

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) revolutionized 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 with 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 recognized 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, DNA, microsatellites, parasitoids, PCR, predators

1 Introduction

The field of biological diagnostics was revolutionized with the advent of the polymerase chain reaction (PCR) in the mid-1980s (Saiki et al. 1985, 1988; Mullis et al. 1986). 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 (Valles et al. 2002; Lange et al. 2004), 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 characterization, 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 diagnostic 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; 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 in the specific DNA region targeted, allowing visualization 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 visualized 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 characterized 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 with 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, nonspecific amplification, and/or loss of sensitivity in comparison with 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 (tables 1–3). 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 with 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

3.1.1 Aphelinidae

Molecular identification using PCR-based assays has been frequently applied to aphid parasitoids in the families Aphelinidae and Braconidae. Many molecular studies of 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 (Hom., Aphididae) in North America (Zhu and Greenstone 1999). The authors designed five sets of PCR primers with specificity for each species and strain investigated (*Aphelinus albipodus* Hayat and Fatima, *Aphelinus varipes* Förster, and two strains of *Aphelinus 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 and Greenstone (1999), amplifying 10^{-3} and 10^{-4} wasp equivalents from *A. hordei* and *A. colemani*, respectively.

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*) parasitize *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

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

Parasitoid	Region	Primary application	Reference
Aphelinidae			
<i>Aphelinus abdominalis</i>	MS*	Identification; discrimination	Vanlerberghe-Masutti and Chavigny (1997)
<i>Aphelinus albipodus</i> , <i>A. asychis</i> , <i>A. varipes</i>	ITS 2	Identification, post-release studies	Zhu and Greenstone (1999)
<i>Aphelinus gossypii</i>	18S	Identification; establishment	Weathersbee et al. (2004)
<i>Aphelinus hordei</i>	ITS 2	Identification, post-release studies	Zhu et al. (2000), Prinsloo et al. (2002)
<i>Encarsia formosa</i> , <i>E. luteola</i> , <i>E. sophia</i>	COI	Identification	Monti et al. (2005)
Encyrtidae			
<i>Agentiaspis citricola</i>	RAPD	Identify cryptic species	Hoy et al. (2000)
<i>Agentiaspis</i> spp.	ITS 2	Identify cryptic species	Alvarez and Hoy (2002)
Eulophidae			
<i>Horismenus butcheri</i> , <i>H. missouriensis</i> , <i>H. depressus</i>	MS*	Population genetics	Aebi et al. (2004)
Eurytomidae			
<i>Eurytoma brumiventris</i>	MS*	Population genetics	Hale et al. (2004)
Mymaridae			
<i>Anaphes tole</i>	ITS 2	Identification, detection	Zhu and Williams (2002)
Pteromalidae			
<i>Muscidifurax raptor</i> , <i>M. raptorellus</i> , <i>M. zaraptor</i>	RAPD	Identification; investigate the origin of gregariousness	Antolin et al. (1996)
<i>Muscidifurax raptor</i> , <i>M. raptorellus</i> , <i>M. uniraptor</i> , <i>M. zaraptor</i>	COI, II	Identification	Taylor et al. (1997)
<i>Muscidifurax raptor</i> , <i>M. raptorellus</i> , <i>M. uniraptor</i> , <i>M. zaraptor</i>	ITS 1	Identification	Taylor and Szalanski (1999)
<i>Muscidifurax raptor</i> , <i>M. raptorellus</i> , <i>M. uniraptor</i> , <i>M. zaraptor</i>	ITS 1, 2	Identification; assess parasitism	Ratcliffe et al. (2002)
<i>Nasonia vitripennis</i>	ITS 1, 2	Identification; assess parasitism	Ratcliffe et al. (2002)
<i>Spalangia cameroni</i> , <i>S. endius</i> , <i>S. nigroaenea</i>	ITS 1, 2	Identification; assess parasitism	Ratcliffe et al. (2002)
<i>Trichomalopsis sarcophagae</i>	ITS 1, 2	Identification; assess parasitism	Ratcliffe et al. (2002)
<i>Urolepis rufipes</i>	ITS 1, 2	Identification; assess parasitism	Ratcliffe et al. (2002)
Trichogrammatidae			
<i>Trichogramma minutum</i> , <i>T. plameri</i>	COII	Identification	Borghuis et al. (2004)
<i>Trichogramma brassicae</i> , <i>T. chilonis</i> , <i>T. dendrolimi</i> , <i>T. ostrinae</i>	ITS 1	Identification	Sappal et al. (1995), Chang et al. (2001)
<i>Trichogramma australicum</i>	ITS 2	Detection	Amornsak et al. (1998)
<i>Trichogramma acactoi</i> , <i>T. atopovirilia</i> , <i>T. bouwarachae</i> , <i>T. brassicae</i> , <i>T. brunii</i> , <i>T. carverae</i> , <i>T. cordubensis</i> , <i>T. deion</i> , <i>T. dendrolimi</i> , <i>T. evanescens</i> , <i>T. funiculatum</i> , <i>T. galloi</i> , <i>T. lasallei</i> , <i>T. pintoi</i> , <i>T. pretiosum</i> , <i>T. rojasi</i> , <i>T. turkestanica</i>	ITS 2	Identification	Silva et al. (1999), Stouthamer et al. (1999), Chang et al. (2001), Ciociola et al. (2001a,b), 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			
<i>Aphidius colemani</i>	ITS 2	Identification, post-release studies	Zhu et al. (2000)
<i>Aphidius colemani</i> , <i>A. matricariae</i> , <i>A. picipes</i>	MS	Identification	Roehrdanz et al. (1993)
<i>Aphidius ervi</i>	MS	Investigation of founder effects	Hufbauer et al. (2004)
<i>Cotesia glomerata</i> , <i>C. rubecula</i>	COI	Identification, assess parasitism	Traugott et al. (2006)
<i>Diaeretiella rapae</i>	MS*	Identification	Roehrdanz et al. (1993)
<i>Diaeretiella rapae</i>	MS*	Investigation of founder effects	Baker et al. (2003)
<i>Dolichogenidia homoeosomae</i>	MS*	Population genetics	Douhovnikoff et al. (2006)
<i>Dolichogenidia tasmanica</i>	18S	Assess host utilization	Suckling et al. (2001)
<i>Leiophron uniformis</i> , <i>L. argentinensis</i>	ITS 2	Identification, assess parasitism	Zhu et al. 2004
<i>Lipolexis scutellaris</i> , <i>Lysiphlebus testaceipes</i>	18S	Identification, assess establishment	Weathersbee et al. (2004)
<i>Lipolexis scutellaris</i> , <i>Lysiphlebus testaceipes</i>	ITS 2	Identification, assess establishment	Persad et al. (2004)
<i>Lysiphlebus testaceipes</i>	16S	Identification, assess parasitism	Jones et al. (2005)
<i>Lysiphlebus testaceipes</i>	MS*	Identification	Roehrdanz et al. (1993)
<i>Lysiphlebus testaceipes</i>	MS*	Population genetics	Fauvergue et al. (2005)
<i>Peristenus conradi</i> , <i>P. digoneutis</i> , <i>P. pallipes</i>	COI	Assess parasitism and dispersal	Tilmon et al. (2000)
<i>Peristenus conradi</i> , <i>P. digoneutis</i> , <i>P. howardi</i> , <i>P. pallipes</i>	COI	Assess parasitism	Mowry and Barbour (2004)
<i>Peristenus digoneutis</i> , <i>P. pallipes</i> , <i>P. stygicus</i>	ITS 1, 2	Identification, assess parasitism	Erlandson et al. (2003), Garipey et al. (2005)
<i>Peristenus howardi</i> , <i>P. pallipes</i> , <i>P. pseudopallipes</i> , <i>P. stygicus</i>	ITS 2	Identification, assess parasitism	Zhu et al. (2004)
Ichneumonidae			
<i>Diadegma semiclausum</i>	COI	Identification, parasitism	Traugott et al. (2006)
<i>Diadegma</i> spp.	ITS 2	Identification, discrimination	Wagener et al. (2004)
<i>Mesochorus curvulus</i>	ITS 2	Detection	Ashfaq et al. (2005)
<i>Tranosema rostrale</i> (based on polydnavirus)	–	Assess multiparasitism	Cusson et al. (2002)

*MS, microsatellite DNA; –, information not available.

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 Hem., Aleyrodidae). Aphelinid wasps in the genus *Encarsia* Förster, in particular *Encarsia 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 *Encarsia luteola* Howard, as well as two populations of *Encarsia 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.

3.1.2 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.

3.1.3 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 (*Horismenus depressus* Gahan, *Horismenus missouriensis* Ashmead, *Horismenus butcheri* Hanson and Aebi) that attack pestiferous bruchid beetles (Col., 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.

3.1.4 Eurytomidae

Hale et al. (2004) developed microsatellite markers for *Eurytoma brunniventris* Ratzeburg, a generalist parasitoid of cynipid oak gall wasps (Hym.,

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

Predator	Region	Primary application	Reference
Araneae: Anyphaenidae <i>Hibana arunda</i> , <i>H. futilis</i>	COI	Predator identification	Greenstone et al. (2005)
Araneae: Linyphiidae <i>Frontinella communis</i> , <i>Grammonota texana</i>	COI	Predator identification	Greenstone et al. (2005)
Various linyphiids	COI	Gut content analysis to detect collembola	Agustí et al. (2003b)
Araneae: Lycosidae <i>Lycosa</i> spp.	ITS 1	Gut content analysis to detect diamondback moth	Ma et al. (2005)
<i>Pardosa milvina</i> , <i>Rabidosia rabida</i>	COI	Predator identification	Greenstone et al. (2005)
Araneae: Miturgidae <i>Cheiracanthium inclusum</i>	COI	Predator identification	Greenstone et al. (2005)
Coleoptera: Carabidae <i>Poecilus versicolor</i>	COI	Effect of scavenging on gut content analysis; detection of scarabaeid prey items	Juen and Traugott (2005, 2006)
<i>Pterostichus cupreus</i>	α -esterase	Gut content analysis to detect digested mosquito over time	Zaidi et al. (1999)
<i>Pterostichus melanarius</i>	COI, 12S	Detection of multiple prey species simultaneously	Harper et al. (2005)
<i>Pterostichus melanarius</i>	COI	Impact of secondary predation on gut content analysis	Sheppard et al. (2005)
Coleoptera: Coccinellidae <i>Coccinella septempunctata</i> , <i>Hippodamia convergens</i>	COII	Gut content analysis to detect aphids	Chen et al. (2000)
<i>Coleomegilla maculata</i>	ITS	Gut content analysis to detect European corn borer and frequency of predation	Hoogendoorn and Heimpel (2001)
<i>Curinus coeruleus</i>	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 <i>Anthocoris tomentosus</i>	COI	Gut content analysis to detect pear psylla	Agustí et al. (2003a)
<i>Orius sauteri</i> , <i>O. minutus</i> , <i>O. strigicollis</i> , <i>O. nagaii</i> , <i>O. tantillus</i>	ITS 1	Predator identification	Hinomoto et al. (2004)
Hemiptera: Nabidae <i>Nabis kinbergii</i>	ITS1	Gut content analysis to detect diamondback moth	Ma et al. (2005)
Mesostigmata: Laelapidae	COI	Gut content analysis to detect soil-dwelling nematodes	Read et al. (2006)
Neuroptera: Chrysopidae <i>Chrysoperla plorabunda</i>	COII	Gut content analysis to detect aphids	Chen et al. (2000)

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.

3.1.5 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 (Hem., Miridae) plant bugs. The assay reliably detected 10^{-5} wasp equivalents, and identified the parasitoid in host eggs as early as 48 h 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.

3.1.6 Pteromalidae

Species-specific molecular markers based on RAPD-PCR were used by Antolin et al. (1996) for species of *Muscidifurax* Girault and Sanders (*Muscidifurax raptor* Girault and Sanders, *Muscidifurax zaraptor* Kogan and Legner, and *Muscidifurax raptorellus* Kogan and Legner), pupal parasitoids of Diptera that breed in animal waste. Although designed to study the origin ofregarious 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) analysed 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, *Trichomalopsis sarcophagae* Gahan and *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 h 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 1 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.

3.1.7 *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 Silva 1993).

Orrego and 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; van Kan et al. 1996, 1997; Pinto et al. 1997; Silva et al. 1999), 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 parasitized by *Trichogramma australicum* Girault, and those that were unparasitized. Sensitivity testing of the PCR assay demonstrated that *T. australicum* eggs were detectable in host eggs in as little as 12 h of parasitoid egg development. However, consistent detection required at least 24 h (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 *Trichogramma deion* Pinto and Oatman complex,

consisting of four species, and the *Trichogramma 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 (*Trichogramma cordubensis* Vargas and Cabello, *Trichogramma turkistanica* Meyer, *Trichogramma evanescens* Westwood, *Trichogramma pinto* Voegelé, and *Trichogramma 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) characterized differences between several *Trichogramma* species in Asia (Chang et al. 2001; Li and Shen 2001, 2002), Brazil (Ciociola et al. 2001a,b), south-eastern Australia (Thomson et al. 2003), and sibling species (*Trichogramma minutum* Riley and *Trichogramma 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 *Trichogrammatidae* (see Pinto and Stouthamer 1994; Pinto 1999; Stouthamer 2006).

3.2 Hymenoptera: Ichneumonoidea

3.2.1 *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, *Aphidius 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 geographical 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 probably 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 probably 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 probably 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 (Hom., 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 Hym., Braconidae) and *Aphelinus gossypii* Timberlake (Hym., Aphelinidae) (Weathersbee et al. 2004). Although this assay was able to detect up to 8% of the parasitoids after only 2 h, 100% detection was only possible by 72 h post-parasitism. 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 h, but suggested that reliable detection (100%) was only possible 24 h post-parasitism. Persad et al. (2004) also indicated that the PCR-based assay is an efficient method for measuring 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. testaceipes*-parasitized aphids at different intervals post-parasitism showed that parasitoid DNA was detectable as early as 48 h 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. testaceipes*. Although their study did not include the use of the markers, the authors suggested that these would be useful for

population studies, particularly because this parasitoid has a broad host range and geographical distribution.

Several molecular identification studies have been directed at the parasitoid community that attack *Lygus* plant bugs, in particular, the parasitoids found in *Lygus* nymphs in Europe and North America. Tilmon et al. (2000) developed molecular markers for *Peristenus pallipes* Curtis, *Peristenus digoneutis* Loan, and *Peristenus 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, *Peristenus howardi* Shaw. Mowry and Barbour (2004) suggested that this molecular assay would facilitate ecological studies of *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; Hym., 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 (*Leiophron argentinensis* Shaw and *Leiophron 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.

Garipey 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 h 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 (Garipey 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 (Lep., 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 whether 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 (Lep., 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 Loan derived from eight geographical populations in South America were released in New Zealand for the control of the Argentine stem weevil, *Listonotus bonariensis* Kuschel (Col., Curculionidae). Winder et al. (2005) developed microsatellite markers that could separate the different geographical populations of *M. hyperodae* 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. (Lep., Plutellidae), *Pieris brassicae* L. and *Pieris rapae* L. (Lep., Pieridae). Primers were developed for two braconids, *Cotesia glomerata* and *Cotesia 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.

3.2.2 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 polydnavirus carried by the parasitoid) to detect *Tranosema rostrale* Brischke in the spruce budworm, *Choristoneura fumiferana* Clemens (Lep., Tortricidae). This tool was used in combination with host dissection to examine multiparasitism and potential competition with another parasitoid, *Actia interrupta* Curran (Dip., 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

3.3.1 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 geographical 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; Lep., 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.

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

4.1.1 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; Dip., 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 h post-consumption when the fragment being amplified is relatively small (<200 bp).

Harper et al. (2005) developed a multiplex PCR system for detecting 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 of 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 *Pterostichus 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. (Col., 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.

4.1.2 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. (Col., Coccinellidae), and one lacewing, *Chrysoperla plorabunda* Fitch (Neu., Chrysopidae). Their study examined the sensitivity and specificity of the primers for two of the six aphid species (*Rhopalosiphum maidis* Fitch and *Rhopalosiphum 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) were able to 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, 2006). This is probably influenced by the predator and/or prey group investigated, primer stability and the fragment size being amplified (Harper et al. 2005; Juen and 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 h, but the half-life for detection of nematode DNA was

approximately 9 h. Prey was detectable in mesostigmatid mites for up to 12 h, with a half-life of approximately 5 h. When used to screen micro-arthropods collected 12 h 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

4.3.1 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 *Orius sauteri* Poppius, *Orius minutus* L., *Orius strigicollis* Poppius, *Orius nagaii* Yasunaga and *Orius 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; Hem., 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.

4.3.2 Nabidae

Ma et al. (2005) developed species-specific PCR primers for the diamondback moth to detect predation by *Nabis kinbergii* Reuter. In laboratory feeding trials, 100% detection was possible within 4 h of consumption, and the half-life for detection of prey DNA was approximately 18 h. 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

4.4.1 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 and

Entomobrya multifasciata Tullberg) that occur in agricultural habitats. The assay detected 100% of ingested prey up to 24 h 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, *Hibana futilis* Banks), two Lycosidae (*Pardosa milvina* Hentz, *Rabidosia 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 for detecting *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 h following consumption, and the half-life for detection of prey DNA was approximately 96 h. 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 with 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. Furthermore, 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 Garipey 2005; Garipey 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 (Hinamoto et al. 2004; Garipey 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 for detecting diamondback moth in predator gut contents (Ma et al. 2005) and 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 for detecting predation on aphids (Chen et al. 2000) could be used in combination with assays for detecting aphid parasitoids (Zhu and Greenstone 1999; Prinsloo et al. 2002; Zhu 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 characterize guild

interactions within a community of natural enemies; however, they can be a powerful tool in demonstrating 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 agricultural sciences have traditionally lagged behind those used in medical sciences. Lévésque (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, microarray 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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