of these, the production of floral nutrients, may influence parasitism of *L. lineolaris* by *P. pseudopallipes* on certain host plants (Shahjahan & Streams, 1973; Price et al., 1980).

Streams et al. (1968) collected *Lygus lineolaris* on *Oenothera biennis* L. (Onagraceae), *Daucus carota* L. (Apiaceae), *Amaranthus retroflexus* L. (Amaranthanaceae), *Chenopodium album* L. (Chenopodiaceae), *Polygonum*

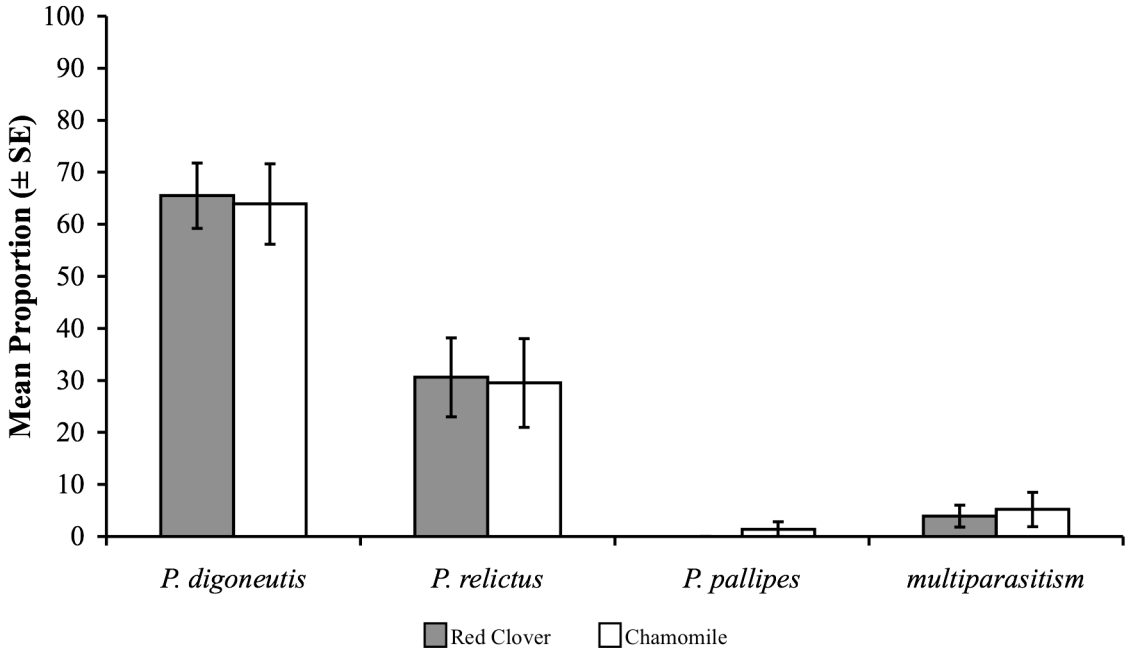

Figure 5.1 Mean proportions of *P. digoneutis*, *P. relictus*, *P. pallipes*, and multiparasitized individuals in the parasitoid community of *L. rugulipennis* in red clover and chamomile in the NTA Ecoregion.

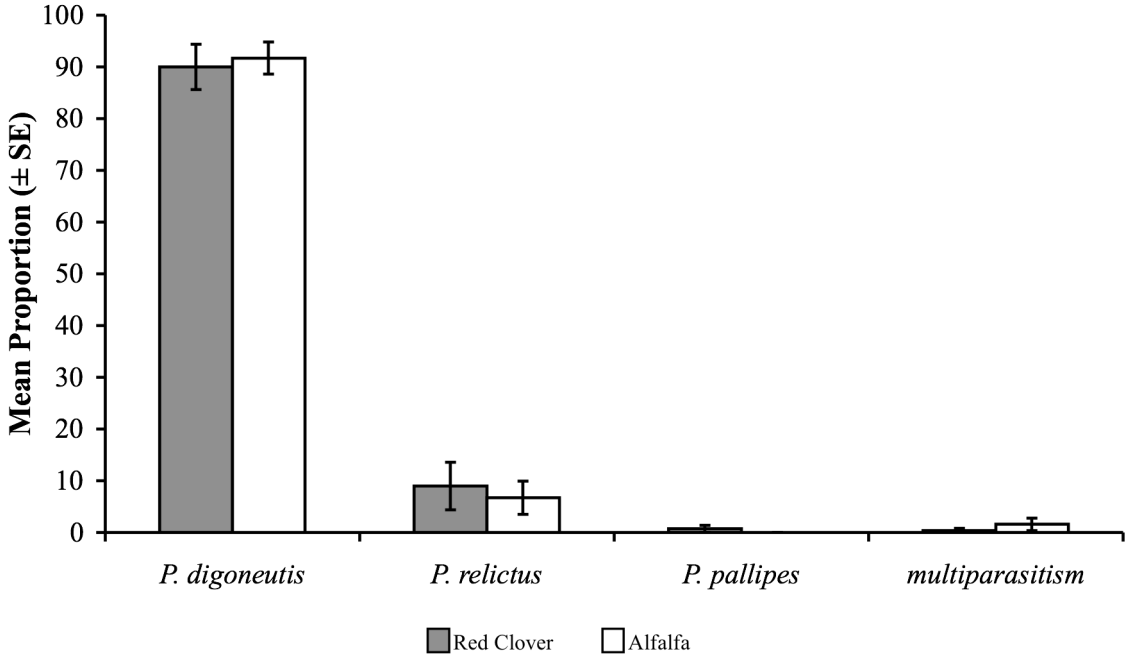

Figure 5.2 Mean proportions of *P. digoneutis*, *P. relictus*, *P. pallipes*, and multiparasitized individuals in the parasitoid community of *L. rugulipennis* in red clover and alfalfa in the WEBF Ecoregion.

pennsylvanicum L. (Polygonaceae), *Medicago sativa*, *Trifolium pratense*, *Erigeron* spp., *Solidago* spp. (Asteraceae) and *Verbena* spp. (Verbenaceae). On most of these host plants, the level of parasitism of *L. lineolaris* nymphs by *P. pseudopallipes* was very low. However, parasitism levels were considerably higher (30% to 60%) when this species was collected on *Erigeron* (Streams et al., 1968).

In the current study, there were no differences in the parasitism level or parasitoid community that attack *L. rugulipennis* in alfalfa, red clover, or chamomile. This lack of significant difference may indicate that European *P. digoneutis, P. relictus,* and *P. pallipes* do not use plant volatiles to locate host and/or food resources. However, olfactometer experiments showed that *P. relictus* responds to volatiles from *L. lineolaris* nymphs feeding on green bean (Condit & Cate, 1982). This suggests that *P. relictus* can detect and respond to volatiles from infested plants, and it is likely that *P. relictus* (and possibly *P. digoneutis*) use these volatiles as long-range cues for host habitat location (Condit & Cate, 1982). If *P. relictus* and *P. digoneutis* respond to volatiles from infested host plants, then parasitism levels in *Lygus* spp. would not necessarily differ based on host plant, but instead may vary with host density. Although this may explain why no significant differences were observed in the current study, further research would be required to confirm this.

Interestingly, the present study showed that *P. digoneutis* and *P. relictus* parasitized *Lygus* nymphs on red clover and alfalfa at a fairly high level. In contrast, native *P. pseudopallipes* rarely parasitized *Lygus* nymphs collected on red clover and alfalfa in North America (Streams et al., 1968). This suggests that European *Peristenus* may be better adapted to search for *Lygus* on these host plants, which would be advantageous for classical biological control programmes in these crops.

In the current study, some of the plant species investigated were closely related. Red clover and alfalfa belong to the same family (Fabaceae) and may be similar in some of the plant characteristics that Price et al. (1980) suggest influence parasitism and parasitoid species composition (e.g., secreted attractants, structural refuges, plant toxins). Future studies should compare parasitism level and parasitoid species composition in *L. rugulipennis* collected from other host plants to determine whether differences occur. In addition, the number of sites sampled in the NTA Ecoregion was

limited (5 sites per host species). It would be prudent to sample a larger number of sites where *L. rugulipennis* occurs on these host plants to conclusively determine whether the parasitoid community in *L. rugulipennis* differs in red clover and chamomile.

European parasitoids for *Lygus* may be released in Canada to reduce pest populations in several economically important crops, including canola. In the Canadian prairies, Braun et al. (2001) noted that parasitism of *Lygus* spp. by native *P. pallipes* was almost insignificant in canola and mustard fields. Therefore, it may be valuable to compare parasitism levels and parasitoid communities in *Lygus* spp. in brassica crops with those in red clover or alfalfa in Europe. The evaluation of parasitism in canola and mustard in Europe may provide insight into whether or not European *Peristenus* attack *Lygus* in these crops. To complement field studies, olfactometer assays could be used to assess parasitoid response to brassica volatiles (i.e., attractive or repellent). Such studies would allow selection of the most appropriate parasitoid species or population (if any) adapted to *Lygus* plant bugs feeding on brassica crops.

In a classical biological control programme for *Lygus*, the availability of host plants that are attractive to *P. digoneutis* and *P. relictus* would be crucial to maintain high parasitoid densities in target crops. In cases where the target crop is not attractive to *Peristenus*, it may be beneficial to intercrop with preferred host plants (such as red clover or alfalfa). This would attract and retain parasitoids in the crop habitat as both food and host resources would be available. However, this may increase the density of *Lygus* plant bugs in the crop habitat, and would not be practical in large-scale fields (such as those seeded to canola in the Canadian prairies).

In-depth knowledge of tritrophic interactions may improve the design of effective biological control strategies (Lewis et al., 1998). In the current context, further investigation of tritrophic interactions between *Lygus* plant bugs, their host plants and parasitoids both in Europe and North America could enhance classical biological control programmes for this pest. Although this study attempted to address the effect of host plant on parasitism level and parasitoid species composition, further investigation is needed to clarify interactions between European *Peristenus* spp. and their host plants.

6. ESTABLISHMENT OF EUROPEAN *PERISTENUS DIGONEUTIS* LOAN IN MIRID POPULATIONS IN SOUTHERN ONTARIO

6.1 Introduction

Lygus lineolaris Palisot de Beauvois (Hemiptera: Miridae) is parasitized by four native parasitoids (Hymenoptera: Braconidae) in eastern Canada (Loan, 1970; Lim & Stewart, 1976; Broadbent et al., 1999; Broadbent et al., 2006; Goulet & Mason, 2006). Two species in the *Peristenus pallipes* Curtis complex, *P. mellipes* Cresson and *P. pseudopallipes* Loan, are univoltine and attack *Lygus* nymphs in the spring and summer, respectively (Lim & Stewart, 1976; Goulet & Mason, 2006). *Leiophron lygivorus* Loan and *L. uniformis* Gahan are multivoltine, but are found primarily in late summer (Broadbent et al., 2006; Goulet & Mason, 2006). However, these species do not suppress *Lygus* plant bug populations in economically important agricultural crops in Canada. As discussed in the previous chapter, this may be due to the fact that these parasitoids prefer habitats that contain a mixture of flowering weeds rather than agricultural crops (Streams et al., 1968; Shahjahan, 1974; Lim & Stewart, 1976; Loan, 1980; Broadbent et al., 2006). The introduction of multivoltine, European *Peristenus* species into Canada is being considered as part of a classical biological control programme for *Lygus* plant bugs. European *Peristenus* species are likely adapted to crop habitats such as red clover and alfalfa (see Chapter 5).

European *P. digoneutis* Loan and *P. relictus* Ruthe were released in New Jersey and Delaware from 1972 to 1980 to suppress populations of *L. lineolaris* in alfalfa (Coulson, 1987). Few *P. relictus* specimens were recovered in subsequent field collections, and it is thought that they failed to establish. However, these releases (and additional releases in the 1980's) led to the establishment of *P. digoneutis* in New Jersey, and subsequent disperal into seven additional states (New York, Vermont,

Maine, New Hampshire, Pennsylvania, Connecticut, Massachusetts) (Day, 1987; Day et al., 1998; Day et al., 2000). Based on its dispersal pattern in the USA, Day et al. (2000) predicted that *P. digoneutis* was likely to spread into southern Ontario.

Peristenus digoneutis has not yet been reared from *Lygus* collected in southern Ontario (Broadbent et al., 1999; Broadbent et al., 2006). However, in 1998 *P. digoneutis* was recorded for the first time in *Lygus* samples collected in Québec (Broadbent et al., 1999), where it likely spread from the northeastern USA. Since this time, *Lygus* populations in Ontario have been monitored for the presence of *P. digoneutis*.

As discussed in Chapter 3, rearing and dissection are generally used to identify parasitoids in mirid populations. However, dissection does not allow identification to the species level due to morphological similarity of larval stages. In addition, rearing can result in high levels of parasitoid mortality, which precludes the identification of some reared specimens. Thus, when a parasitoid is rare in a host population, as may be the case with recently dispersed or introduced parasitoids, conventional methods may not detect its presence. Molecular methods may be better suited to detect the presence of newly introduced or dispersed parasitoids, as PCR can provide more complete information on the parasitoid community associated with different host species (see Chapter 3).

In New Jersey, molecular methods have been used to evaluate the impact of *P. digoneutis* on *Lygus* populations in alfalfa, and to document the dispersal of this species into additional crop habitats (Tilmon et al., 2000; Tilmon & Hoffmann, 2003). However, molecular methods were not used in earlier phases of introduction to assess establishment of European *Peristenus* in North America. In fact, there is only one example in which molecular techniques were used to identify and assess the establishment of an exotic biological control agent: Prinsloo et al. (2002) used a PCR-based technique to confirm the establishment of *Aphelinus hordei* Kurdjumov (Hymenoptera: Chalcidoidea), a parasitoid of aphids, in the Free State province of South Africa. This method was favored over conventional methods because accurate identification of a parasitoid following its release can be difficult when a complex of congeners (native and exotic) occur in the area of introduction (Prinsloo et al., 2002). A similar situation exists in North America where native *Peristenus* spp. and *Leiophron*

spp. occur in the same host species and at the same times as exotic *P. digoneutis*. Thus, molecular methods may be useful to assess the establishment of this species in Canada.

Peristenus digoneutis has not been reared from field-collected mirids in Ontario; however, it is suspected that this species is established in *Lygus* spp. populations at low levels. In fact, individual adult specimens have been collected in sweep net samples in Ontario (Goulet & Mason, 2006). *Peristenus relictus* was released in New Jersey (Coulson, 1987), but this species has not been collected in sweep net samples or recorded from *Lygus* populations in eastern North America,.

In the present study, molecular methods were used to determine whether European *Peristenus* spp. could be detected among parasitoid larvae dissected from field-collected Miridae in southern Ontario. The confirmation of European *Peristenus* species in Canada may facilitate and expedite further introductions.

6.2 Materials and Methods

6.2.1 Parasitoid Samples for Molecular Analysis

A.B. Broadbent (Research Scientist, Agriculture and Agri-Food Canada, London, ON) provided parasitoid larvae that were dissected from mirid nymphs and adults (primarily *Lygus* spp. nymphs) collected in southern Ontario in 2004 and 2005 (Appendix C and D). The 2004 samples were collected between 15 June 2004 and 29 July 2004 near London, ON, and the 2005 samples were collected between 26 May 2005 and 9 September 2005 near London and St. Catherines, ON. Collections were made in alfalfa fields, mixed weeds and grasses.

The larvae supplied had been sorted by date of collection and field site, and were preserved in 95% ethanol. However, as all larvae dissected from hosts collected at a given site were combined in a single vial, information on the host species for each larva was not always available.

In 2004, 46 parasitoid larvae were obtained for identification. The majority of these larvae were dissected from *Lygus* spp. nymphs (40); however, 1 was dissected from a *Lygus* spp. adult, 2 from *Adelphocoris* spp., 2 from *Polymerus* spp., and 1 from an unidentified mirid nymph. In 2005, 177 parasitoid larvae were obtained for identification. Of these, 153 larvae were dissected from *Lygus* spp. nymphs, 6 from

Lygus spp. adults, 9 from *Leptopterna* spp. nymphs, 6 from *Trigonotylus* spp. nymphs, and 2 from *Adelphocoris* spp. nymphs.

6.2.2 Detection and Identification of Parasitoid DNA in Larval Samples from Southern Ontario

DNA was extracted and amplified from each parasitoid larva using the multiplex PCR assay described in Chapter 2. Multiplex PCR analysis allows the identification of two European species, *P. digoneutis* and *P. relictus*, and holarctic *P. pallipes* complex.

A second PCR reaction using the TL2-N-3014 and Peristenus Ia primers that target the COI gene (Tilmon et al., 2000) was carried out with those parasitoid DNA samples that did not produce a PCR product with the multiplex PCR assay. These COI primers were designed for *Peristenus* spp., but have also amplified DNA from several other Hymenoptera, including a variety of braconids and ichneumonids. However, Tilmon et al. (2000) clearly point out that the primers do not universally amplify species of Ichneumonoidea. Thus, to determine whether species of *Leiophron* are among the Ichneumonoidea that are not amplified with the COI primers developed by Tilmon et al. (2000), DNA from six voucher specimens was analyzed with the COI-targeted PCR protocol of Tilmon et al. (2000).

In addition, universal insect primers (inDNA 44 and inDNA 45) that target the rRNA ITS region were used to determine whether or not insect DNA was present and amplifiable for those samples that were negative with the multiplex and COI assays. The protocol described by Erlandson et al. (2003) was used to amplify DNA using these PCR primers.

6.3 Results

Of the 46 larvae collected in 2004, 29 were identified as members of the *P. pallipes* complex, and 17 produced no PCR product. *Peristenus digoneutis* and *P. relictus* were not identified from these samples.

Of the samples collected in 2005, 146 provided positive results with the multiplex PCR assay, and 31 had no visible PCR product. *Peristenus pallipes*-specific PCR products were obtained from 143 of the DNA samples, and *P. digoneutis*-specific

PCR products were obtained from 3 of the DNA samples (Figure 6.1). *Peristenus relictus* DNA was not detected from any of the larvae analyzed.

Peristenus digoneutis DNA was detected only from *Lygus* nymphs collected near St. Catherines, ON. This species was identified from samples collected in mid-June and originated from two sites in the same area. Larvae dissected from all mirids collected in London were identified as *P. pallipes* or were unidentified.

In samples from both years, unidentified specimens were generally from the same sites and collection dates, with the majority from collections in late summer (July – August).

When the 48 unidentified samples (17 from 2004 and 31 from 2005) were analyzed using PCR primers for the COI gene, no PCR product was generated. In addition, DNA from voucher specimens of *L. lygivorus* did not yield PCR products upon amplification with the COI primers developed by Tilmon et al. (2000).

Amplification of the unidentified specimens with universal primers for the ITS region yielded PCR products for 41 of the 48 unidentified samples.

6.4 Discussion

This study demonstrates for the first time that *P. digoneutis* is present in *Lygus* plant bug populations in southern Ontario; however, it is present in fairly low numbers. Some of the parasitoid larvae (48 of 223) were not identified using the multiplex PCR assay. It is possible that DNA extracted from these parasitoids was not of sufficient quality for PCR analysis, or that these individuals are parasitoids other than *P. digoneutis*, *P. relictus*, or *P. pallipes*. To address this, primers for the COI gene were used. These primers are known to amplify the DNA of several braconid and ichneumonid parasitoids, including seven *Peristenus* species and a hyperparasitoid of *Peristenus* (Tilmon et al., 2000). However, no PCR product was obtained from these unidentified samples when the COI primers were used. In contrast, the use of the universal insect primers described by Erlandson et al. (2003) yielded PCR products for

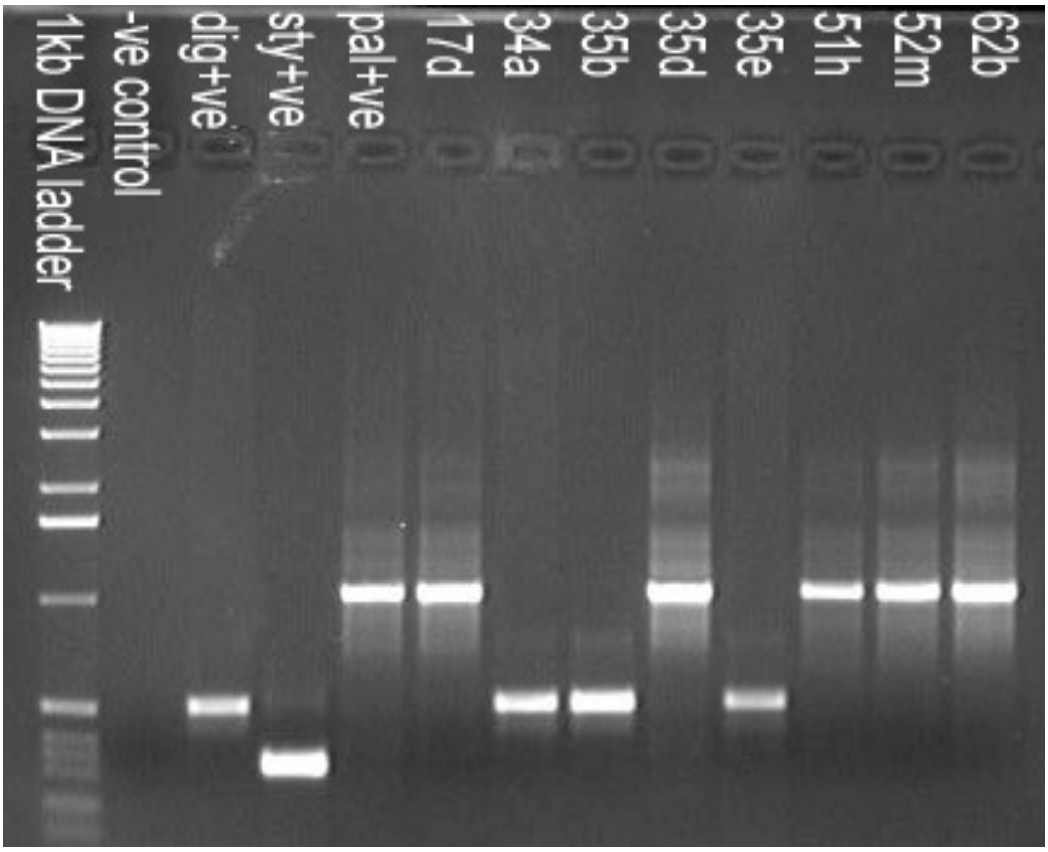

Figure 6.1 Multiplex PCR analysis of DNA extracted from selected parasitoid larvae. A negative control (-ve control; no DNA) and positive controls for *P. digoneutis* (dig+ve), *P. relictus* (sty+ve) and *P. pallipes* (pal+ve) are included to demonstrate that the PCR worked, and amplified representative species-specific fragments for the three species.

approximately 84% of the previously unidentified specimens. This indicates that in most samples the DNA was of sufficient quality for PCR analysis and suggests that additional parasitoid species were present.

Tilmon et al. (2000) stated that their COI primers do not work universally within the Ichneumonoidea. Species of *Leiophron* Nees (Hymenoptera: Braconidae) are associated with *Lygus* spp. in North America (Loan, 1970, 1980; Goulet & Mason, 2006), but it was not previously known whether the COI primers described by Tilmon et al. (2000) would amplify members of this genus. In the current study, PCR analysis of *L. lygivorus* voucher specimens demonstrated that the COI primers designed by Tilmon et al. (2000) do not amplify DNA from *L. lygivorus*, and likely do not amplify DNA from other *Leiophron* species either.

In southern Ontario, *L. lygivorus* was commonly reared from *L. lineolaris* collections from July to September (Broadbent et al., 2006). DNA from parasitoid larvae that failed to amplify with the multiplex and COI assays could be species of *Leiophron*. In most cases, the parasitoid DNA that failed to amplify originated from nymphs that were collected in mid-July through September. This corresponds with the time of occurrence of *L. lygivorus* in the field. DNA amplified using the universal ITS primers should be sequenced and compared to sequences from voucher specimens of *Leiophron lygivorus*, as this may confirm the identity of these larvae.

This demonstrates one of the pitfalls encountered in the use of species-specific PCR primers, as they do not identify new or unexpected parasitoid species. Thus, rearing of host material to obtain parasitoid adults for identification is invaluable in these types of studies. However, as an additional safeguard, parasitoid DNA that does not amplify with species-specific primers should be re-amplified with universal primers, and the PCR products sequenced to confirm the identity.

Here, molecular diagnostic assays to identify parasitoids in a host population were used in a different context than in the previous chapters. In this study, the multiplex PCR assay was used to identify parasitoid larvae that were dissected from field-collected mirids. Thus, specimens that were dissected and preserved several years prior to the release of European *Peristenus* could potentially be identified and the parasitoid species composition in different mirid hosts over time could be investigated. This would provide

baseline information on parasitism levels and parasitoid species composition prior to the arrival of an exotic species. Such analysis would facilitate post-release studies to evaluate the success of the biological control programme for *Lygus* plant bugs, detect non-target parasitism by *P. digoneutis*, and assess competitive interactions between native and exotic parasitoids of *Lygus*. Post-release studies are an important component of non-target risk assessment, as they can be used to evaluate whether pre-release studies were predictive of the realized host range of a natural enemy (Van Driesche & Hoddle, 1997; Louda et al., 2003). Further, retrospective analysis can be a powerful tool to refine or improve current risk assessment protocols (Barlow et al., 2004). The *Lygus*-*Peristenus* system would be an ideal model system to investigate post-release impacts of an exotic parasitoid. Pre-release data on non-target effects in the area of origin are available (Haye et al., 2005b; Haye et al., 2006a), and historical information in the area introduction would be accessible through DNA analysis of parasitoid larvae or nymphs collected and preserved prior to the arrival of exotic *Peristenus*.

Low parasitism of *Lygus* plant bugs in Ontario and Québec is likely due to the recent establishment of *P. digoneutis* in these areas, and a significant impact on *Lygus* plant bug populations may not occur for several years (Goulet & Mason, 2006). However, the confirmation of *P. digoneutis* in Canada will facilitate further introductions in Ontario and Québec to supplement the population that has dispersed from the USA. Ecological host-range studies have concluded that *P. digoneutis* has minimal impact on non-target mirids (Haye et al., 2005b; Chapter 4). Thus, *P. digoneutis* could be mass-reared and released in agroecosystems to augment already-established populations in Eastern Canada.

7. CONCLUSIONS AND FUTURE DIRECTIONS

Lygus Hahn (Hemiptera: Miridae) are pests of a broad range of crops throughout North America (Kelton, 1975; Young, 1986; Broadbent et al., 2002). North American *Peristenus* Förster and *Leiophron* Nees (Hymenoptera: Braconidae) are considered ineffective as biological control agents for *Lygus* plant bugs, as nymphs of the first generation are attacked, but subsequent *Lygus* generations are not significantly parasitized (Clancy & Pierce, 1966; Day, 1987; Braun et al., 2001; Broadbent et al., 2006). To increase overall parasitism of native *Lygus* spp., exotic *Peristenus* spp. were imported from Europe and released against *Lygus* spp. in the northeastern USA and California (Day et al., 1990; Pickett et al., 2005). Previous attempts to introduce European *Peristenus* spp. in Canada have failed. However, the success of European *Peristenus* spp. in the USA, coupled with ongoing problems with *Lygus* spp. in Canada and concerns regarding insecticide resistance in *Lygus* populations, have renewed interest in deliberate releases of European *Peristenus* spp. in Canada (Broadbent et al., 2002).

In this thesis, I describe my pre- and post-release studies on exotic parasitoids for biological control of native North American *Lygus* species. This research takes a unique approach in that molecular methods (as opposed to conventional rearing and dissection methods) were used to address some of the key ecological questions regarding host-parasitoid associations. These include: the evaluation of target and non-target parasitism by candidate biological control agents, the assessment of target parasitism on different host plants, and the confirmation of agent establishment in the area of introduction. The use of molecular diagnostics in pre- and post-release studies is a novel approach. This approach could be valuable in further ecological studies on the parasitoids investigated here, and could serve as a model for similar studies on other host-parasitoid systems.

The development of a specific and sensitive molecular diagnostic tool for *P. digoneutis* Loan, *P. relictus* Ruthe, and *P. pallipes* Curtis has facilitated the detection and identification of immature stages of these species within their mirid hosts (Chapter 2). This precludes the need for tedious and time-consuming rearing and dissection methods and can provide more complete information on parasitoid species composition in host populations. The single-step multiplex assay developed for *Peristenus* also improves upon previous molecular assays used to detect and identify these parasitoids in host material. The ability to screen DNA from individual mirid nymphs for the presence of three parasitoid species in a single step reduces processing time and costs considerably. This makes the use of molecular diagnostic tools in large-scale ecological studies more practical than processing individual samples multiple times with different PCR primer sets. It should be noted that molecular methods do not completely preclude the need for conventional rearing methods. The multiplex assay can detect *P. digoneutis*, *P. relictus*, and *P. pallipes* complex; however, new or unexpected species are not detected.

A large-scale comparative assessment of conventional and molecular methods validated the utility of the multiplex assay in the estimation of parasitism level and parasitoid species composition (Chapter 3). In general, parasitism levels estimated by rearing, dissection, and molecular analysis were not significantly different; however, molecular analysis may detect parasitism earlier and more efficiently than either of the two conventional methods. Further, molecular detection methods have the potential to provide information on parasitoid species composition that cannot be obtained with conventional methods. Hence, when applied in risk-assessment studies, molecular diagnostics may be better able to detect non-target parasitism by candidate biological control agents. For example, in the present study pupal mortality and hyperparasitism in reared samples precluded the identification of up to 66% of the parasitoids obtained from non-target hosts. The loss of this information in non-target studies is problematic, as it could lead to an underestimation of non-target parasitism if these unidentified individuals are the candidate biological control agents in question. In fact, parasitism of non-target mirids by *P. relictus* was underestimated by rearing. Although parasitism was still low, the proportion of *P. relictus* detected in two of the three non-target species was

significantly higher by molecular analysis than by rearing (Chapter 3). Despite the importance of identifying all parasitoids collected from a non-target host, the impact of pupal mortality on accurate estimation of non-target parasitism is largely neglected in ecological host-range studies. This is perhaps due to the fact that methods that could account for the loss of species-composition information were previously unavailable. The demonstrated utility of molecular methods in ecological host-range studies on *Peristenus* spp. shows that there is room for improvement in protocols used to assess potential non-target risk. Further, it provides an alternative that may increase the standard for future risk-assessment studies.

With the knowledge that the multiplex assay could provide comparable or superior information to conventional rearing methods, the assay was applied in risk-assessment studies in two ecoregions in Europe (Chapter 4). Although a limited number of non-target species were included in this study, the molecular data compared favorably with published data on the host ranges of *P. digoneutis* and *P. relictus* (Haye et al., 2005b; Haye et al., 2006a) and suggested that non-target parasitism was generally low. In addition, several mirid species were recorded as hosts for *P. digoneutis* and *P. relictus* for the first time. This is likely due to the fact that the current host-range studies were extended over two geographically distinct ecoregions, one of which had not been surveyed for non-target parasitism by *P. digoneutis* and *P. relictus*. This confirms the utility of an ecoregion approach to non-target risk-assessment studies and should promote the inclusion of multiple ecoregions in ecological host-range studies in the future. The use of molecular diagnostics should facilitate the analysis of samples collected from broad geographic areas because it precludes the need to transport and rear live specimens (samples can be promptly preserved in ethanol, then transported and processed when convenient).

Surveys in different ecoregions may also be useful to select parasitoid biotypes that are best suited for release against the target pest in the proposed area of introduction. In the current study, parasitism of *L. rugulipennis* Poppius by *P. relictus* was almost insignificant in the WEBF Ecoregion (Chapter 4). Thus, biotypes from this region would likely not be promising biological control agents for *Lygus* spp. in North America. Previous attempts to introduce European *P. digoneutis* and *P. relictus* into

Saskatchewan between 1978 and 1981 were unsuccessful (Craig & Loan, 1987). This may have been due to the selection of parasitoid biotypes that were not adapted to the climate and failed to successfully overwinter. The introduced parasitoids originated from *L. rugulipennis* collected in Austria (part of the WEBF Ecoregion of the Temperate Broadleaf and Mixed Forests Biome); however, the release area (near Saskatoon, SK) is within the Temperate Grasslands, Savannas and Shrubland Biome. If future releases are planned for the Canadian prairies, parasitoids should be collected from analogous ecoregions in the same biome in Europe. Potentially, surveys for *Lygus* parasitoids in Ukraine and western Russia may yield biotypes that are better adapted the Canadian prairies.

Tritrophic interactions may be an important factor in the successful establishment of *P. digoneutis* and *P. relictus* in North America. Tritrophic interactions between *P. pseudopallipes* Loan, *L. lineolaris* Palisot de Beauvois, and *Erigeron* spp. are known to occur (Streams et al., 1968; Shahjahan & Streams, 1973; Shahjahan, 1974; Price et al., 1980; Godfray, 1994). Further, olfactometer experiments suggested that *P. relictus* is attracted to volatiles from *Lygus*-infested plants (Condit & Cate, 1982). Coutinot & Hoelmer (1999) indicated that host-plant attraction and association among European *Lygus* parasitoids are poorly understood. Evaluation of parasitism level and/or parasitoid species composition in *Lygus* spp. associated with different host plants may help select appropriate crops in which to release these parasitoids. In addition, it may improve the design of management strategies for *Lygus* plant bugs because it can identify suitable plants that can be intercropped with the target crop to attract and retain parasitoids. In the areas investigated, parasitism levels in alfalfa and red clover were generally high (Chapter 5). As *P. digoneutis* has reduced *Lygus* spp. populations in alfalfa in the USA (Day, 1996) and appears to be migrating into this crop in Québec (Broadbent et al., 1999), releases in red clover and/or alfalfa would appear to be the logical choice for initial releases in Canada. The current study merely touched upon the topic of *Peristenus* – *Lygus* – host-plant associations, and no significant differences were found in the host plants investigated. Further research is recommended to determine how *Peristenus* spp. locate their hosts, and whether they forage for *Lygus* on other economically important host plants (e.g., canola).

The confirmed establishment of *P. digoneutis* in southern Ontario (Chapter 6) and Québec (Broadbent et al., 1999) will facilitate further introductions in eastern Canada to supplement the population that has dispersed from the USA. Currently, *Lygus* plant bugs are not highly parasitized in Ontario and Québec; however, this is likely due to the recent establishment of *P. digoneutis* in these areas (Goulet & Mason, 2006). Ecological host-range studies have concluded that *P. digoneutis* has minimal impact on non-target mirids and should be approved for release (Haye et al., 2005b; Chapter 4). Thus, *P. digoneutis* could be mass-reared and released in agroecosystems to augment already-established populations in eastern Canada. Protocols and facilities for mass-rearing of *P. digoneutis* in *L. lineolaris* have already been established in London, ON (Whistlecraft et al., 2000), and releases are planned for Ontario in 2007 (T. Haye, personal communication)[2].

Several obstacles may prevent the release of European *Peristenus* in western Canada. In British Columbia, concern has been expressed regarding the potential dispersal of European *Peristenus* to high-elevation areas. Holarctic *L. rugulipennis* occurs only in mountain meadows in British Columbia and is not considered a pest (Maw et al., 2000). Preliminary surveys in Switzerland showed that *P. digoneutis* is the dominant parasitoid species in *L. rugulipennis* collected from high-elevation areas (Gariepy, unpublished). Thus, non-target parasitism of *L. rugulipennis* by *P. digoneutis* in North America would constitute a threat to the biodiversity in sensitive, high-elevation areas (D. Gillespie, personal communication)[3]. In Saskatchewan, previous attempts to establish *P. digoneutis* and *P. relictus* have failed. Studies on the cold-hardiness of European *Peristenus* concluded that severe overwintering mortality of *P. digoneutis* in the Canadian prairies is likely (particularly in central Saskatchewan) due to lengthy periods of continuous sub-zero soil temperatures (Haye, 2004). However, *P. relictus* is more resistant to low temperatures (Haye, 2004) and is recorded as far north as central Scandinavia, where *P. digoneutis* is absent (Rämert et al., 2005). Thus, *P. relictus* may be more likely to successfully overwinter in the Canadian prairies if biotypes are selected from areas with similar winter conditions. These areas may include

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Scandinavia (Coutinot & Hoelmer, 1999; Haye, 2004) or areas in Ukraine and Russia (as previously discussed with respect to ecoregion similarities).

Although relatively low non-target parasitism was detected in the current study, future attempts to introduce *P. relictus* are unlikely. Fundamental and ecological host-range studies suggested this species has the potential to use non-target host populations for reproduction and is more likely to cause non-target effects than *P. digoneutis* (Haye et al., 2006a). Further, there has been some controversy regarding the release of multiple natural enemies for the same insect pest. Some critics attribute poor agent establishment and/or impact to interspecific competition among introduced natural enemies (Ehler & Hall, 1982, 1984; De Moraes & Mescher, 2005; Batchelor et al., 2006; Muli et al., 2006). Myers et al. (1989) suggested that more agents are not necessarily better; in the majority of successful biological control programmes, a single agent was responsible for pest suppression even when several species were released. Although multiparasitism by *P. digoneutis* and *P. relictus* was relatively low (Chapter 3 and 4), it does occur and indicates the potential for competitive interactions between these two species. In addition, *P. relictus* females readily oviposit in hosts that have already been parasitized by conspecific (superparasitism) and congeneric parasitoids (multiparasitism) (Lachance et al., 2001; Haye, 2004; Haye et al., 2005a).

In addition to being used to confirm the establishment of a parasitoid in the area of introduction, molecular methods would facilitate post-release studies to evaluate the success of the biological control programme for *Lygus* plant bugs. These methods could also be used to detect non-target parasitism by *P. digoneutis* and assess competitive interactions between native and exotic parasitoids of *Lygus*. Post-release studies are an important component of non-target risk assessment, as they can be used to evaluate whether pre-release studies were predictive of the realized host range of a natural enemy (Van Driesche & Hoddle, 1997; Louda et al., 2003). Further, retrospective analysis can be a powerful tool to refine or improve current risk-assessment protocols (Barlow et al., 2004).

Several studies present post-release data on European *Peristenus* in the USA. The majority of these studies focus on confirmation of agent establishment and estimation of parasitism levels (Day et al., 1990; Day, 1996; Tilmon & Hoffmann,

2003). Two additional studies (Day, 1999; Day, 2005) addressed post-release non-target effects of *P. digoneutis*. However, these studies were limited in that they focused on only five non-target species that occur in alfalfa. Further, four of the five non-target species investigated were accidentally introduced into North America from Europe. The impact of exotic *Peristenus* on native North American mirids in various habitats has been neglected and is worthwhile revisiting with more thorough and objective post-release studies. The multiplex assay could be used in large-scale post-release studies to study parasitoid dispersal, estimate parasitism levels, investigate host-parasitoid associations, and estimate impacts on non-target species. This would be especially useful in the northeastern USA where *P. digoneutis* has been established for several years and non-target effects (if present) would likely be expressed. However, this tool would also be useful in Canada, where *P. digoneutis* has only recently become established.

Post-release studies could be extended to include the evaluation of potential indirect non-target effects. One of the central questions in biological control is whether interspecific competition between natural enemies with the same niche impacts the fitness of individuals at the population level (Myers et al., 1989; Heinz & Nelson, 1996; Perez-Lachaud et al., 2004; De Moraes & Mescher, 2005; Muli et al., 2006). Lachance et al. (2001) studied in-host competition between exotic and native *Lygus* parasitoids in laboratory experiments, and demonstrated that *P. digoneutis* and *P. relictus* were competitively superior to *P. pallipes*, *P. pseudopallipes*, and *L. lygivorus* Loan. Laboratory studies on competition between native and exotic parasitoids are not always predictive of the outcome in the field, and may be misleading as they do not take into account the influence of environmental factors (Urbaneja et al., 2003). Thus, it would be valuable to address interactions between native and introduced *Peristenus* under field conditions. Although the multiplex PCR assay amplifies DNA from *P. digoneutis* and native members of the *P. pallipes* complex, it may be useful to develop and incorporate PCR primers for species of *Leiophron.* This would increase the diagnostic utility of the assay for post-release studies in North America, as it would permit the detection of an additional native species. If this modified multiplex assay were applied to field-collected *Lygus* plant bugs, it may be possible investigate interspecific competition, multiparasitism, and displacement of native parasitoids. Extensive baseline data on

parasitism of *Lygus* spp. by native parasitoids in Canada is available (Broadbent et al., 1999; Braun et al., 2001; Broadbent et al., 2006). Further, historical information from the region of introduction may be accessible through DNA analysis of parasitoid larvae or nymphs collected and preserved prior to the arrival of exotic *Peristenus*. Thus, it would be possible to estimate the parasitoid species composition before and after introduction to monitor fluctuations in the abundance of native and introduced parasitoids. The clarification of trophic links between natural enemies may be crucial in the development and application of effective pest-control strategies and provides a better understanding of potentially disruptive interactions between biological control agents. Molecular techniques have not yet been used to characterise guild interactions within a community of natural enemies; however they could be a powerful tool to demonstrate the occurrence of parasitic interactions and may help detect indirect non-target effects (Messing et al., 2006).

Although pre- and post-release studies on *Peristenus* spp. are the primary focus here, another critical issue that needs to be addressed in the near future is the taxonomy of *Peristenus* spp. Recently, Goulet & Mason (2006) provided a taxonomic key for the *P. pallipes* complex associated with *Lygus* in North America. Members of the *P. pallipes* complex are known to attack many mirid species in North America (Loan, 1980) and Europe (Brindley, 1939; Waloff, 1967; Bilewicz-Pawinska, 1982; Haye, 2004). However, the *P. pallipes* complex associated with mirid species other than *Lygus* is undergoing taxonomic revision, and keys are not yet available. Currently, species are separated based on their ecological and biological attributes; however, DNA-based methods may facilitate the separation of species in the *P. pallipes* complex. Thus, DNA sequencing of the COI gene and ITS regions is recommended for members of this species complex. Although genetic variation among members may be slight, alignment and comparison of DNA sequences may reveal regions that are conserved within a species, but in which consistent nucleotide differences occur between species.

Taxonomic resolution of these species would be valuable, as it would clarify host-parasitoid associations in the Miridae. In a biological control context, this may be important in the identification of species-specific parasitoids that attack Miridae of economic importance in other countries (see Wheeler, 2000). For example,

Closterotomus norwegicus Gmelin (Hemiptera: Miridae) was accidentally introduced into New Zealand where it is now considered a major pest of alfalfa, lotus, and white clover seed crops (Eyles, 1999). A recent survey to obtain parasitoids of this host in Europe identified a member of the *P. pallipes* complex, *P. closterotomae* n. sp., that shows promise as a classical biological control agent for *C. norwegicus* in New Zealand (Haye et al., 2006b). *Stenotus binotatus* F. and *Megaloceraea recticornis* Geoffroy (Hemiptera: Miridae) are also of concern in New Zealand, but have not yet reached pest status (Eyles, 1999). Members of the *P. pallipes* complex are associated with these mirids in Europe (Haye, 2004). However, clarification of species boundaries is necessary before any of these species are given serious consideration as classical biological control agents.

The use of molecular diagnostic techniques in arthropod biological control programmes is in its infancy, and the application of these techniques to ecological studies on biological control agents is far from routine. However, the demonstrated utility of these techniques, coupled with the fact that they often provide information unavailable with conventional techniques, should lead to their increased adoption in the near future. My thesis presents some of the ways in which these techniques can be used to estimate non-target effects in pre-release studies, clarify host-parasitoid and host-plant-parasitoid associations, and confirm the establishment of a parasitoid following its release and dispersal. In the future, such research may serve as a basis to improve non-target risk-assessment studies. Further, molecular assays can provide an alternative to conventional methods used to assess parasitism and parasitoid species composition in a target or non-target host population. Molecular diagnostic techniques for parasitoid detection and identification may help address some of the key ecological questions that arise when an exotic natural enemy is imported into a new environment. Techniques that allow a better understanding of host-parasitoid associations as well as trophic and guild interactions among natural enemies may help improve the success and safety of classical biological control.

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APPENDIX

A. Parasitoids, predators and PCR: the use of diagnostic molecular markers in biological control of arthropods

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Abstract: The polymerase chain reaction (PCR) revolutionised the field of diagnostics, and today it has routine applications in medical, veterinary, forensic, and botanical sciences. The fields of biological control and insect pest management have generally been slow to adopt PCR-based diagnostics in comparison to other fields of science. However, there has been increasing interest in the use of molecular diagnostic tools in arthropod biological control. In applied entomology, molecular techniques have generally been used for insect identification and systematics; however, PCR-based techniques are increasingly becoming recognised as valuable tools in ecological studies. Here, we review research that has used PCR-based techniques for parasitoid and predator/prey identification and detection, and place these studies in the context of their contributions to biological control of arthropods. The status and future directions of diagnostic molecular markers in applied entomology and insect pest management are also discussed.

Keywords: biological control, parasitoids, predators, DNA, PCR, microsatellites

1 Introduction

The field of biological diagnostics was revolutionised with the advent of the polymerase chain reaction (PCR) in the mid-1980s (Saiki et al. 1985; Mullis et al. 1986; Saiki et al. 1988). The ability to amplify numerous copies of a gene or genomic region of interest opened up a world of possibilities in terms of, for example, the identification of organisms, genes, genotypes, mutations, and populations. Now one of the fundamental tools in molecular biology, PCR has diagnostic applications in the food industry as well as in forensic, medical, veterinary, and botanical sciences (Rossiter and Caskey 1993; Newton and Graham 1997).

In entomology, DNA-based techniques have proven useful in many areas of research, particularly in the study of taxonomic and phylogenetic relationships (reviewed by Caterino et al. 2000) and population genetics (see Behura 2006). However, PCR-based techniques are also being used in an insect pest management context to identify and distinguish between different insect vectors of disease (Phuc et al. 2003), to

diagnose various insect pathogens (Lange et al. 2004; Valles et al. 2002), and to identify and detect insect pests of quarantine concern (Armstrong and Ball 2005). Molecular techniques have also been used to clarify phylogenetic relationships among parasitic Hymenoptera (Dowton and Austin 1994) and to facilitate ecological studies on parasitoids and predators used in classical, augmentative, and conservation biological control programmes (Symondson 2002; Greenstone 2006).

Accurate identification of natural enemies is the cornerstone of biological control, and lack of adequate techniques for identification can lead to failure of biological control programmes. In their guidelines for the importation and release of exotic invertebrate biological control agents, Bigler et al. (2005) listed taxonomy and characterisation, including molecular analysis, as essential information required prior to importation and release of a natural enemy. However, despite the importance of sound taxonomy in biological control, Menalled et al. (2004) indicated that 'tools for accurate parasitoid identification are not well developed'.

The need to accurately identify natural enemies, understand their population dynamics, and ensure minimal non-target impacts has spurred the development of molecular diagnostics tools. PCR using species-specific primers has the potential to facilitate ecological studies on natural enemies used in classical, augmentative, and conservation biological control programmes. Additionally, microsatellite analysis can be used to separate strains and evaluate genetic diversity of natural enemy populations. Beyond agent identification, molecular phylogeny may be able to predict natural enemy host range, climatic adaptability, and other important biological traits that can be used in the selection of efficient candidate biological control agents (Unruh and Woolley 1999). Menalled et al. (2004) suggested that new molecular technologies could lead to increased adoption and success of biological control agents, as they provide taxonomic accuracy, precise identification, and a more thorough understanding of population genetics and gene flow in natural enemies.

Recently, Greenstone (2006) and Stouthamer (2006) reviewed the molecular methods used in the assessment of insect parasitism, providing a thorough account of the proteins and DNA regions most useful in the detection and identification of parasitoids. The purpose of this review is to take a critical look at research that has used DNA-based techniques for studies on parasitoids and predators, and place these studies in the context of their contribution to biological control programmes. First, techniques and gene regions commonly used in molecular studies in entomology are described, followed by a review of molecular diagnostics in parasitoid and predator research.

2 Techniques and Gene Regions Used in Insect Molecular Diagnostics

Several gene regions have been used in insect molecular studies (Simon et al. 1994, Caterino et al. 2000, Greenstone 2006, and Stouthamer 2006). The two regions most often targeted for sequencing in insect systematics are mitochondrial DNA (mtDNA) and nuclear ribosomal DNA (rDNA). For mtDNA, the genes most commonly used include cytochrome oxidase I and II (COI, COII), and the 16S and 12S subunits of

rDNA. When sequencing nuclear rDNA, the 18S and 28S subunits of rRNA and the first and second internal transcribed spacer regions (ITS 1 and ITS 2) are commonly used. All these genetic elements have multiple copies per cell, which increases the target density for DNA analysis.

The molecular diagnostic approaches referred to in this review include: specific PCR, PCR followed by restriction endonuclease (REN) digestion and gel electrophoretic analysis of fragment length polymorphisms (PCR-RFLP), random amplified polymorphic DNA (RAPD-PCR), DNA barcoding, and microsatellite analysis. All these methods rely on PCR-based techniques that result in over a million-fold increase of the specific DNA region targeted, allowing visualisation of the product by gel electrophoresis. Factors that affect the specificity, sensitivity, and efficiency of PCR primers, as well as some of the advantages and disadvantages of specific genomic targets for primer design are discussed by Erlandson and Gariepy (2005).

As stated above, there are some variations on the PCR technique that depend on different primer design and DNA target strategies. The RAPD-PCR technique uses random primers (available commercially as kits) in a PCR reaction to amplify multiple regions of genomic DNA without prior knowledge of DNA sequences for the organism in question. The products of RAPD-PCR can be visualised by gel electrophoresis and the fragment patterns can be diagnostic for a given species. However, RAPD-PCR suffers from a lack of reproducibility and has seen limited use as a system for detection of biological control agents (Greenstone 2006).

DNA barcoding involves the sequencing of a particular fragment of DNA (generally the COI gene) as a way of identifying an organism. Comparison of the COI sequence for an unidentified specimen to DNA sequence databases of identified and characterised species may allow the identification and phylogenetic classification of the specimen. As universal primers are used in the initial PCR assay, barcoding can identify new or previously unknown species, cryptic species, or strains. However, as a single character (i.e. one portion of a gene) is used in the identification of a specimen, this technique may have limited phylogenetic resolution unless combined with morphological and/or ecological information (Moritz and Cicero 2004; Dasmahapatra and Mallet 2006).

Microsatellites refer to tandem repeats of short segments of nuclear DNA that can be used as molecular markers for genetic, kinship, and population studies. Their utility in such studies is due to the fact that they have a high mutation rate in comparison to other regions of DNA, and so are useful in detecting polymorphic loci. Microsatellites are also based on the use of PCR; however, PCR primers are developed that flank microsatellite repeats, allowing amplification and subsequent electrophoresis of fragments that form a characteristic DNA fingerprint of the individual being examined. For a thorough discussion of the features and drawbacks of microsatellite isolation, see Zane et al. (2002).

The more commonly used PCR strategy for organism identification and detection involves the use of species-specific PCR primer sets, although some challenges can be

encountered in their development (Erlandson and Gariepy 2005). In addition, PCR assays can be designed to incorporate primers for several species in a single reaction (multiplex PCR), which can provide a more rapid and cost-effective method for screening a single DNA sample for multiple targets. However, the design of multiple primer pairs that do not anneal to each other and amplify different fragment sizes for each target species can be complicated. This is particularly problematic for closely-related species, as lack of unique sequence may limit the regions amenable to primer design. Preferential amplification of one target over another, non-specific amplification, and/or loss of sensitivity in comparison to singleplex PCR can also be encountered. Optimization of the PCR conditions can alleviate such difficulties, but can be tedious and time-consuming for inexperienced users. Nonetheless, once a multiplex assay is developed and thoroughly tested for specificity and sensitivity, it can be a very efficient tool for use in ecological studies.

Regardless of whether singleplex or multiplex assays are being used, thorough primer-specificity testing is necessary to eliminate the possibility of false positives due to the cross-reactivity of primers, as discussed by Admassu et al. (2006). This is of particular concern when dealing with generalist predators that have access to a wide variety of prey items in the natural environment, and may require testing of an extensive list of non-target species. However, as parasitoids are more host-specific and rely on a complex suite of behavioural and chemical cues for host acceptance, there is usually a limited number of parasitoid species associated with a given host species. As such, it is generally acceptable to challenge the primers against closely-related parasitoid species and/or parasitoid species which are known to attack closely-related hosts. However, it must be noted that molecular assays that rely on species-specific PCR primers are only capable of detecting those particular species; new or previously unknown species or strains would not be detected or identified.

3 Molecular Diagnostics in Parasitoid Research

Traditional methods for identification of parasitoids and quantification of parasitism in a host population generally rely on rearing and dissection of host material. However, there has been increasing interest in the use of molecular methods to identify parasitoids and assess parasitism in host populations. Stouthamer et al. (1999) noted that the advantage of molecular identification over traditional morphological methods is that non-specialists are able to identify individual specimens quickly and cheaply. In fact, numerous studies have already used molecular methods for this purpose, as shown in Tables 1 - 2. These methods may provide data that are not easily obtained by rearing and dissection. In systems where host dissection provides no information on parasitoid species composition, molecular diagnostics using species-specific PCR primers for parasitoid species of interest could be helpful. Like dissection, they provide a rapid estimate of parasitism level, but also give the species composition data lacking in host dissection. Molecular markers for parasitoids are capable of detecting minute quantities of DNA. This enables the detection of parasitoid eggs within a host, which are often missed by dissection. Additionally, molecular techniques can provide information more rapidly than rearing, which is often delayed by lengthy diapause periods prior to

parasitoid emergence. Furthermore, in comparison to rearing, molecular analysis is not affected by host and parasitoid mortality; thus, a more accurate estimate of parasitism level may be possible.

However, detection of parasitoid DNA in a host does not necessarily indicate parasitoid survival, as host immune response may neutralize immature stages of the natural enemy. As such, overestimation of the parasitism level may occur when using molecular methods. In addition, molecular analysis is only capable of determining the presence or absence of parasitoid DNA. It is unable to identify the parasitoid stage or the presence of multiple parasitoids of the same species within a single host. For this reason, data obtained by traditional dissection and rearing methods should be used to supplement molecular data.

3.1 Hymenoptera: Chalcidoidea

Aphelinidae

Molecular identification using PCR-based assays has been frequently applied to aphid parasitoids in the Families Aphelinidae and Braconidae. Many molecular studies on aphid parasitoids investigated members of both families because they share common host species. As such, there may be some overlap between this section and the section on the braconid subfamily Aphidiinae.

The first molecular markers described for aphelinids were microsatellites in *Aphelinus abdominalis* Dalman (Vanlerberghe-Masutti and Chavigny 1997). Although this study reported only on the development of this tool, the authors suggested these microsatellites be used to separate sibling species and strains of biological control agents. When applied in this context, the microsatellites could be used to evaluate the impact of a given parasitoid strain on pest population dynamics after release (Vanlerberghe-Masutti and Chavigny 1997).

The lack of distinctive morphological characteristics for reliably distinguishing *Aphelinus* species has led to difficulties in evaluating the success of biological control programmes for the Russian wheat aphid *Diuraphis noxia* Kurdjumov (Homoptera: Aphididae) in North America (Zhu and Greenstone 1999). The authors designed five sets of PCR primers with specificity for each species and strain investigated (*A. albipodus* Hayat and Fatima, *A. varipes* Förster, and two strains of *A. asychis* Walker). Primer sensitivity tests indicated that 10^{-9} wasp equivalents could be detected in a PCR reaction.

Additional diagnostic assays with species-specific PCR primers have been used to detect the aphelinid *Aphelinus hordei* Kurdjumov and the braconid *Aphidius colemani* Viereck. Both species have been used as biological control agents for the Russian wheat aphid (Zhu et al. 2000). The PCR assay was less sensitive than that described by Zhu et al. (1999), amplifying 10^{-3} and 10^{-4} wasp equivalents from *A. hordei* and *A. colemani*, respectively.

Table 1. PCR-based assays developed for Hymenoptera: Chalcidoidea

Parasitoid	Region	Primary Application	Reference
APHELINIDAE			
Aphelinus abdominalis	MS*	Identification; discrimination	Vanlerberghe-Matussi and Chavigny, 1997
Aphelinus albipodus, A. asychis, A. varipes	ITS 2	Identification, post-release studies	Zhu and Greenstone 1999;
Aphelinus gossypii	18S	Identification; establishment	Weathersbee et al. 2004
Aphelinus hordei	ITS 2	Identification, post-release studies	Zhu et al. 2000; Prinsloo et al. 2002
Encarsia formosa, E. luteola, E. sophia	COI	Identification	Monti et al. 2005
ENCYRTIDAE			
Ageniaspis citricola,	RAPD	Identify cryptic species	Hoy et al. 2000
Ageniaspis spp.	ITS 2	Identify cryptic species	Alvarez and Hoy 2002
EULOPHIDAE			
Horismenus butcheri, H. missouriensis, H. depressus	MS*	Population genetics	Aebi et al. 2004
EURYTOMIDAE			
Eurytoma brunniventris	MS*	Population genetics	Hale et al. 2004
MYMARIDAE			
Anaphes iole	ITS 2	Identification, detection	Zhu and Williams 2002
PTEROMALIDAE			
Muscidifurax raptor, M. raptorellus, M. zaraptor	RAPD	Identification; investigate the origin of gregariousness	Antolin et al. 1996
Muscidifurax raptor, M. raptorellus, M. uniraptor, M, zaraptor	COI, II	Identification	Taylor et al. 1997
Muscidifurax raptor, M. raptorellus, M. uniraptor, M, zaraptor	ITS 1	Identification	Taylor and Szalanski 1999
Muscidifurax raptor, M. raptorellus, M. uniraptor, M. zaraptor	ITS 1, 2	Identification; assess parasitism	Ratcliffe et al. 2002
Nasonia vitripennis	ITS 1, 2	Identification; assess parasitism	Ratcliffe et al. 2002
Spalangia cameroni, S. endius, S. nigroaenea,	ITS 1, 2	Identification; assess parasitism	Ratcliffe et al. 2002
Trichomalopsis sarcophagae	ITS 1, 2	Identification; assess parasitism	Ratcliffe et al. 2002
Urolepis rufipes	ITS 1, 2	Identification; assess parasitism	Ratcliffe et al. 2002
TRICHOGRAMMATIDAE			
Trichogramma minutum, T. platneri	COII	Identification	Borghuis et al. 2004
Trichogramma brassicae T. chilonis, T. dendrolimi, T. ostrinae,	ITS 1	Identification	Chang et al. 2001; Sappal et al. 1995
Trichogramma australicum	ITS 2	Detection	Amornsak et al. 1998
Trichogramma acacioi, T. atopovirilia, T. bourarachae, T. brassicae, T. bruni, T. carverae, T. cordubensis, T. deion, T. dendrolimi, T. evanescens, T. funiculatum, T. galloi, T. lasallei, T. pintoi, T. pretiosum, T. rojasi, T. turkestanica	ITS 2	Identification	Silva et al. 1999; Stouthamer et al. 1999; Ciociola et al. 2001a, b; Chang et al. 2001; Li and Shen, 2001, 2002; Thomson et al. 2003

MS* = Microsatellite DNA

Table 2. PCR-based assays developed for Hymenoptera: Ichneumonoidea

Parasitoid	Region	Primary Application	Reference
BRACONIDAE			
Aphidius colemani	ITS 2	Identification, post-release studies	Zhu et al. 2000
Aphidius colemani, A. matricariae, A. picipes	MS	Identification	Roehrdanz et al. 1993
Aphidius ervi	MS	Investigation of founder effects	Hufbauer et al. 2004
Cotesia glomerata, C. rubecula	COI	Identification, assess parasitism	Traugott et al. 2006
Diaeretiella rapae	MS*	Identification	Roehrdanz et al. 1993
Diaeretiella rapae	MS*	Investigation of founder effects	Baker et al. 2003
Dolichogenidia homoeosomae	MS*	Population genetics	Douhovnikoff et al. 2006
Dolichogenidia tasmanica	18S	Assess host utilization	Suckling et al. 2001
Leiophron uniformis, L. argentinensis	ITS 2	Identification, assess parasitism	Zhu et al. 2004
Lipolexis scutellaris, Lysiphlebus testaceipes	18S	Identification; assess establishment	Weathersbee et al. 2004
Lipolexis scutellaris, Lysiphlebus testaceipes	ITS 2	Identification; assess establishment	Persad et al. 2004
Lysiphlebus testaceipes	16S	Identification; assess parasitism	Jones et al. 2005
Lysiphlebus testaceipes	MS*	Identification	Roehrdanz et al. 1993
Lysiphlebus testaceipes	MS*	Population genetics	Fauvergue et al. 2005
Peristenus conradi, P. digoneutis, P. pallipes	COI	Assess parasitism and dispersal	Tilmon et al. 2000
Peristenus conradi, P. digoneutis, P. howardi, P. pallipes	COI	Assess parasitism	Mowry and Barbour 2004
Peristenus digoneutis, P. pallipes, P. stygicus	ITS 1, 2	Identification, assess parasitism	Erlandson et al. 2003; Gariepy et al. 2005
Peristenus howardi, P. pallipes, P. pseudopallipes, P. stygicus	ITS 2	Identification, assess parasitism	Zhu et al. 2004
ICHNEUMONIDAE			
Diadegma semiclausum	COI	Identification; parasitism	Traugott et al. 2006
Diadegma spp.	ITS 2	Identification, discrimination	Wagener et al. 2004
Mesochorus curvulus	ITS 2	Detection	Ashfaq et al. 2005
Tranosema rostrale (based on polydna virus)	-	Assess multiparasitism	Cusson et al. 2002

*MS = Microsatellite DNA; - = information not available

Prinsloo et al. (2002) conducted similar research on *A. hordei* to determine establishment following its release in South Africa. The authors indicated that accurate identification of a parasitoid following its release is often difficult when a complex of congeners (native and imported) occur in the area of introduction. This is the case in South Africa where two additional *Aphelinus* species (*A. varipes* and *A. asychis*) parasitise *D. noxia* (Prinsloo et al. 2002). Difficulty in separating these species (particularly *A. hordei* and *A. varipes*) based on morphological characteristics delayed confirmation of establishment of *A. hordei*. Molecular methods were used to address release and recovery issues using PCR assays (PCR-RFLP) to distinguish between *A. hordei* and *A. varipes* in *D. noxia* in South Africa. By applying this assay to field-collected aphid mummies, Prinsloo et al. (2002) confirmed the establishment of *A. hordei* and determined that this parasitoid had dispersed over a wide area from the point of release.

Between these three studies, molecular techniques have been developed to identify six species and/or strains of parasitoids attacking the Russian wheat aphid. In a biological control context, these molecular tools could be used to detect the presence or absence of *Aphelinus* species in a host population, confirm establishment of introduced *Aphelinus* species, and evaluate the success of augmentative and classical biological control programmes against the Russian wheat aphid.

In greenhouse biological control, the major target pests are often whiteflies (*Trialeurodes vaporariorum* Westwood and *Bemisia tabaci* Gennadius; both Hemiptera: Aleyrodidae). Aphelinid wasps in the genus *Encarsia* Förster, in particular *E. formosa* Gahan, are important biological control agents in whitefly control worldwide (van Lenteren et al. 1997). Several studies have investigated the phylogeny of *Encarsia* and developed molecular methods to distinguish closely related species (Babcock and Heraty, 2000; Babcock et al. 2001; Manzari et al. 2002; Polaszek et al. 2004; Monti et al. 2005). Using PCR-RFLP, Monti et al. (2005) developed a method to separate *Encarsia* species that are difficult or impossible to distinguish morphologically, including *E. formosa* and *E. luteola* Howard, as well as two populations of *E. sophia* Girault and Dodd. Given the importance of *Encarsia* in greenhouse biological control of whiteflies, accurate identification is critical, particularly when screening field-collected specimens and conducting quality control measures in mass-rearing efforts.

Encyrtidae

To determine whether two colonies of *Ageniaspis citricola* Logvinovskaya that differ slightly in their behaviour and biology were cryptic species, Hoy et al. (2000) employed RAPD-PCR. Significant differences in RAPD profiles and actin sequences in the two populations suggested the existence of two separate, but cryptic species. Similarly, Alvarez and Hoy (2002) used ITS 2 sequences to distinguish two geographically distinct *Ageniaspis* Dahlbom populations, and indicated that the populations represent two cryptic species.

Eulophidae

Only one study has resulted in the development of molecular markers for eulophid parasitoids in a biological control context. Aebi et al. (2004) developed

microsatellite markers for three species of *Horismenus* Walker (*H. depressus* Gahan, *H. missouriensis* Ashmead, *H. butcheri* Hanson and Aebi) that attack pestiferous bruchid beetles (Coleoptera: Chrysomelidae) in bean crops (*Phaseolus* L.). The authors suggested these markers be used to investigate the effect of host-plant species on the population genetic structure of the parasitoid community that attack bruchid beetles.

Eurytomidae

Hale et al. (2004) developed microsatellite markers for *Eurytoma brunniventris* Ratzeburg, a generalist parasitoid of cynipid oak gall wasps (Hymenoptera: Cynipidae). Hale et al. (2004) reported only on the development of the markers, but suggested their value in analysis of parasitoid population genetic structure among host-gall species and among host-plant species.

Mymaridae

Mymarid egg parasitoids are among the smallest parasitoids found. Unlike trichogrammatids, mymarids have been the subject of only one molecular diagnostic study. Zhu and Williams (2002) developed PCR primers specific for *Anaphes iole* Girault, an egg parasitoid of *Lygus* Hahn (Hemiptera: Miridae) plant bugs. The assay reliably detected 10^{-5} wasp equivalents, and identified the parasitoid in host eggs as early as 48 hours post-parasitism. Because of the small size of *A. iole* and difficulty in finding adults in the field, this PCR assay was developed to facilitate studies on the impact of this parasitoid on *Lygus* populations. Its use could preclude the need for tedious and time-consuming rearing and dissection protocols, and provide more accurate parasitism data.

Pteromalidae

Species-specific molecular markers based on RAPD-PCR were used by Antolin et al. (1996) for species of *Muscidifurax* Girault and Sanders (*M. raptor* Girault and Sanders, *M. zaraptor* Kogan and Legner, and *M. raptorellus* Kogan and Legner), pupal parasitoids of Diptera that breed in animal waste. Although designed to study the origin of gregarious oviposition in different populations of *Muscidifurax*, these molecular markers would facilitate identification of specimens, particularly in view of their important role as biological control agents for coprobiont Diptera. Taylor et al. (1997) analyzed mitochondrial DNA variation of four *Muscidifurax* species (*M. raptor, M. zaraptor, M. raptorellus, M. uniraptor* Kogan and Legner). Although it was necessary to follow the initial PCR with an enzyme digest, the authors were able to separate three of the species. Additional restriction digests were necessary to distinguish between all four. Taylor and Szalanski (1999) conducted a similar analysis on the same four species using a different gene region (Table 1). In this case, PCR followed by a single restriction enzyme digest provided diagnostic fragment patterns for all four *Muscidifurax* species.

Ratcliffe et al. (2002) investigated parasitism of house flies (*Musca domestica* L.) and stable flies (*Stomoxys calcitrans* L.) by 5 genera and 10 species of pteromalid (*Muscidifurax raptor*, *M. uniraptor*, *M. zaraptor*, *M. raptorellus*, *Spalangia nigroaenea* Curtis, *S. cameroni* Perkins, *S. endius* Walker, *Urolepis rufipes* Ashmead, and *Trichomalopsis sarcophagae* Gahan, *Nasonia vitripennis* Walker). Parasitoid-specific PCR primers were developed that would amplify all parasitoid species mentioned above

without amplifying DNA from the host species. Ratcliffe et al. (2002) tested the sensitivity of the assay, and reported that parasitoids could be detected in their muscid hosts within 24 hours post-parasitism. PCR products were digested with RENs to identify parasitoids to the species level. Although the selected RENs failed to produce unique banding patterns for all species, one enzyme (*TaqI*) allowed identification of 3 of the 10 parasitoids to the species level, and one to the genus level. This demonstrates the difficulties in separating numerous species using REN digestion, as the generation of unique fragment patterns is not always possible due to limited sequence variation.

Trichogrammatidae

Species of *Trichogramma* Westwood have been prime candidates for molecular diagnostics, mainly due to their small size, morphological similarity, and important role in biological control of lepidopteran pests. The lack of morphological distinctiveness among species of *Trichogramma* is an obstacle in understanding the effects of these parasitoids on pest populations (Orrego and Agudelo-Silva 1993).

Orrego and Agudelo-Silva (1993) examined ITS 1 sequence and length differences in *Trichogramma* spp. to distinguish adults from three different sources. The results indicated that amplification and sequencing of this region could be valuable to identify and monitor genetic variation in *Trichogramma* species. Several other studies focused on molecular phylogeny and identification (Vanlerberghe-Masutti 1994; Sappal et al. 1995; Silva et al. 1995; van Kan et al. 1996; Pinto et al. 1997; van Kan et al. 1997), but it was Amornsak et al. (1998) who first used species-specific primers to detect *Trichogramma* parasitoids within host eggs. They used PCR to discriminate between *Helicoverpa* Hardwick eggs that were parasitised by *T. australicum* Girault, and those that were unparasitised. Sensitivity testing of the PCR assay demonstrated that *T. australicum* eggs were detectable in host eggs in as little as 12 hours of parasitoid egg development. However, consistent detection required at least 24 hours (Amornsak et al., 1998).

Subsequent research examined the use of DNA sequences to distinguish sibling species of *Trichogramma*, and stressed the importance of accurate identification to ensure the correct species are being used in mass-rearing and release efforts (Stouthamer et al. 1999). Two *Trichogramma* complexes were studied, the *T. deion* Pinto and Oatman complex, consisting of four species, and the *T. pretiosum* Riley complex, consisting of two species. Species-specific primers were developed, and PCR or PCR followed by REN digestion allowed separation of all six *Trichogramma* species studied. Similarly, Silva et al. (1999) amplified DNA from five species of *Trichogramma* occurring in Portugal (*T. cordubensis* Vargas and Cabello, *T. turkestanica* Meyer, *T. evanescens* Westwood, *T. pintoi* Voegelé, and *T. bourarachae* Pintureau and Babault) using the same primer sequences as Stouthamer et al. (1999). The authors successfully distinguished these species using PCR or PCR followed by REN digestion.

Additional molecular studies (see Table 1) characterised differences between several *Trichogramma* species in Asia (Chang et al. 2001; Li and Shen 2001; Li and Shen 2002), Brazil (Ciociola et al. 2001a, b), south-eastern Australia (Thomson et al. 2003), and sibling species (*T. minutum* Riley and *T. platneri* Nagarkatti) in North

America (Borghuis et al. 2004). In the last 10 years, molecular diagnostic techniques have been investigated for the identification of at least 27 different *Trichogramma* species, far more than for any other parasitoid genus. In addition, many more studies have focused on the molecular systematics of the Trichogrammatidae (see Pinto 1999; Stouthamer 2006; Pinto and Stouthamer 1994).

3.2 Hymenoptera: Ichneumonoidea

Braconidae

Roehrdanz et al. (1993) used RAPD-PCR to distinguish between five species of braconid parasitoids that attack aphids (*Diaeretiella rapae* M'Intosh, *Aphidius matricariae* Haliday, *A. picipes* Nees, *A. colemani*, and *Lysiphlebus testaceipes* Cresson). In addition to being able to separate the species, this method also distinguished some of the strains from different geographic areas. When applied in the context of a biological control programme, this RAPD-PCR assay could be used to monitor the success of different parasitoid strains and species on pest suppression, as well as to assess the 'genetic purity' of laboratory-reared strains (Roehrdanz et al. 1993).

Microsatellite analysis has also been used to study founder effects and genetic bottlenecks that occur after the release of exotic parasitoids. Low genetic diversity in *D. rapae* in western Australia suggested that the introduced populations were likely derived from a small number of founders (Baker et al., 2003). Hufbauer et al. (2004) suggested that there was less genetic diversity in introduced populations of *Aphidius ervi* Haliday in North America than in native populations in Europe and that a mild genetic bottleneck likely occurred. Both studies discussed the fact that a reduced ability to respond to potential hosts and/or overcome host immune response is often related to the amount of genetic diversity in the parasitoid population. This emphasizes the need for detailed knowledge of the genetic variability of native and introduced parasitoid species and strains in biological control programmes, as it is likely linked to the ability of a parasitoid to suppress pest populations and adapt to changes in host population dynamics (Hopper et al. 1993).

The brown citrus aphid, *Toxoptera citricida* Kirkaldy (Homoptera: Aphididae) and associated parasitoids have been the subject of several molecular identification assays. One native and two exotic parasitoids have been recovered from this exotic pest in Florida. According to Weathersbee et al. (2004), difficulties are often encountered when parasitism levels are monitored by traditional rearing and dissection techniques, particularly when multiple parasitoid species occur. Species-specific primers were designed to detect and identify *Lysiphlebus testaceipes* Cresson, *Lipolexis scutellaris* Mackauer (both Hymenoptera: Braconidae) and *Aphelinus gossypii* Timberlake (Hymenoptera: Aphelinidae) (Weathersbee et al. 2004). Although this assay was able to detect up to 8% of the parasitoids after only 2 hours, 100% detection was only possible by 72 hours post-parasitsm. The authors stated that this technique identifies parasitoids earlier and more precisely than conventional rearing and dissection techniques and may be used to monitor parasitoid establishment and assess competition between native and exotic parasitoids.

Persad et al. (2004) also developed a PCR-based assay to separate two parasitoid species that attack the brown citrus aphid, *L. testaceipes* and *Lipolexis oregmae* Gahan (= *L. scutellaris*). Although primers amplified unique PCR fragments for each species, they were not used in multiplex and therefore multiple PCRs were required for conclusive identification of a given specimen. Using this assay, Persad et al. (2004) were able to detect parasitoid DNA within a host in 6 hours, but suggested that reliable detection (100%) was only possible 24 hours post-parasitism. Persad et al. (2004) also indicated that the PCR-based assay is an efficient method to measure the success of biological control agents released against the brown citrus aphid, and that it is a relatively inexpensive, sensitive and specific tool.

Jones et al. (2005) investigated parasitism by hymenopteran wasps (primarily *L. testaceipes*) in four different species of cereal aphids using a two-step identification process. In a first step, general primers that amplified DNA from different parasitoid species were used to identify hosts which contained DNA of these parasitoids. This was followed by a second step in which samples that were positive with the first set of primers were then screened with a set of *L. testaceipes*-specific primers. Screening of *L. testiceipes*-parasitized aphids at different intervals post-parasitism showed that parasitoid DNA was detectable as early as 48 hours post-parasitism. To provide additional information regarding the utility of the molecular assay, Jones et al. (2005) compared parasitism levels obtained by molecular analysis and dissections. Results showed that there was no significant difference between parasitism estimates using the two methods.

Using microsatellite DNA Fauvergue et al. (2005) developed markers for *L. testiceipes*. Although their study did not include the use of the markers, the authors suggested these would be useful for population studies, particularly because this parasitoid has a broad host range and geographic distribution.

Several molecular identification studies have been directed at the parasitoid community that attack *Lygus* plant bugs (Hemiptera: Miridae), in particular, the parasitoids found in *Lygus* nymphs in Europe and North America. Tilmon et al. (2000 developed molecular markers for *Peristenus pallipes* Curtis, *P. digoneutis* Loan, and *P. conradi* Marsh. The PCR assay was based on a two-step approach: PCR with primers that amplified DNA from all species studied, followed by REN analysis to reveal species-specific banding patterns. The authors showed that as little as 0.01% *P. digoneutis* DNA in 99.99% host DNA could be detected. This two-step assay was later used to assess parasitism by *Peristenus* spp. in strawberry to determine whether *P. digoneutis* had dispersed into new cropping habitats from the original releases in alfalfa (Tilmon and Hoffmann 2003). Mowry and Barbour (2004) used the same primers with a different restriction enzyme to distinguish the same three species as Tilmon et al. (2000) and an additional native species, *P. howardi* Shaw. Mowry and Barbour (2004) suggested that this molecular assay would facilitate ecological studies on *Lygus* and *P. howardi* in the Pacific Northwest region of the USA.

Erlandson et al. (2003) developed species-specific PCR primers for *P. digoneutis*, *P. stygicus* Loan (= *P. relictus* Ruthe), and *P. pallipes*, each amplifying

approximately the same fragment size. Ashfaq et al. (2004) applied this assay to estimate parasitism levels in *Lygus* species and compared results based on rearing, dissection and molecular analysis. The authors found that *P. pallipes* was the only parasitoid species present in the *Lygus* population sampled. In addition, molecular analysis provided higher estimates of parasitism than rearing or dissection. As a further step, Ashfaq et al. (2005) developed species-specific PCR primers to detect a hyperparasitoid (*Mesochorus curvulus* Thomson; Hymenoptera: Ichneumonidae) of *P. pallipes*.

Zhu et al. (2004) used PCR primers to distinguish four species of *Peristenus* (*P. pallipes*, *P. pseudopallipes*, *P. stygicus*, and *P. howardi*) and two species of *Leiophron* Nees (*L. argentinensis* Shaw and *L. uniformis* Gahan). The authors designed species-specific primers for *P. stygicus*, *P. howardi*, *L. argentinensis*, and *L. uniformis*. In addition, a series of single, short oligonucleotide primers were used to create banding patterns that could potentially be used to separate *P. howardi*, *P. pallipes*, and *P. pseudopallipes* from one another. In primer sensitivity tests, the species-specific PCR primers for *P. stygicus* and *L. uniformis* were capable of detecting 7.5×10^{-7} and 1.3×10^{-5} wasp equivalents.

Gariepy et al. (2005) developed species-specific PCR primers for *P. digoneutis*, *P. stygicus*, and *P. pallipes*, with each primer set amplifying a uniquely sized PCR fragment for each species. The differences in fragment size allowed the primers to be applied in multiplex PCR, and permitted multiple species to be identified in a single PCR reaction. In sensitivity tests, the multiplex assay was capable of detecting parasitoid DNA in *Lygus* nymphs at least 72 hours post-parasitism. A single-step multiplex PCR approach improves upon previous molecular assays for *Lygus* parasitoids as processing times and costs are substantially reduced. This provides a more efficient technique for screening a large number of mirid nymphs for the presence of multiple parasitoid species, and could facilitate and expedite pre- and post-release host-range studies (Gariepy et al. 2005).

DNA-based assays have also been used to study the effect of host plant and host insect on selection by braconids (Suckling et al., 2001). The authors analysed host selection by *Dolichogenidea tasmanica* Cameron using larvae of three leafroller (Lepidoptera: Tortricidae) species that feed on four species of host plant. Leafroller larvae were identified to species using PCR-RFLP, and the same DNA extract was then re-analysed using parasitoid-specific PCR primers to detect the presence or absence of a parasitoid in each larva. Suckling et al. (2001) applied this tool to determine if host-use by leafroller parasitoids is influenced by host species and host plant.

Douhovnikoff et al. (2006) developed microsatellite markers for *D. homoeosomae* Muesebeck, a parasitoid of the sunflower moth, *Homoeosoma electellum* Hulst (Lepidoptera: Pyralidae). Although the markers were developed with the intention of studying the genetic structure of the parasitoid population, their use in this context has not yet been reported.

Microctonus hyperodae derived from eight geographical populations in South America were released in New Zealand for the control of the Argentine stem weevil,

Listonotus bonariensis Kuschel (Coleoptera: Curculionidae). Winder et al. (2005) developed microsatellite markers that could separate the different geographical populations of *Microctonus hyperodae* Loan and suggested that they be applied to *M. hyperodae* specimens recaptured in New Zealand. When used in this context, microsatellite analysis would be able to identify the origins of the established parasitoids, and provide information regarding the impact of different parasitoid populations on the target pest (Winder et al. 2005). Such studies would allow subsequent release efforts to focus on those species or strains which are best suited for the climate and host species in the area of introduction.

Traugott et al. (2006) developed species-specific PCR primers, some of which could be used in multiplex PCR, to identify parasitoids that attack the lepidopteran pests, *Plutella xylostella* L. (Lepidoptera: Plutellidae), *Pieris brassicae* L. and *P. rapae* L (Lepidoptera: Pieridae). Primers were developed for two braconids, *Cotesia glomerata* and *C. rubecula*, and one ichneumonid, *Diadegma semiclausum* Hellén. The authors also developed PCR primers for *P. xylostella*, as immature stages of some lepidopteran cabbage pests are virtually indistinguishable from one another. Multiplexing these primers could enable identification of host and parasitoid species simultaneously. However, the primers for *P. xylostella* proved unspecific, amplifying the DNA of diamondback moth, as well as that of two parasitoid species (*C. glomerata* and *Microplitis mediator* Haliday) which commonly attack Lepidoptera in the same habitat. Traugott et al. (2006) determined the sensitivity of their assay using serial dilutions and demonstrated that as little as 1 pg and 11.8 pg of parasitoid DNA was detectable in singleplex PCR using primers for *C. glomerata* and *D. semiclausum*, respectively. Using PCR primers for *C. glomerata* and *C. rubecula* in multiplex, as little as 2.2 pg of parasitoid DNA was detectable for each species.

Ichneumonidae

Wagener et al. (2004) examined the potential of molecular techniques to distinguish between seven *Diadegma* Förster species that are known to attack the diamondback moth, *P. xylostella*. The authors noted that the taxonomic confusion surrounding *Diadegma* has resulted in the introduction of ineffective species as biological control agents. Further, despite the availability of identification keys, differentiation of *Diadegma* species can be time-consuming and requires taxonomic expertise. Alternative methods (specifically those that are rapid, accurate and inexpensive) would facilitate identification of *Diadegma* species and strains (Wagener et al. 2004). In this study, the authors used PCR with conserved 'universal' primers, followed by REN digestion to separate the seven species of interest and one unidentified species. In the process of screening 11 RENs, Wagener et al. (2004) identified one that produced diagnostic banding patterns for all species investigated. This method provides an economical, rapid and accurate means of identifying the important *Diadegma* species that attack the diamondback moth and can be used by biocontrol workers to assess post-release establishment (Wagener et al. 2004).

Cusson et al. (2002) used species-specific PCR primers (based on gene sequences of a polydna virus carried by the parasitoid) to detect *Tranosema rostrale* Brischke in the spruce budworm, *Choristoneura fumiferana* Clemens (Lepidoptera:

Tortricidae). This tool was used in combination with host dissection to examine multiparasitism and potential competition with another parasitoid, *Actia interrupta* Curran (Diptera: Tachinidae). The molecular tool was essential to confirm the presence of the ichneumonid, which is very difficult to detect by dissection. The study clearly demonstrated that an underestimation of parasitism level and multiparasitism was likely to occur when using dissection to detect *T. rostrale*. Accurate measurement of multiparasitism is important as it could provide information on parasitoid competition for a given host species. As interspecific competition for host resources can be detrimental to achieving biological control of a pest species (Heinz and Nelson 1996; Urbaneja et al. 2003; Batchelor et al. 2006; Muli et al. 2006), a better understanding of multiparasitism could provide insight into the level of interspecific competition in the field.

3.3 Diptera

Tachinidae

Despite the importance of some dipteran groups, notably Tachinidae, as parasitoids, molecular diagnostic tools have been used on only a handful of occasions.

To determine parasitism levels and geographic distribution of two tachinid parasitoids, *Lydella thompsoni* Herting and *Pseudoperichaeta nigrolineata* Walker, Agustí et al. (2005) developed species-specific PCR primers and applied them to field-collected European corn borer (*Ostrinia nubilalis* Hübner; Lepidoptera: Crambidae). With this assay, Agustí et al. (2005) demonstrated that there was no significant difference in parasitism of *O. nubilalis* collected from different geographical areas in France.

Smith et al. (2006) used DNA barcodes to investigate the host specificity of several species of the tachinid *Belvosia* Robineau-Desvoidy. *Belvosia* spp. are widely assumed to be generalist parasitoids. However, extensive host-rearing records and DNA barcoding (of both the COI and ITS1 regions) of approximately 20 morphospecies of *Belvosia* revealed that there are 32 species, many being cryptic species with a high degree of specificity for their lepidopteran host (Smith et al. 2006). The use of molecular markers to evaluate the host specificity of biological control agents is a novel approach which could be of value in future studies to ensure selection and release of agents that have minimal impact on non-target host species.

4 Molecular Diagnostics in Predator Research

A number of studies have addressed the potential of PCR-based techniques to identify predators and detect prey remains within a predator (Table 3). Symondson (2002) reviewed the use of molecular diagnostics for prey identification in predator diets, and described the techniques used to identify consumed prey. Symondson (2002) noted that although the most common technique is visual examination of gut contents, the process of digestion often leaves material unidentifiable and, because many insect predators are fluid feeders, no identifiable remains are present in the gut.

Table 3. Development of PCR-based techniques for use in predator-prey identification and ecological studies

Predator	Region	Primary Application	Reference
ARANEAE: ANYPHAENIDAE			
Hibana arunda, H. futilis	COI	Predator identification	Greenstone et al. 2005
ARANEAE: LINYPHIIDAE			
Frontinella communis, Grammonota texana	COI	Predator identification	Greenstone et al. 2005
Various lyniphiids	COI	Gut content analysis to detect collembola	Agustí et al. 2003b
ARANEAE: LYCOSIDAE			
Lycosa spp.	ITS 1	Gut content analysis to detect diamond back moth	Ma et al. 2005
Pardosa milvina, Rabidosa rabida	COI	Predator identification	Greenstone et al. 2005
ARANEAE: MITURGIDAE			
Cheiracanthium inclusum	COI	Predator identification	Greenstone et al. 2005
COLEOPTERA: CARABIDAE			
Poecilus versicolor	COI	Effect of scavenging on gut content analysis; detection of scarabaeid prey items	Juen and Traugott 2005; Juen and Traugott 2006
Pterostichus cupreus	α-esterase	Gut content analysis to detect digested mosquito over time	Zaidi et al. 1999
Pterostichus melanarius	COI, 12S	Detection of multiple prey species simultaneously	Harper et al. 2005
Pterostichus melanarius	COI	Impact of secondary predation on gut content analysis	Sheppard et al. 2005
COLEOPTERA: COCCINELLIDAE			
Coccinella septempunctata, Hippodamia convergens	COII	Gut content analysis to detect aphids	Chen et al. 2000
Coleomegilla maculata	ITS	Gut content analysis to detect European corn borer and frequency of predation	Hoogendoorn & Heimpel 2001
Curinus coeruleus	COI	Gut content analysis to detect geometrids; potential infiltration of exotic predators in native foodwebs	Sheppard et al. 2004
COLLEMBOLA: ISOTOMIDAE	COI	Gut content analysis to detect soil-dwelling nematodes	Read et al. 2006
HEMIPTERA: ANTHOCORIDAE			
Anthocoris tomentosus	COI	Gut content analysis to detect pear psylla	Agustí et al. 2003a
Orius sauteri, O. minutus, O. strigicollis, O. nagaii, O. tantillus	ITS 1	Predator identification	Hinomoto et al. 2004
HEMIPTERA: NABIDAE			
Nabis kinbergii	ITS1	Gut content analysis to detect diamond back moth	Ma et al. 2005
MESOSTIGMATA: LAELAPIDAE	COI	Gut content analysis to detect soil-dwelling nematodes	Read et al. 2006
NEUROPTERA: CHRYSOPIDAE			
Chrysoperla plorabunda	COII	Gut content analysis to detect aphids	Chen et al. 2000

Because of their high specificity and sensitivity, monoclonal antibodies have frequently been used to identify prey items in predator gut contents. However, their design can be highly time-consuming, complicated, and costly (Chen et al. 2000; Symondson 2002). According to Symondson (2002), PCR-based methods are likely to replace current techniques for assessing predation due to the efficacy and versatility of PCR.

4.1 Coleoptera

Carabidae

Using an artificial predator-prey combination, Zaidi et al. (1999) screened the gut contents of laboratory-fed carabid beetles (*Pterostichus cupreus* L.) for the remains of mosquito larvae (*Culex quinquefasciatus* Say; Diptera: Culicidae) using PCR with species-specific primers. This was the first report of a PCR-based technique for the detection of one invertebrate consumed by another. Zaidi et al. (1999) showed that semi-digested prey material could be detected for up to 28 hours post-consumption when the fragment being amplified is relatively small (less than 200 bp).

Harper et al. (2005) developed a multiplex PCR system to detect multiple prey items simultaneously using species-specific PCR primers for 14 potential prey species. Additional 'general' primers were designed for aphids, earthworms, and slugs. Laboratory and field experiments showed that multiple species could be detected simultaneously, which would allow conclusions to be drawn regarding predator consumption and preferences (Harper et al. 2005). However, the authors cautioned that prey items consumed directly cannot be distinguished from other items that were ingested as a result of secondary predation or scavenging. Nonetheless, the clear advantage to this system is the detection of multiple prey species simultaneously in the same predator. This is especially important in the analysis of field-collected material, as a predator may have consumed a number of different prey items prior to collection, and processing each predator sample individually for each potential prey species would be time-consuming and inefficient.

To address the concern that screening DNA from predator gut contents may detect secondary predation or scavenging, Sheppard et al. (2005) investigated the potential error that may result from such events using an aphid-spider-carabid model. DNA analysis of *P. melanarius* that ingested spiders that had previously eaten aphids suggested that secondary predation may be a source of error. However, as aphids were detectable in carabid gut contents only if recently consumed by the spider, this source of error is likely minimal (Sheppard et al. 2005). Similarly, Juen and Traugott (2005) studied the effect of scavenging by the carabid *Poecilus versicolor* Sturm on gut-content analysis using PCR-based assays. Their assays revealed that consumption of 9-day-old prey cadavers and 'fresh' prey items were equally detectable in the predator gut. However, Juen and Traugott (2005) suggested that this problem may be overcome by combining electrophoretic and DNA-based techniques. Despite the fact that secondary predation and scavenging may provide a source of error when quantifying prey consumption, the detection of such events may offer the opportunity to investigate intraguild predation and energy transfer between trophic levels (Sheppard et al. 2005).

Juen and Traugott (2006) developed a multiplex assay for a predatory carabid (*P. versicolor*) and a potential prey species, *Amphimallon solstitiale* L. (Coleoptera: Scarabaeidae). Extensive specificity tests against other soil-dwelling invertebrates demonstrated that the primers for *A. solstitiale* were highly specific, whereas the primers for *P. versicolor* amplified the DNA of two other carabid species. Nonetheless, the use of universal or predator-specific primers in a multiplex assay can serve as an internal positive control to confirm the DNA in each reaction is amplifiable, regardless of whether it is positive or negative for the prey species of interest. This provides an overall indication of DNA quality, as the presence of PCR inhibitors can result in false negatives. Juen and Traugott (2006) also showed that the addition of bovine serum albumin to the PCR reaction significantly reduced the number of false negatives.

Coccinellidae

Chen et al. (2000) used species-specific PCR-primers to detect remnants of six cereal aphid species in the gut contents of two lady beetle species, *Hippodamia convergens* Guerin-Meneville and *Coccinella septempunctata* L. (Coleoptera: Coccinellidae), and one lacewing, *Chrysoperla plorabunda* Fitch (Neuroptera: Chrysopidae). Their study examined the sensitivity and specificity of the primers for two of the six aphid species (*Rhopalosiphum maidis* Fitch and *R. padi* L.) to demonstrate the efficacy of their system.

Hoogendoorn and Heimpel (2001) also used PCR-based techniques to estimate the frequency of predation. The ability to detect remains of European corn borer in the gut contents of a coccinellid predator, *Coleomegilla maculata* DeGeer, was assessed over time using PCR primers that amplified different-sized fragments. Hoogendoorn and Heimpel (2001) could determine the approximate time of prey consumption based on the fragment sizes generated. This system could be applied to field-collected predators to estimate predation rate and to provide an indication of the minimum and maximum times since consumption (Hoogendoorn and Heimpel 2001). However, this approach is not always possible, as some studies reported no significant correlation between fragment length and detection period (Chen et al., 2000; Juen and Traugott, 2005; Juen and Traugott, 2006). This is likely influenced by the predator and/or prey group investigated, primer stability, and the fragment size being amplified (Harper et al., 2005; Juen & Traugott, 2006).

Sheppard et al. (2004) suggested the use of PCR-based techniques to investigate the infiltration of exotic predators into invertebrate food chains. Specifically, Sheppard et al. (2004) used PCR primers to screen the gut contents of an exotic biological control agent, *Curinus coeruleus* Mulsant, for the presence of two endemic Lepidoptera (Geometridae) of conservational concern (*Scotorythra rara* and *Eupithecia monticolens* Butler). Although this assay was only challenged in the laboratory, this was the first attempt to use molecular techniques in arthropod conservation ecology to evaluate non-target effects of introduced biological control agents.

4.2 Collembola

Species-specific PCR primers for two species of entomopathogenic nematode (*Heterorhabditis megidis* Poinar, Jackson and Klein and *Steinernema feltiae* Filipjev) and one species of slug-pathogenic nematode (*Phasmarhabditis hermaphrodita* Schneider) were developed by Read et al. (2006). The primers were used to screen the gut contents of collembolans and mesostigmatid mites to determine whether micro-arthropod predators are capable of reducing nematode densities. Laboratory feeding trials demonstrated that prey was detectable in collembolans for up to 24 hours, but the half-life for detection of nematode DNA was approximately 9 hours. Prey was detectable in mesostigmatid mites for up to 12 hours, with a half-life of approximately 5 hours. When used to screen micro-arthropods collected 12 hours after field-application of *H. megidis*, the PCR assay was capable of detecting *H. megidis* in two of three collembolan species and in unspecified mesostigmatid mites. The ability to screen predator gut contents for the presence of another natural enemy can be important, as it would allow the discovery and investigation of interactions between biological control agents.

4.3 Hemiptera

Anthocoridae

Several species of *Orius* Wolff are used to manage insect pests in greenhouse and field crops. As identification of *Orius* species can be difficult, Hinomoto et al. (2004) designed species-specific PCR primers to be used in multiplex to identify *O. sauteri* Poppius, *O. minutus* L., *O. strigicollis* Poppius, *O. nagaii* Yasunaga, and *O. tantillus* Motschulsky. The authors suggested that the primers could be used in the evaluation of *Orius* species as biological control agents.

Agustí et al. (2003b) used PCR with species-specific primers to detect pear psylla (*Cacopsylla pyricola* Förster; Hemiptera: Miridae) in the gut contents of *Anthocoris tomentosus* Pericart. The aim of the study was to develop a system that could be used to provide information on predator-prey dynamics and the ecological role of key psylla predators. Previous methods for studying such relationships provided only weak estimates or poor understanding of trophic relationships involving pear psylla.

Nabidae

Ma et al. (2005) developed species-specific PCR primers for the diamondback moth (*P. xylostella*) to detect predation by *Nabis kinbergii* Reuter. In laboratory feeding trials, 100% detection was possible within 4 hours of consumption, and the half-life for detection of prey DNA was approximately 18 hours. When used to screen DNA extracted from field-collected *N. kinbergii*, 68% and 10% of the samples collected in fields of broccoli and cauliflower, respectively, generated *P. xylostella*-specific products. In a biological control context, this type of study can help identify key predators and clarify their role in the reduction of natural populations of a given pest.

4.4 Araneae

Anyphaenidae, Lycosidae, Linyphiidae, and Miturgidae

In addition to their use with insect predators, molecular techniques have also been applied to studies on predatory arachnids. For example, Agustí et al. (2003a) developed PCR primers (based on COI gene sequences) for three collembola species (*Isotoma anglicana* Lubbock*, Lepidocyrtus cyaneus* Tullberg*, Entomobrya multifasciata* Tullberg) that occur in agricultural habitats. The assay could detect 100% of ingested prey up to 24 hours post-consumption (Agustí et al. 2003a). Application of these primers to DNA extracted from 50 field-collected spiders (primarily linyphiids) enabled Agustí et al. (2003a) to determine the number of spiders that contained remnants of each collembola species. With this information, the authors demonstrated that spiders preferred one collembola species over the others.

Greenstone et al. (2005) used PCR and DNA barcoding of the COI gene to separate two Anyphaenidae (*Hibana arunda* Platnick, *H. futilis* Banks), two Lycosidae (*Pardosa milvina* Hentz, *Rabidosa rabida* Walckenaer), two Linyphiidae (*Frontinella communis* Hentz, *Grammonota texana* Banks), and one species of Miturgidae (*Cheiracanthium inclusum* Hentz). The assay facilitated the identification of immature specimens to the species level, which is often tenuous due to morphological similarity of immature stages.

Ma et al. (2005) developed a species-specific PCR assay to detect *P. xylostella* DNA in predator gut contents. This assay was used to screen species of *Lycosa* Latreille for the presence of diamondback moth to determine the potential impact of spiders on populations of this pest. Prey detection was consistent (100%) in the 48 hours following consumption, and the half life for detection of prey DNA was approximately 96 hours. When applied to DNA extracted from field-collected *Lycosa* spp., 76.6% and 24.6% of *Lycosa* samples collected in fields of broccoli and cauliflower, respectively, were positive for *P. xylostella*.

5 Conclusions

It is clear from the studies outlined above that molecular diagnostic tools have earned their place in biological control research. The last few years have seen a tremendous increase in the number of studies using diagnostic molecular markers for parasitoid and predator research. From 1993 to 2000, 14 studies reported the development of molecular markers for this purpose. In contrast, three times as many articles on this topic were published between 2001 and 2005. The efficiency, speed, and cost-effectiveness of molecular diagnostics (in comparison to traditional methods) have been the driving force behind their increased application.

In a biological control context, diagnostic molecular markers have been used to identify morphologically similar parasitoid species, cryptic species, and strains. This has allowed the evaluation of natural enemy establishment and/or impact on pest populations, as well as the investigation of factors affecting parasitoid population

genetics. Molecular diagnostic tools have been instrumental in the analysis of predator gut-contents, particularly in the identification of prey items, evaluation of predator preferences, and frequency of predation. In addition, such tools can help investigate host-parasitoid associations, predator – prey population dynamics, intraguild predation, and trophic interactions. Further, molecular markers could potentially be used to screen natural enemies for resistance to insecticides (Edwards and Hoy 1993; MacDonald and Loxdale 2004) and to detect the occurrence of interbreeding between native and introduced parasitoids (Hopper et al. 2006). Such applications would be useful in assessing fitness and evolution of natural enemies used in biological control programmes.

Many of the PCR-based techniques described in the literature require two steps (notably PCR followed by REN digestion) or require several PCR reactions on a single DNA sample to obtain species-level identification. Single-step assays for multiple prey or parasitoid species are more efficient and more suitable for large-scale ecological studies (Erlandson and Gariepy 2005; Gariepy et al. 2005; Traugott et al. 2006). Such single-step multiplex PCR assays are being increasingly used in molecular diagnostics in applied entomology and are already used routinely in many other fields of molecular diagnostics. Lopez et al. (2003) listed multiplex PCR among the innovative tools in use for pathogen diagnosis because it offers a highly sensitive technique capable of detecting multiple DNA targets in a single reaction. The use of multiplex PCR in the identification of arthropod biological control agents has been fairly limited, and to date only five studies (Hinomoto et al. 2004; Gariepy et al. 2005; Harper et al. 2005; Juen and Traugott, 2006; Traugott et al. 2006) have used multiplex PCR in this context. Despite the limited number of multiplex assays used in parasitoid and predator research, Greenstone (2006) suggested that techniques such as multiplex PCR would simplify the study of multi-parasitoid systems.

The combination of multiple PCR assays to detect different prey and/or parasitoid species could be extremely useful in the study of intraguild interactions. Although it would be unrealistic to assume that multiplex PCR would be possible in all cases, DNA samples could be processed with each PCR assay separately to obtain similar information. A practical scenario for studying intraguild interactions among biological control agents would involve the investigation of predation on parasitized pest insects. Assays to detect diamondback moth in predator gut contents (Ma et al. 2005), and to detect parasitoids of diamondback moth (Wagener et al. 2004; Juen and Traugott 2006) could theoretically be combined in a one- or two-step process to screen predator DNA for the presence of parasitized diamondback moth. Similarly, PCR assays to detect predation on aphids (Chen et al. 2000) could be used in combination with assays to detect aphid parasitoids (Zhu and Greenstone 1999; Zhu et al. 2000; Prinsloo et al. 2000; Weathersbee et al. 2004). This could reveal the occurrence of intraguild predation in the field, and determine whether predators preferentially prey on parasitized individuals. The clarification of trophic links between natural enemies may be crucial in the development and application of effective pest control strategies and may provide a better understanding of potentially disruptive interactions between biological control agents. Molecular techniques have not been used to characterise guild interactions within a

community of natural enemies; however they can be a powerful tool to demonstrate the occurrence of predatory and/or parasitic interactions (Messing et al. 2006).

Although many DNA-based techniques are available for use in parasitoid and predator research, their application in ecological studies on biological control agents is far from routine. Diagnostic techniques employed in the agricultural sciences have traditionally lagged behind those used in the medical sciences. Lévesque (2001) and Behura (2006) have described future directions for the use of molecular techniques in pest detection, identification, and control strategies. These authors based their assessment of future developments on the rapid advances in genomics, microarrray technology, and the miniaturization and automation of processes such as DNA extraction, PCR, and DNA sequencing. Recognition of molecular diagnostics as a valid and integral part of ecological and epidemiological studies in biological control and pest management is essential. This, coupled with the affordability that accrues from routine use and automation of procedures should facilitate the adoption of such techniques in applied entomology in the near future.

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B. Collection details and GPS coordinates

Mirid Species	Site Name	Region, Country*	Plant or Habitat	GPS Coordinates
Lygus rugulipennis	Gettorf	Schleswig-Holstein, D	Chamomile	N 54°23.923' E 10°00.438'
	Heikendorf	Schleswig-Holstein, D	Chamomile	N 54°22.437' E 10°13.642'
	Probsteierhagen	Schleswig-Holstein, D	Chamomile	N 54°21.262' E 10°16.420'
	Schilksee	Schleswig-Holstein, D	Chamomile	N 54°24.160' E 10°09.506'
	Wulfshagen	Schleswig-Holstein, D	Chamomile	N 54°22.872' E 10°00.105'
	Busdorf	Schleswig-Holstein, D	Red clover	N 54°05.190' E 10°04.354'
	Estruphof	Schleswig-Holstein, D	Red clover	N 54°43.052' E 09°33.071'
	Idstedt	Schleswig-Holstein, D	Red clover	N 54°35.254' E 09°31.280'
	Osdorf	Schleswig-Holstein, D	Red clover	N 54°25.484' E 10°01.349'
	Schirnau	Schleswig-Holstein, D	Red clover	N 54°20.433' E 09°44.454'
	Farnern	Bern, CH	Alfalfa	N 47°16.098' E 07°36.609'
	Pieterlen	Bern, CH	Alfalfa	N 47°10.257' E 07°18.989'
	Delémont	Jura, CH	Alfalfa	N 47°21.543' E 07°19.542'
	Porrentruy	Jura, CH	Alfalfa	N 47°23.456' E 07°03.476'
	Develier 11	Jura, CH	Alfalfa	N 47°22.086' E 07°15.943'
	Develier 8	Jura, CH	Alfalfa	N 47°21.835' E 07°17.319'
	Hagendorf	Solothurn, CH	Alfalfa	N 47°18.694' E 07°46.110'
	Kappel	Solothurn, CH	Alfalfa	N 47°18.867' E 07°50.895'
	Oberbuchsiten	Solothurn, CH	Alfalfa	N 47°18.384' E 07°45.510'
	Oensingen	Solothurn, CH	Alfalfa	N 47°17.733' E 07°44.322'
	Pfetterhouse 3	Alsace, F	Red clover	N 47°30.578' E 07°09.112'
	Pfetterhouse 4	Alsace, F	Red clover	N 47°30.465' E 07°09.664'
	Mappach	Baden-Württemberg, D	Red clover	N 47°41.495' E 07°35.561'
	Attiswil	Bern, CH	Red clover	N 47°14.989' E 07°37.826'
	Biel	Bern, CH	Red clover	N 47°06.387' E 07°07.346'
	Oberbipp	Bern, CH	Red clover	N 47°15.377' E 07°38.994'
	Coeuve	Jura, CH	Red clover	N 47°27.478' E 07°06.008'
	Porrentruy	Jura, CH	Red clover	N 47°23.308' E 07°03.675'
	Bettlach	Solothurn, CH	Red clover	N 47°12.486' E 07°25.099'
	Selzach	Solothurn, CH	Red clover	N 47°12.203' E 07°28.367'
Closterotomus norwegicus	Bohnert	Schleswig-Holstein, D	Fallow field	N 54°31.620' E 09°46.284'
	Scharnhagen	Schleswig-Holstein, D	Fallow field	N 54°26.972' E 10°06.306'
	Gettorf	Schleswig-Holstein, D	Chamomile	N 54°23.923' E 10°00.438'
	Laböe	Schleswig-Holstein, D	Chamomile	N 54°23.672' E 10°14.308'
	Lütterbek	Schleswig-Holstein, D	Chamomile	N 54°23.302' E 10°16.287
	Rastorf	Schleswig-Holstein, D	Chamomile	N 54°16.554' E 10°17.212'
	Wulfshagen	Schleswig-Holstein, D	Chamomile	N 54°22.872' E 10°00.105'
	Kragholm	Schleswig-Holstein, D	Mustard	N 54°48.384' E 09°34.770'
	Kiel-Holtenau	Schleswig-Holstein, D	Phacelia	N 54°22.762' E 10°07.969'
	Attiswil	Bern, CH	Fallow field	N 47°14.989' E 07°37.826'
	Corban	Jura, CH	Fallow field	N 47°20.718' E 07°28.142'
	Courcelon	Jura, CH	Fallow field	N 47°21.240' E 07°23.514'
	Courroux	Jura, CH	Mixed grasses	N 47°22.181' E 07°22.423'
	Courtetelle	Jura, CH	Fallow field	N 47°21.145' E 07°19.919'
	Delémont	Jura, CH	Fallow field	N 47°21.326' E 07°20.040'
	Kleinlützel	Baselland, CH	Mixed grasses	N 47°25.101' E 07°28.172'
	Niederbuchsiten	Solothurn, CH	Fallow field	N 47°17.990' E 07°47.025'
	Porrentruy	Jura, CH	Mixed grasses	N 47°23.712' E 07°03.752'
	Recolaine	Jura, CH	Fallow field	N 47°20.950' E 07°25.399'
Leptopterna dolobrata	Altenholz	Schleswig-Holstein, D	Orchard grass	N 54°23.609' E 10°07.759'
	Dänischenhagen	Schleswig-Holstein, D	Orchard grass	N 54°25.251' E 10°07.161'
	Kiel-Holtenau	Schleswig-Holstein, D	Orchard grass	N 54°22.762' E 10°07.969'
	Lehmkaten	Schleswig-Holstein, D	Orchard grass	N 54°24.836' E 10°07.770'
	Lindhof	Schleswig-Holstein, D	Orchard grass	N 54°27.541' E 09°58.635'
	Osdorf	Schleswig-Holstein, D	Orchard grass	N 54°25.484' E 10°01.349'
	Scharnhagen	Schleswig-Holstein, D	Orchard grass	N 54°26.972' E 10°06.306'
	Schwedeneck	Schleswig-Holstein, D	Orchard grass	N 54°28.427' E 10°08.764'
	Wellsee	Schleswig-Holstein, D	Orchard grass	N 54°05.743' E 10°08.497'
	Florimont	Franche-Compté, F	Orchard grass	N 47°30.849' E 07°04.110'
	Bamlach	Baden-Württemberg, D	Orchard grass	N 47°42.470' E 07°32.832'
	Neuenburg	Baden-Württemberg, D	Orchard grass	N 47°49.297' E 07°34.242'
	Courgenay	Jura, CH	Orchard grass	N 47°24.187' E 07°08.921'
	Courroux	Jura, CH	Orchard grass	N 47°22.181' E 07°22.423'
	Delémont	Jura, CH	Orchard grass	N 47°21.591' E 07°19.912'
	Pleigne 2	Jura, CH	Orchard grass	N 47°24.710' E 07°17.108'
	Pleigne 4	Jura, CH	Orchard grass	N 47°24.935' E 07°16.761'
	Rossemaison	Jura, CH	Orchard grass	N 47°21.199' E 07°20.994'

Mirid Species	Site Name	Region, Country*	Plant or Habitat	GPS Coordinates
Liocoris tripustulatus	Dänischenhagen	Schleswig-Holstein, D	Stinging nettle	N 54°25.401' E 10°07.480'
	Grevenkrug	Schleswig-Holstein, D	Stinging nettle	N 54°13.591' E 10°01.843'
	Kiel-University	Schleswig-Holstein, D	Stinging nettle	N 54°19.600' E 10°07.604'
	Krusendorf	Schleswig-Holstein, D	Stinging nettle	N 54°28.155' E 10°03.951
	Lindhöft	Schleswig-Holstein, D	Stinging nettle	N 54°27.541' E 09°58.635'
	Osterrönfeld	Schleswig-Holstein, D	Stinging nettle	N 54°17.939' E 09°42.397'
	Schierensee	Schleswig-Holstein, D	Stinging nettle	N 54°15.667' E 09°58.032'
	Schilksee	Schleswig-Holstein, D	Stinging nettle	N 54°24.757' E 10°09.919'
	Steinfurt	Schleswig-Holstein, D	Stinging nettle	N 54°17.798' E 10°00.870'
	Westensee	Schleswig-Holstein, D	Stinging nettle	N 54°16.517' E 09°53.829'
	Mooslargue	Alsace, F	Stinging nettle	N 47°30.103' E 07°12.676'
	Pfetterhouse	Alsace, F	Stinging nettle	N 47°30.885' E 07°09.091'
	Seppois-le-bas	Alsace, F	Stinging nettle	N 47°31.856' E 07°08.220'
	Mappach	Baden-Württemberg, D	Stinging nettle	N 47°41.396' E 07°35.438'
	Kleinlützel	Baselland, CH	Stinging nettle	N 47°25.101' E 07°28.172'
	Galmiz	Freibourg, CH	Stinging nettle	N 46°57.772' E 07°09.791'
	Bellerive	Jura, CH	Stinging nettle	N 47°23.039' E 07°21.794'
	Courroux	Jura, CH	Stinging nettle	N 47°22.068' E 07°21.767'
	Develier	Jura, CH	Stinging nettle	N 47°21.444' E 07°17.318'
	Porrentruy	Jura, CH	Stinging nettle	N 47°23.120' E 07°03.352'
Adelphocoris lineolatus	Auggen	Baden-Württemberg, D	Alfalfa	N 47°47.427' E 07°34.682'
	Buggingen 4	Baden-Württemberg, D	Alfalfa	N 47°52.863' E 07°36.864'
	Buggingen 6	Baden-Württemberg, D	Alfalfa	N 47°51.358' E 07°36.293'
	Hügelheim	Baden-Württemberg, D	Alfalfa	N 47°49.479' E 07°36.764'
	Neuenburg 5	Baden-Württemberg, D	Alfalfa	N 47°47.108' E 07°33.354'
	Neuenburg 6	Baden-Württemberg, D	Alfalfa	N 47°47.265' E 07°35.032'
	Zienken	Baden-Württemberg, D	Alfalfa	N 47°51.114' E 07°35.946'
	Farnern	Bern, CH	Alfalfa	N 47°16.098' E 07°36.609'
	Niederbipp	Bern, CH	Alfalfa	N 47°17.730' E 07°44.320'
Adelphocoris seticornis	Farnern	Bern, CH	Alfalfa	N 47°16.098' E 07°36.609'
	Niederbipp	Bern, CH	Alfalfa	N 47°17.730' E 07°44.320'
Notostira elongata	Ausackerholz	Schleswig-Holstein, D	Mixed grasses	N 54°42.580' E 09°35.320'
	Bohnert	Schleswig-Holstein, D	Mixed grasses	N 54°31.620' E 09°46.284'
	Schilksee	Schleswig-Holstein, D	Mixed grasses	N 54°24.160' E 10°09.503'
Stenodema calcerata	Dagebüll	Schleswig-Holstein, D	Mixed grasses	N 54°43.976' E 08°42.255'
Trigonotylus caelestialium	Pfetterhouse 4	Alsace, F	Mixed grasses	N 47°30.465’ E 07°09.664’
	Courcelon	Jura, CH	Fallow field	N 47°21.240' E 07°23.514'

*D = Germany; CH = Switzerland; F = France

C. Information on parasitoid larvae from 2004 collections in southern Ontario. Numbers in brackets indicate the number of larvae dissected from mirid hosts and the number identified as a given parasitoid species.

Vial #	Date	Location	Habitat	Mirid Hosts	Identification
13	15/06/04	London	Canola	Lygus (1)	*P. pallipes* (1)
14	15/06/04	London	Weeds	Lygus (2)	*P. pallipes* (1)
				Other (1)	Unidentified (2)
18	15/06/04	London	Weeds	Lygus (1)	*P. pallipes* (1)
				Lygus (1)*	Unidentified (1)
19	15/06/04	London	Weeds	Lygus (7)	*P. pallipes* (9)
				Adelphocoris (1)	Unidentified (1)
				Polymerus (2)	
23	15/06/04	London	Alfalfa	Lygus (1)	*P. pallipes* (1)
24	15/06/04	London	Alfalfa	Lygus (2)	*P. pallipes* (2)
26	23/06/04	London	Grass, weeds	Lygus (1)	*P. pallipes* (1)
27	23/06/04	London	Fallow field	Lygus (1)	*P. pallipes* (1)
34	29/06/04	London	Weeds	Lygus (1)	*P. pallipes* (1)
35	29/06/04	London	Weeds	Lygus (5)	*P. pallipes* (4)
					Unidentified (1)
36	29/06/04	London	Weeds	Lygus (3)	*P. pallipes* (2)
					Unidentified (1)
38	29/06/04	London	Alfalfa	Lygus (3)	*P. pallipes* (2)
					Unidentified (1)
39	29/06/04	London	Weeds	Lygus (1)	*P. pallipes* (1)
76	29/07/04	London	Fallow field	Lygus (2)	*P. pallipes* (1)
					Unidentified (1)
77	29/07/04	London	Alfalfa	Lygus (4)	Unidentified (4)
78	29/07/04	London	Weeds	Lygus (1)	Unidentified (1)
80	29/07/04	London	Weeds	Lygus (1)	Unidentified (1)
81	29/07/04	London	Alfalfa	Lygus (1)	Unidentified (1)
82	29/07/04	London	Weeds	Lygus (3)	*P. pallipes* (1)
					Unidentified (2)

* indicates that the parasitoid larvae were dissected from adult mirids.

D. Information on parasitoid larvae from 2005 collections in southern Ontario. Numbers in brackets indicate the number of larvae dissected from mirid hosts and the number identified as a given parasitoid species.

Vial #	Date	Location	Habitat	Mirid Hosts	Identification
6	26/05/05	London	Weeds	*Leptopterna* (1)	*P. pallipes* (1)
10	31/05/05	London	Weeds	*Leptopterna* (2)	*P. pallipes* (2)
17	31/05/05	London	Alfalfa	*Leptopterna* (6), *Lygus* (2)	*P. pallipes* (7) Unidentified (1)
24	07/06/05	London	Weeds	*Trigonotylus* (1)	Unidentified (1)
26	08/06/05	London	Alfalfa	*Trigonotylus* (5)	*P. pallipes* (5)
32	14/06/05	London	Weeds	*Lygus* (1)	*P. pallipes* (1)
33	14/06/05	London	Weeds	*Lygus* (21)	*P. pallipes* (20) Unidentified (1)
34	17/06/05	St. Catherine's	Alfalfa	*Lygus* (1)	*P. digoneutis* (1)
35	17/06/05	St. Catherine's	Grass, weeds	*Lygus* (6)	*P. pallipes* (4) *P. digoneutis* (2)
36	17/06/05	St. Catherine's	Grass, weeds	*Lygus* (3)*	*P. pallipes* (3)
37	17/06/05	St. Catherine's	Grass, weeds	*Lygus* (2)*	*P. pallipes* (2)
38	17/06/05	St. Catherine's	Grass, weeds	*Lygus* (1)*	*P. pallipes* (1)
39	21/06/05	London	Weeds	*Lygus* (6)	*P. pallipes* (6)
40	21/06/05	London	Grass	*Lygus* (2)*	*P. pallipes* (2)
41	21/06/05	London	Weeds	*Lygus* (2)	*P. pallipes* (1) Unidentified (1)
51	21/06/05	London	Weeds	*Lygus* (12) *Adelphocoris* (1)*	*P. pallipes* (13)
52	21/06/05	London	Alfalfa	*Lygus* (21)	*P. pallipes* (19) Unidentified (2)
55	27/06/05	London	Alfalfa	*Lygus* (10)	*P. pallipes* (10)
62	04/07/05	London	Weeds	*Lygus* (7)	*P. pallipes* (7)
70	04/07/05	London	Alfalfa	*Lygus* (5)	*P. pallipes* (5)
74	11/07/05	London	Alfalfa	*Lygus* (6)	Unidentified (6)
76	11/07/05	London	Weeds	*Lygus* (5)	*P. pallipes* (1) Unidentified (4)
83	19/07/05	London	Alfalfa	*Lygus* (6)	Unidentified (6)
84	19/07/05	London	Alfalfa	*Lygus* (7) *Adelphocoris* (1)	*P. pallipes* (2) Unidentified (6)
86	19/07/05	London	Weeds	*Lygus* (7)	*P. pallipes* (6) Unidentified (1)
92	28/07/05	London	Buckwheat	*Lygus* (6)	*P. pallipes* (6)
96	02/08/05	London	Weeds	*Lygus* (12)	*P. pallipes* (12)
97	02/08/05	London	Alfalfa	*Lygus* (2)	*P. pallipes* (2)
102	08/08/05	London	Alfalfa	*Lygus* (2)	*P. pallipes* (2)
103	08/08/05	London	Weeds	*Lygus* (4)	*P. pallipes* (3) Unidentified (1)
115	09/09/05	London	Weeds	*Lygus* (1)	Unidentified (1)

* indicates that the parasitoid larvae were dissected from adult mirids.