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REVIEW ARTICLE

Molecular methods for assessing insect parasitism

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Abstract

Determining insect parasitism rates is problematic due to the small size and lack of useful distinguishing morphological characters of many parasitoid taxa. To solve this problem, entomologists have employed one of four general methods to detect parasitoid protein or nucleic acid markers: serological assay; random amplified polymorphic DNA–polymerase chain reaction (RAPD-PCR); allozyme electrophoresis; or specific PCR. Serological methods, especially with monoclonal antibodies, are unrivalled for specificity, enabling discrimination at the stage as well as species level. However, they have not found favour with many workers, possibly due to complexity and expense. RAPD–PCR has been widely used, but can only be recommended for restricted applications because of its poor reproducibility. Allozyme electrophoresis provides reproducible detection and discrimination of closely related species. Specific-PCR is highly specific and reproducible, and also has the shortest latency for detection, usually 24 h or less after parasitization. The substantial existing literature on allozyme electrophoresis and specific PCR is used to support recommendations on what are apt to be fruitful enzyme systems or genomic regions for detecting and discriminating parasitoids in untried parasitoid–host assemblages.

Keywords: allozyme electrophoresis, isozyme, specific PCR, parasitoids, RAPD–PCR

Introduction

Parasitioids are parasites that kill their hosts as a normal part of their development (Lafferty & Kuris, 2002). This functional group comprises a broad taxonomic array of both parasitoid and host organisms, e.g. flagellates attacking diatoms (Kühn & Hoffmann, 1999) and flatworms attacking decapod crustaceans (Kuris *et al*., 2002). To practising entomologists, however, parasitoids are insects, primarily in the orders Hymenoptera and Diptera, whose larvae develop in or upon the bodies of other insects. They destroy billions of pest insects annually in crops worldwide and are key players in biological control. While most parasitism is ambient, parasitoids are also produced in vast quantities for intentional inundative release to control pest infestations (Pinto *et al*., 1993; Ram *et al*., 1995; Burks & Pinto, 2002; Borghuis *et al*., 2004). Numerous parasitoid species have also been released and established on new continents to rejoin their hosts in what had been 'enemy-free space' (Jeffries & Lawton, 1984; Loxdale & Lushai, 1999), in classical biological control programmes to suppress exotic invasive pest species (Takada, 1998; Kimani-Njogu *et al*., 1998; Hufbauer *et al*., 2001; Prinsloo *et al*., 2002; Iline & Phillips, 2004; Persad *et al*., 2004).

Understanding and managing parasitoid–host interactions pose daunting technical challenges. Most parasitoids are small, even minute, and many groups are speciose and evolving rapidly. Related species often have few or no known sufficiently invariant distinguishing morphological characters for reliable discrimination (Pungerl, 1986; Landry *et al*., 1993; Pinto *et al*., 1993; Demichelis & Manino, 1998;

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Kimani-Njogu *et al*., 1998; Stouthamer *et al*., 1999; Barnay *et al*., 2001; Chang *et al*., 2001). Detecting and differentiating endoparasitic stages within their hosts is particularly difficult. Parasitoids have been visualized within hosts by conventional X-ray (Biever & Boldt, 1970) and magnetic resonance imaging (MRI; Geogehegan *et al*., 2000). While these techniques might enable detection of parasitism *per se*, current resolution would be insufficient to discriminate among closely related parasitoid species (Hart *et al*., 2003), if indeed morphological differences could be found among the immature stages of related species (Persad *et al*., 2004). Another visualization technique, near-infrared spectroscopy, has been used to separate adults, but not immatures, of sibling species (Cole *et al*., 2003). Hagler & Jackson (1998) used exogenous molecules, vertebrate immunoglobulins, to mark adult parasitoids for later identification.

Most biological control workers employ one of two methods for detection and discrimination of parasitoids: holding field-collected hosts in the laboratory for parasitoid emergence, or dissecting hosts and examining endoparasitic stages microscopically. Holding for emergence requires provisioning of artificial diet or living plant material to sustain the hosts until parasitoid emergence, usually under temperature-controlled conditions. Because of the time lag between host collection and parasitoid emergence, the results become available too late to be of use for management decisions. In the worst case, some species may have a prolonged post-emergence diapause (Keen *et al*., 2001), sometimes approaching a full year (Tilmon *et al*., 2000). Even when this is not a problem, there is almost always a failure of some hosts to produce parasitoids, sometimes due to patent microbial infections (Greenstone & Edwards, 1998; Ratcliffe *et al*., 2002), but often due to unknown causes, and for the latter there is no way to determine the status of these so-called 'duds' (Wool *et al*., 1978; Stuart & Greenstone, 1996; Ratcliffe *et al*., 2002). Whatever the cause, this leads to unknown biases in percentage parasitism estimates. Dissection on the other hand does provide unequivocal and timely estimates of parasitization rate, but it is tedious and requires special training, and species-specific identification can be challenging where members of the same genus or even family are present (e.g. Persad *et al*., 2004).

This has led to a search for stable, unequivocal, species-specific characters to support evolutionary, ecological, and applied research. Such characters are generally drawn from two classes of organic molecules: proteins and nucleic acids. These have the virtue of abundance in tissues, ubiquity within animals in general, and, in many cases, assignability to loci inherited in simple Mendelian fashion. Furthermore, formats for sensitive and reproducible assays of these molecules are available. This has made it possible to use them for studies of phylogeny (Campbell *et al*., 1993; Kimani-Njogu *et al*., 1998), genetic variability (Landry *et al*., 1993; Baldanza *et al*., 2001; Baker *et al*., 2003; Chaterjee *et al*., 2003; Baer *et al*., 2004; Hufbauer *et al*., 2004), hybridization (Atanassova *et al*., 1998), population dynamics (Vaughn & Antolin, 1998; Schneider *et al*., 2003; Macdonald & Loxdale, 2004), effectiveness of release and establishment (Edwards & Hoy, 1995; Prinsloo *et al*., 2002; Ratcliffe *et al*., 2002) and quality control (Yazlovetskii *et al*., 1981; Unruh *et al*., 1983; Morgan *et al*., 1988; Stuart & Burkholder, 1991; Landry *et al*., 1993; Roehrdanz *et al*., 1993), as well as for our present purposes, differentiation and detection within the host.

With the large literature presently available, it is possible to gain an impression of which proteins and which DNA regions are potentially most useful for detecting and discriminating parasitoids. Here I summarize this literature to determine which molecules represent sufficiently variable and detectable characters for parasitoid detection and discrimination; I then provide some guidelines on which specific characters appear most useful for these tasks.

Proteins

Animal tissues contain an abundance and diversity of enzymes and structural proteins. Techniques for detecting and characterizing both protein classes are well established and both have been used to discriminate and detect parasitoids.

Enzymes

Enzymes may be found as multiple forms, produced by different loci, referred to as isozymes; when there is more than one allele at an isozyme locus, each is referred to as an allozyme (Richardson *et al*., 1986). These different enzyme forms usually migrate to different positions on electrophoresis gels and are visualized as bands that become stained when exposed to appropriate substrates and linking systems (enzymes, coenzymes); if they do not differ in mobility, they can often be differentiated by means of specific stains (e.g. Wynne *et al*., 1992). The genetics, chemistry, and interpretation of allozyme electrophoresis are thoroughly explained by Richardson *et al*. (1986). The mobility and number of bands may be influenced by a number of factors, including pH, viscosity of the matrix, and ionic strength of the electrophoresis buffer.

Allozymes by definition reveal simple Mendelian traits (Kimani-Njogu *et al*., 1998), permitting genetic analysis (Atanassova *et al*., 1998), and most practitioners assume that electrophoretic banding patterns reflect such inheritance. In fact this is not always the case, because the number of alleles may not be revealed due to non-detectable amino acid substitutions (Richardson *et al*., 1986). Bands may also differ in staining intensity due to differences in allele number (homozygosity versus heterozygosity), subunit structure of enzymes affecting expression of bands in homozygous and heterozygous condition (Richardson *et al*., 1986), and gene duplication. Post-transcriptional modifications of the polypeptide chains can also complicate the interpretation of banding patterns (e.g. Cameron *et al*., 1984). If the assumption of Mendelian inheritance is critical to the analysis, formal genetic crosses should be made to confirm it (for examples, see Hung & Huo, 1985, and Pinto *et al*., 1992).

Because Mendelian inheritance cannot be assumed, the most conservative authors refer to electrophoretic variants as electromorphs rather than alleles. For our purposes, these bands will be useful as diagnostic markers provided they confer the specificity and sensitivity required for detection and discrimination, regardless of the mode of inheritance. Whether particular electromorphs are reliable species identifiers, i.e. invariant within species but differing between species, must be determined empirically. This can produce some surprises. For example, host switching may lead to loss of esterase bands (Cameron *et al*., 1984), indicating that allozyme expression is subject to rapid evolution.

Scores of enzymes have been used in animal isozyme studies (Richardson *et al*., 1986). Numerous authors have assayed a dozen or more enzyme systems in their search for sufficiently polymorphic bands/loci to enable discrimination of parasitoid species within particular parasitoid–host systems (Castrovillo & Stock, 1981; Castañera *et al*., 1983; Pinto *et al*., 1992; Atanassova *et al*., 1998; Kimani-Njogu *et al*., 1998; Burks & Pinto, 2002). Wynne *et al*. (1992) provide a list of eight enzymes they have found to be generally polymorphic in insects.

Table 1 provides an overview of enzyme systems that have been used as markers in all manner of parasitoid research. In each case, the table identifies those that the authors found most useful for differentiating parasitoids in the particular host–parasitoid systems under study. Not surprisingly, allozymes of hyperparasites as well as primary parastoids are detectable and may be species-specific (Cameron *et al*., 1984).

There have been relatively few assessments of the temporal threshold for detection of allozymes of parasitoids within their hosts. Castañera *et al*. (1983) were able to detect 'nearly full grown' larvae of *Aphidius uzbekistanus* (Luzhetski) (Hymenoptera: Aphidiidae) in *Sitobion avenae* (Fabricius) (Hemiptera: Aphididae). Walton *et al*. (1990a) detected parasitism of *S. avenae* after 84 h by *Praon volucre* (Haliday) (Hymenoptera: Braconidae) (24 h later than by dissection); after 156 h by *Ephedrus plagiator* Nees (Hymenoptera: Aphidiidae) (72 h later than by dissection); and after 132 h in *Aphidius rhopalosiphi* De Stefani Perez (Hymenoptera: Aphidiidae) (24 h earlier than by dissection).

Structural proteins

Proteins may be detected and characterized by a number of standard biochemical techniques, e.g. native and denaturing polyacrylamide gel electrophoresis (PAGE and SDS–PAGE, respectively), and isoelectric focusing (IEF). SDS–PAGE has been used to differentiate closely related parasitoid species (Baldanza *et al*., 2001). However, the most useful approach for parasitoid differentiation and detection has been immunoassay.

The vertebrate immune system, which is capable of recognizing proteins and protein substructures with exquisite specificity (Greenstone, 1996), can provide antibodies as reagents for use in standardized assays. Recognition of the parasitoid antigen protein by the antibody is made visible by an enzyme-substrate reporter system, in an immunodot assay or enzyme-linked immunosorbent assay (ELISA).

Within an immunized vertebrate, antibodies are produced by clones of lymphocytes, each clone secreting into the blood stream a population of identical antibody molecules recognizing a single region of an antigenic molecule, the so-called antigenic determinant (or epitope). Since proteins typically contain more than one determinant, more than one lymphocyte clone will be activated and the resulting polyclonal antiserum will recognize numerous determinants. Therefore when antiserum from an immunized animal is used as the reagent in a parasitoid assay, the assay is apt to cross react (produce false positives) with related species due to the presence of shared antigenic determinants, a problem that may not be solved by absorption with specific antigen (e.g. Keen *et al*., 2001). The surest route to avoiding cross-reactivity is the use of monoclonal antibodies, which are produced by lymphocyte clones removed from the immunized vertebrate host, immortalized by fusion with a myeloma cell, isolated in tissue culture, and selected to secrete antibodies that recognize only unique determinants (Greenstone, 1996). Since determinants vary in their distribution across taxa, monoclonals may be specific at almost any taxonomic level, and even different developmental stages of a single species. For example, Stuart & Greenstone (1996) developed a monoclonal that distinguished first from second instars of just one braconid species, and another (Stuart & Greenstone, 1997) that recognizes all hymenopterous parasitoids tested, but not their hosts. This was due to the determinant being a hymenopteran-specific variant of elongation factor-1α (Stuart, 1998), a protein common to all animals.

In addition to their own structural proteins, parasitoids may also secrete specific proteins into their hosts (e.g. Soldevila & Jones, 1991; Hochuli & Lanzrein, 2001). Although these do not appear to have been used as markers of parasitization, they should in principle work as well as parasitoid structural proteins.

Immunoassays are widely used for diagnosis but have not been much used to document parasitoid–host interactions. Consequently there are few data on the time course of detection of parasitoid antigens within hosts. A polyclonal ELISA for a pteromalid parasitoid (Hymenoptera: Pteromalidae) of house fly pupae (*Musca domestica* Linnaeus (Diptera: Muscidae)) could not detect larvae until 7 days post-parasitization (Keen *et al*., 2001), and another for a braconid wasp could not detect 'early stage' larvae (Allen *et al*., 1992) in its lepidopteran leaf roller host. A monoclonal-based immunodot assay of Stuart & Greenstone (1996) was able to detect first instars of a braconid in its caterpillar host.

Nucleic acids

An advantage of nucleic acids is the large number of loci revealed, far in excess of those available for protein analysis (Edwards & Hoy, 1993; Landry *et al*., 1993). Although ribonucleic acid (RNA) could in principle be used and would enable stage- as well as species-specificity, only deoxyribonucleic acid (DNA) has been employed to date. Thanks to the ability of the polymerase chain reaction (PCR; Ehrlich, 1989) to amplify minute quantities of DNA, scores of reactions can be performed on a single individual. Protein detection generally requires a larger quantity of target, so that fewer reactions can be run on a single animal (Edwards & Hoy, 1993; Vanlerberghe & Chavigny, 1997).

The sensitivity of a molecular assay depends upon the number of copies of the target molecule in the sample. DNA targets may be either non-coding sequences or specific genes. Multiple copies of a given sequence are found throughout the genome, variously arrayed among chromosomes in the nucleus or within cellular organelles. Both within and among individual animals and populations, these sequences may be identical or variable to a greater or lesser degree; they also display varying rates of evolution (Hillis & Dixon, 1991; Elder & Turner, 1995; Hoy, 2003), suggesting that some are better suited than others for species-level discrimination. Nevertheless, all of these classes of sequences have been successfully used to discriminate and detect closely related parasitoid species.

Table 1. Most useful enzyme systems used in parasitoid allozyme electrophoresis.

Enzyme	Application	References
Acid phosphatase	Discrimination	Burks & Pinto, 2002
		Pinto *et al*., 1993
Aconitase	Discrimination	Pinto *et al*., 1993
		Unruh *et al*., 1983
	Discrimination and detection	Castrovillo & Stock, 1981
Esterases	Discrimination	Burks & Pinto, 2002
		Castañera *et al*., 1983
		Dawah, 1989
		Dawah *et al*., 2002
		Cameron *et al*., 1984
		Kimani-Njogu *et al*., 1998
		Silva *et al*., 1999
		Pintureau, 1993
		Ram *et al*., 1995
		Pungerl, 1986
		Ram *et al*., 1995
		Silva *et al*., 1999
		Takada, 1998
		Unruh *et al*., 1983
		Yazlovetskii *et al*., 1981
	Discrimination and detection	Castrovillo & Stock, 1981
		Walton *et al*., 1990a,b
		Yazlovetskii *et al*., 1981
Calcium binding protein	Discrimination	Iline & Phillips, 2004
Creatine kinase	Discrimination	Morgan *et al*., 1988
Fumarase	Discrimination	Pinto *et al*., 1993
		Unruh *et al*., 1983
Glucose-phosphate isomerase	Discrimination	Burks & Pinto, 2002
		Kimani-Njogu *et al*., 1998
		Kitthawee *et al*., 1999
		Pinto *et al*., 1993
Glucose-6-phosphate dehydrogenase	Differentiation	Burks & Pinto, 2002
		Pinto *et al*., 1993
Glutamate-oxaloacetate transaminase	Discrimination and detection	Walton *et al*., 1990a
		Dawah *et al*., 2002
Glyceraldehyde-3-phosphate dehydrogenase	Discrimination	Morgan *et al*., 1988
α-glycerol-phosphate dehydrogenase	Discrimination	Burks & Pinto, 2002
		Morgan *et al*., 1988
	Discrimination and detection	Demichelis & Manino, 1998
Glycerol-3-phosphate dehydrogenase	Discrimination	Kitthawee *et al*., 1999
Hexokinase	Discrimination	Dawah, 1989
		Kimani-Njogu *et al*., 1998
Sorbital dehydrogenase	Discrimination	Kimani-Njogu *et al*., 1998
Isocitrate dehydrogenase	Discrimination	Atanassova *et al*., 1998
		Burks & Pinto, 2002
		Kitthawee *et al*., 1999
		Morgan *et al*., 1988
		Pinto *et al*., 1993
		Unruh *et al*., 1983
Lactate dehydrogenase	Discrimination and detection	May *et al*., 1977
Leucine aminopeptidase	Discrimination and detection	Castrovillo & Stock, 1981
Dihydrolipoamide dehydrogenase	Discrimination	Iline & Phillips, 2004
Malate dehydrogenase	Discrimination	Iline & Phillips, 2004
		Kitthawee *et al*., 1999
		Morgan *et al*., 1988
		Pinto *et al*., 1993
		Tomiuk *et al*., 1979
		Wool *et al*., 1978
	Detection	Castañera *et al*., 1983
		Walton *et al*., 1990a
Malic enzyme	Discrimination and detection	Castañera *et al*., 1983
	Discrimination	Unruh *et al*., 1983
		Walton *et al*., 1990a
		Burks & Pinto, 2002
		Pinto *et al*., 1993
		Pungerl, 1986
		Kitthawee *et al*., 1999

Table 1. Continued.

Enzyme	Application	References
Mannose phosphate isomerase	Discrimination	Kitthawee *et al*., 1999
Peptidase	Discrimination	Atanassova *et al*., 1998
Phosphoglucomutase	Discrimination	Atanassova *et al*., 1998 Morgan *et al*., 1988 Burks & Pinto, 2002 Dawah, 1989 Hung and Huo, 1985 Kitthawee *et al*., 1999 Pinto *et al*., 1993 Pinto *et al*., 2003
Phosphogluconate dehydrogenase	Discrimination	Kimani-Njogu *et al*., 1998 Kitthawee *et al*., 1999 Unruh *et al*., 1983
6-phosphoglucose dehydrogenase	Discrimination and detection	Castrovillo & Stock, 1981
Phosphoglucose isomerase	Discrimination	Atanassova *et al*., 1998 Hung & Huo, 1985 Unruh *et al*., 1983

Genomic sequences tend to repeat

Repetitive DNA sequences exist in several to millions of copies per cell (Hoy, 2003), which makes them useful for PCR targeting. For reasons that are not well understood, they tend to exhibit concerted evolution, i.e. non-independent evolution resulting in sequence similarity that is greater within than among species (Elder & Turner, 1995). They are therefore also useful for species differentiation.

Ribosomal RNA genes (rDNA) exist in arrays on one or more chromosomes and typically number in the thousands of copies per cell (Elder & Turner, 1995). The nuclear ribosomal DNA array in eukaryotes comprises three genes named on the basis of their sedimentation rates, 18S, 5.8S and 28S, preceded by an external transcribed spacer and separated by the ITS-1 and ITS-2 internal transcribed spacers; adjacent copies of the array are separated by a non-transcribed spacer (NTS) or intergenic spacer (Hillis & Dixon, 1991). 12S and 16S rDNA genes are found in the mitochondrial genome. Because different regions of the array evolve at different rates, they are useful for resolving differences at different taxonomic levels, with 16–18S genes being highly conserved and the 28S less so. The non-coding sequences also evolve, the NTS very rapidly, the nuclear ITS regions a little less so (Hillis & Dixon, 1991). One potential drawback is that, at least for ITS-2, there may be significant intraspecific variation in sequences (Allemand *et al*., 2002). Furthermore, intra-individual sequence variation may sometimes exceed inter-individual differences (Stouthamer *et al*., 1999; Alvarez & Hoy, 2002 and references cited therein), making discrimination of closely related populations problematical. The ITS-2 region discriminated some but not all of a suite of closely related *Trichogramma* (Hymenoptera: Trichogrammatidae) species (Stouthamer *et al*., 1999, 2000).

Mitochondrial sequences (mtDNA) are also found at high copy numbers per cell, typically hundreds or thousands (Hoy, 2003). A catalogue of conserved primers (Simon *et al*., 1994) makes it a simple matter to 'fish out' specific mtDNA regions for sequencing to support specific primer design. Mitochondrial DNAs usually evolve three times faster than nuclear DNA because of inefficient repair mechanisms (however, ITS-1 may diverge at a more rapid rate than mtDNA; Elder & Turner, 1995). Since there is a finite risk that a putative amplified mtDNA sequence could actually be a nuclear 'pseudogene' evolving at a different rate than its mitochondrial ancestor, phylogenetic inferences and hypotheses about divergence times (cf. Chen *et al*., 2002) employing mtDNA sequences must be made with care (Sunnucks & Hales, 1996; Benasson *et al*., 2001).

Rapidly evolving tandem simple sequence repeats (SSRs) of 1–6 bp, sometimes referred to as microsatellites, are found throughout the coding and non-coding portions of genomes of all organisms. Their functions and mechanisms of evolution, and neutrality or selectivity, appear to be varied (Li *et al*., 2002); they also differ from other repeating sequences in being non-homologous, so that different copies within a given genome have different flanking sequences. Vanlerberghe-Masutti & Chavigny (1997) developed a species-specific microsatellite-based PCR assay for aphelinind parasitism; Hufbauer *et al*. (2001, 2004) used microsatellites to document reduced genetic variability in introduced populations of *Aphidius ervi* Haliday (Hymenoptera: Braconidae). Microsatellites of *Diaretiella rapae* (M'Intosh) (Hymenoptera: Aphidiidae) were used by Baer *et al*. (2004) to demonstrate lack of polyphagy, and by Loxdale & Macdonald (2004) to study movement of winged individuals at the farm scale.

In addition to microsatellites, the genome contains DNA comprising tandem arrays rich in A + T or C + G sequences, largely associated with chromosomal heterochromatin and centromeres (Elder & Turner, 1995; Hoy, 2003), and variously referred to as satellite or moderately repetitive DNA. Greenstone & Edwards (1998) used a 438 bp squash-blot hybridization probe from a genomic library containing 53, 42, and 29 bp repeats to detect braconid endoparasitism. Similarly, Landais *et al*. (2000) used a genomic library to devise a 385 bp squash-blot hybridization probe containing 8–43 bp repeating motifs to differentiate and detect a suite of trichogrammatid parasitoids.

DNA assays

The simplest assays utilize complementary strands of the target sequences as hybridization probes. Such assays can detect small quantities of DNA if they employ probes

comprising moderately repetitive sequences from the target species. Greenstone & Edwards (1998) developed such a probe that was able to detect early first instars, but not eggs, of the braconid *Microplitis croceipes* (Cresson) in lepidopteran host larvae in a squash-blot assay. Using the same approach, Landais *et al*. (2000) could detect the egg of *Trichogramma brassicae* (Bezdenko) 7 days post-parasitization within the host egg.

The most sensitive DNA assays employ PCR, which can amplify just a few copies of the target sequence. PCR using both random and specific primers has been applied to both parasitoid species discrimination and detection.

Random amplified polymorphic DNA markers (RAPDs)

RAPD markers are generally thought to sample highly polymorphic non-coding DNA (Black, 1993), to be phenotypically dominant or less often co-dominant, and to conform to Mendelian segregation patterns (Vaughn & Antolin, 1998); if co-dominant, i.e. having one strand with a large insertion or deletion, they can be detected using single-strand conformation polymorphism (SSCP) techniques (Sunnucks *et al*., 2000). This permits population genetic analyses (but see Kazmer *et al*., 1995, for caveats).

RAPDs were more successful than isozyme analysis in separating three *Muscidifurax* species (Hymenoptera: Pteromalidae) (Antolin *et al*., 1996). RAPDs have effectively differentiated closely related congeneric species and strains within species (Baldanza *et al*., 2001; Kirk *et al*., 2000; Barnay *et al*., 2001). Edwards & Hopper (1999) used RAPDs to differentiate hymenopterous parasitoids of differing genotypes emerging from individual host larvae, while Edwards & Hoy (1995) used them to distinguish pesticide-resistant from wild-type parasitoids of the same species. Edwards & Hoy (1993) looked for presence-absence rather than fragment-size polymorphisms, which greatly facilitates the scoring of differences between populations. Crossing studies showed that some but not all bands were inherited in Mendelian fashion as dominant traits.

RAPDs suffer from a reputation for lack of reproducibility both within one laboratory and from one laboratory to another (Black, 1993; Kazmer *et al*., 1995; Taylor & Szalanski, 1999; Hoy *et al*., 2000). Occasional contaminants, e.g. bacterial or protozoan symbionts, may produce bands that can be mistakenly identified as polymorphisms for the targets of interest (Black, 1993). There is also no reason to expect that similar-sized bands in different species are homologous (Black, 1993), a problem that does not arise with specific PCR approaches (below). RAPDs can be used as a discovery tool to find single-locus, species-specific sequences (see 'SCARS,' below under 'Specific PCR').

Black *et al*. (1992) provide the only report of latency in detection of parasitization by RAPD-PCR; parasitism in their aphid–parasitoid system could not be detected until six days post-parasitization.

Specific PCR

The availability of universal primer sequences for numerous genomic regions (Simon *et al*., 1994) has facilitated the isolation and sequencing of DNA to enable design of parasitoid species-specific PCR primers. Table 2 summarizes the use of various genomic regions for species-specific differentiation and detection of parasitoid DNA. Not surprisingly, DNAs of hyperparasites as well as of primary parasitoids are detectable by PCR (Chen *et al*., in press). Table 3 lists parasitoid species for which specific primers have been designed.

Although it is the newest technology to be applied to this field, specific PCR has been used more than any of the other approaches for parasitoid detection. Consequently, there is quite a bit of information on the sensitivity and time course of detectability of parasitization. Sensitivity is typically measured as wasp-equivalents of DNA detectable, generally in a reaction mixture containing host DNA (Zhu & Greenstone, 1999). Sensitivities on the order of 10^{-3} to 10^{-4} wasp equivalents are common (Zhu & Greenstone, 1999; Zhu *et al*., 2000; Ratcliffe *et al*., 2002), and sensitivities as low as 10^{-6} to 10^{-7} have been achieved (Zhu *et al*., 2004b; Chen *et al*., in press). This makes it possible, for example, to detect a single *Trichogramma* egg in the egg of its host (Li & Shen, 2002).

These high sensitivities also correspond to very short detection latencies, usually no more than 24 h post-parasitization (Zhu & Greenstone, 1999; Zhu *et al*., 2000; Ratcliffe *et al*., 2002; Zhu *et al*., 2004b). Latencies less than 24 h have frequently been found (Amornsak *et al*., 1998; Zhu & Williams, 2002; Persad *et al*., 2004; Weathersbee *et al*., 2004), and latencies longer than 24 h appear to be the exception (Zhu & Williams, 2002; Jones *et al*., 2005).

Although specific PCR is very powerful, often enabling detection of just a single base-pair difference, it is not always possible to adjust PCR conditions to enable discrimination of all species of interest. In such cases, cutting the PCR product with a restriction enzyme may resolve these near-identical sequences. This process, known as restriction fragment length polymorphism (RFLP) analysis (also called restriction endonucease (REN) analysis; Escribano *et al*., 2000), requires knowledge of the target sequence so that restriction sites can be mapped. RFLP of a variety of coding and non-coding sequences has been widely used to differentiate closely related parasitoid species (Sappal *et al*., 1995; Silva *et al*., 1999; Taylor & Szalanski, 1999; Stouthamer *et al*., 1999; Tilmon *et al*., 2000; Allemand *et al*., 2002; Asfaq *et al*., 2004; Borghuis *et al*., 2004). Prinsloo *et al*. (2002) used RFLP and two genomic regions, ITS-2 and 16s rDNA, to distinguish three sibling species of *Aphelinus* (Hymenoptera: Aphelinidae).

In 1993, Paran & Michelmore published a technique, which they dubbed sequence characterized amplified regions (SCARS), in which PCR products are excised from a RAPD gel, sequenced, and used for primer design. This approach was used by Zhu *et al*. (2004a) to distinguish pesticide-resistant and -susceptible strains of a pteromalid parasitoid by isolating and cloning strain-specific fragments amplified by RAPD–PCR, and then making PCR primers to amplify these specific but theretofore unknown fragments.

Recommendations

This review has identified four distinct molecular approaches that have been applied to the discrimination and detection of insect parasitoids, and that may be appropriate for assessing the extent of insect parasitism. Immunoassay, especially with monoclonal antibodies, is sensitive and has great powers of discrimination, enabling stage- as well as species-specificity. Nevertheless, the relative paucity of studies employing it, especially given the

Table 2. DNA regions used in specific PCR and PCR–RFLP.

Region	Application	References
Cytochrome B	Discrimination	Daza-Bustamente *et al*., 2002
Cytochrome oxidase I	Discrimination	Baer *et al*., 2004
		Hufbauer *et al*., 2004
	Discrimination and detection	Ashfaq *et al*., 2004
		Agustí *et al*., 2005
		Persad *et al*., 2004
		Tilmon *et al*., 2000
Cytochrome oxidase II	Discrimination	Baer *et al*., 2004
		Borghuis *et al*., 2004
		Hufbauer *et al*., 2004
ITS-1	Discrimination	Chang *et al*., 2001
		Hufbauer *et al*., 2004
		Orrego & Agudelo-Silva, 1993
		Sappal *et al*., 1995
		Taylor & Szalansi, 1999
	Discrimination and detection	Gariepy *et al*., 2005
		Ratcliffe *et al*., 2002
Cytochrome oxidase I/II	Discrimination	Daza-Bustamente *et al*., 2002
		Schneider *et al*., 2003
ITS-2	Discrimination	Allemand *et al*., 2002
		Alvarez & Hoy, 2002
		Campbell *et al*., 1993
		Hufbauer *et al*., 2004
		Persad *et al*., 2004
		Sappal *et al*., 1995
		Silva *et al*., 1999
		Stouthamer *et al*., 1999
		Stouthamer *et al*., 2000
	Discrimination and detection	Amornsak *et al*., 1998
		Ashfaq *et al*., 2004
		Erlandson *et al*., 2003
		Gariepy *et al*., 2005
		Li & Shen, 2002
		Prinsloo *et al*., 2002
		Zhu & Greenstone, 1999
		Zhu & Williams, 2002
		Zhu *et al*., 2000
		Zhu *et al*., 2004b
28s rDNA	Discrimination	Campbell *et al*., 1993
		Persad *et al*., 2004
		Sappal *et al*., 1995
18s rDNA	Discrimination and detection	Suckling *et al*., 2001
		Weathersbee *et al*., 2004
16s rDNA	Discrimination	Baer *et al*., 2004
		Chen *et al*., 2002
		Persad *et al*., 2004
	Discrimination and detection	Chen *et al*., in press
		Jones *et al*., 2005
		Prinsloo *et al*., 2002
12s rDNA	Discrimination	Persad *et al*., 2004
NADH	Discrimination	Persad *et al*., 2004
Actin genes	Discrimination	Hoy *et al*., 2000
Esterase-like enzyme	Discrimination	Zhu *et al*., 1999
Satellites	Discrimination and detection	Greenstone & Edwards, 1998
		Landais *et al*., 2000
Microsatellites	Discrimination	Amornsak *et al*., 1998
		Hufbauer *et al*., 2001
		Jensen *et al*., 2002
		Loxdale & MacDonald, 2004
		MacDonald *et al*., 2003
		Vanlerbeghe & Masutti, 1997
Unknown sequences	Discrimination	Zhu *et al*., 2004a

maturity and broad application of the technology to other disciplines, suggests that it may not be practical for biocontrol practitioners. RAPDs have been used for many applications, but only once (Black *et al*., 1992) for detection, and exhibited low sensitivity in that instance. Given its poor laboratory-to-laboratory reproducibility, it is most useful for

Table 3. Parasitoid species for which specific PCR primers have been published.

Species	Family	Region	References
Ageniapsis citricola	Encyrtidae	Actin genes	Hoy *et al*., 2000
Alloxysta xanthopis	Charipidae	16s rDNA	Chen *et al*., in press
Anaphes iole	Mymaridae	ITS-2	Zhu & Williams, 2002
Anisopteromalus calandrae	Pteromalidae	Esterase-like enzyme	Zhu *et al*., 1999
		Uncharacterized	Zhu *et al*., 2004a
Aphelinus abdominalis	Aphelinidae	Microsatellite	Vanlerberghe-Masutti & Chavigny, 1997
A. albipodus		ITS-2	Zhu & Greenstone, 1999
A. asychis			
A. gossypii		18s rDNA	Weathersbee *et al*., 2004
A. hordei		ITS-2	Zhu *et al*., 2000
		ITS-2, 16s rDNA	Prinsloo *et al*., 2002
A. varipes		ITS-2	Zhu & Greenstone, 1999
Aphidius colemani	Aphidiidae	ITS-2	Zhu *et al*., 2000
A. ervi		CO I-II, CO B	Daza-Bustamante *et al*., 2002
		Microsatellite	Hufbauer *et al*., 2001
Cotesia congregata	Braconidae	Microsatellite	Jensen *et al*., 2002
Diaretiella rapae		Microsatellite	Loxdale & Macdonald, 2004
Dendrocerus carpenteri	Megaspilidae	16s rDNA	Chen *et al*., in press
Dolichogenidia tasmanica	Braconidae	18s rDNA	Suckling *et al*., 2001
Leiophron argentinensis		ITS-2	Zhu *et al*., 2004a
L. uniformis			
Lipolexis oregmae	Aphidiidae	ITS-2	Persad *et al*., 2004
L. scutellaris		18s rDNA	Weathersbee *et al*., 2004
Lydella thompsoni	Tachinidae	CO I	Agustí *et al*., 2005
Lysiphlebus testaceipes	Aphidiidae	ITS-2	Persad *et al*., 2004
		18s rDNA	Weathersbee *et al*., 2004
		16s rDNA	Jones *et al*., 2005
Muscidifurax raptor	Pteromalidae	ITS-1	Ratcliffe *et al*., 2002
M. raptorellus			
M. zaraptor			
Nasonia vitripennis	Pteromalidae	ITS-1	Ratcliffe *et al*., 2002
Peristenus digoneutis	Pteromalidae	ITS-1, ITS-2	Erlandson *et al*., 2003
P. howardi			Gariepy *et al*., 2005
P. pallipes			Zhu *et al*., 2004b
P. pseudopallipes			Erlandson *et al*., 2003
			Gariepy *et al*., 2005
			Zhu *et al*., 2004b
P. stygicus			Erlandson *et al*., 2003
			Gariepy *et al*., 2005
			Zhu *et al*., 2004b
Pseudoperichaeta nigrolineata	Tachinidae	CO I	Agustí *et al*., 2005
Spalangia calcitrans	Pteromalidae	ITS-1	Ratcliffe *et al*., 2002
S. cameroni			
S. endius			
S. nigroaena			
Trichomalopsis sarcophagae	Pteromalidae	ITS-1	Ratcliffe *et al*., 2002
Urolepis rufipes	Pteromalidae	ITS-1	Ratcliffe *et al*., 2002
Trichogramma australicum	Trichogrammatidae	ITS-2	Amornsak *et al*., 1998
T. brassicae		Satellite	Landais *et al*., 2000
		ITS-1, ITS-2	Sappal *et al*., 1995
T. chilonis		ITS-1	Chang *et al*., 2001
T. deion		ITS-2	Stouthamer *et al*., 1999
T. dendrolimi		ITS-2	Li & Shen, 2002
T. minutum		ITS-1, ITS-2	Sappal *et al*., 1995
T. ostriniae		ITS-1	Chang *et al*., 2001
T. pretiosum		ITS-2	Stouthamer *et al*., 1999
		ITS-2	Li & Shen, 2002
T. turkestanica		ITS-2	Silva *et al*., 1999

a particular short-term project, or for long-term studies of a particular system and run by a single laboratory.

Allozyme electrophoresis and specific PCR, on the other hand, exhibit excellent reproducibility, and, unlike RAPDs, use markers whose homologies across species can be verified by activity and sequence, respectively. The compilation in table 1 suggests that perhaps a half dozen enzyme classes, led by esterases, phosphoglucomutase, and malate dehydrogenase, are adequate for separating most parasitoid taxa by allozyme electrophoresis. When faced with having to make a choice of which enzyme system to use to assay a large number of field-collected hosts, Walton *et al*. (1990b)

chose the esterase system as the 'most versatile' system for parasitoid detection in their system. The few studies of sensitivity, all involving aphid parasitoids, have given detection times greater than 3 days post-parasitization. As indicated by Richardson *et al*. (1986), many factors influence the appearance and interpretation of banding patterns. Furthermore, particular host-parasitoid systems may have their own quirks, requiring a certain amount of experience for correct interpretation (H.D. Loxdale, personal communication).

As table 2 shows, much of the genome has been used for discrimination and/or detection of parasitoids by specific PCR. Despite some concerns about intra-individual variation, ITS-2 has been most used for detection, followed by cytochrome oxidase. Cytochrome oxidase I (COI) has been proposed as a universal barcode for all animals, and there is general agreement that COI will achieve this for most animal species (see http://www.barcodinglife.com). Therefore, broader employment of COI would increase the utility of markers developed for biocontrol use. In cases where sequence similarity is so high, for example in some groups of parasitoid sibling species, that COI cannot resolve differences (e.g. see Alvarez & Hoy, 2002), RFLP of COI targets, or amplification of other genomic sequences, may then be necessary.

Specific PCR has exhibited extraordinary sensitivity, detecting as little as 10^{-7} wasp equivalents, enabling detection of parasitoid eggs or larvae in their hosts within 24–48 h of oviposition in all studied cases, hence an advance over allozymes analysis. The fact that many pests, and in some cases their parasitoids, are nearly cosmopolitan, means that primer sequences once developed in any laboratory may be used by other biocontrol practitioners in their field situations. Finally, the maturity of the PCR technique, and the very large pool of talented entomologists able to develop, employ, and trouble-shoot this methodology, augurs well for PCR to become the dominant assay method in the near term. Optimization of multiplex PCR protocols (Gariepy *et al*., 2005), also utilized in predator–prey research (e.g. Harper *et al*., 2005), will simplify application of the methodology to multi-parasitoid systems.

Even so, DNA technologies are very rapidly evolving, and we may expect before this first decade of the new millennium is out to see DNA hybridization assays employing nanotechnology approaches exhibiting very high sensitivities and specificities (e.g. see Taton *et al*., 2000 and Parks *et al*., 2002). When these approaches are standardized and applied to parasitism research, we will not have to run PCR assays to assess parasitism rates: we shall use our thermocyclers only to fish out species-specific primers, which we shall in turn use to design species-specific probes for use in sensitive, 'quick-and-dirty' hybridization assays.

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References

Agustí, N., Bourguet, D., Spataro, T., Delos, M., Eychenne, N., Folcher, L. & Arditi, R. (2005) Detection, identification, and geographical distribution of European corn borer larval parasitoids using molecular markers. *Molecular Ecology* **14**, 3267–3274.

Allemand, R., Lematre, C., Frey, F., Bouletreau, M., Vavre, F., Nordlander, G., van Alphen, J. & Carton, Y. (2002) Phylogeny of six African *Leptopilina* species (Hymenoptera: Cynipoidea, Figitidae), parasitoids of *Drosophila*, with description of three new species. *Annales de la Société Entomologique de France* **38**, 319–332.

Allen, W.R., Trimble, R.M. & Vickers, P.M. (1992) ELISA used without trituration to detect larvae of *Phyllonorycter blancardella* (Leidoptera: Gracillariidae) parasitized by *Pholetesor ornigis* (Hymeoptera: Braconidae). *Environmental Entomology* **21**, 50–56.

Alvarez, J.M. & Hoy, M.A. (2002) Evaluation of the ribosomal ITS2 DA sequences in separating closely related populations of the parasitoid *Ageniaspis* (Hymenoptera: Encyrtidae). *Annals of the Entomological Society of America* **95**, 250–256.

Amornsak, W., Gordh, G. & Graham, G. (1998) Detecting parasitized eggs with polymerase chain reaction and DNA sequence of *Trichogramma australicum* Girault (Hymenoptera: Trichogrammatidae). *Australian Journal of Entomology* **37**, 174–179.

Antolin, M.F., Guertin, D.S. & Petersen, J.J. (1996) The origin of gregarious *Muscidifurax* (Hymenoptera: Pteromalidae) in North America: an analysis using molecular markers. *Biological Control* **6**, 76–82.

Ashfaq, M., Braun, L., Hegedus, D. & Erlandson, M. (2004) Estimating parasitism levels in *Lygus* spp. (Hemiptera: Miridae) field populations using standard and molecular techniques. *Biocontrol Science and Technology* **14**, 731–735.

Atanassova, P., Brookes, C.P., Loxdale, H.D. & Powell, L. (1998) Electrophoretic study of five aphid parasitoid species of the genus *Aphidius* (Hymenoptera: Braconidae), including evidence for reproductively isolated sympatric populations and cryptic species. *Bulletin of Entomological Research* **88**, 3–13.

Baer, C.F., Tripp, D.W., Bjorksten, A. & Antolin, M.F. (2004) Phylogeography of a parasitoid wasp (*Diaretiella rapae*): no evidence of host-associated lineages. *Molecular Ecology* **13**, 1859–1869.

Baker, D.A., Loxdale, H.D. & Edwards, O.R. (2003) Genetic variation and founder effects in the parasitoid wasp, *Diaretiella rapae* (M'Intosh) (Hymenoptera: Braconidae: Aphidiidae), affecting its potential as a biological control agent. *Molecular Ecology* **12**, 3303–3311.

Baldanza, F., Aceto, S., Gaudio, L. & Viggiani, G. (2001) Soluble protein profile variability in four populations of *Encarsia citrina* (Craw) (Hymenoptera: Aphelinidae) obtained by SDS–PAGE technique. *Bollettino del Laboratorio di Entomologia Agraria Filippo Silvestri* **56**, 5–14.

Barnay, O., Hommay, G., Gertz, C., Kienlen, J.C., Schubert, G., Marro, J.P., Pizzol, J. & Chavigny, P. (2001). Survey of natural populations of *Trichogramma* (Hym., Trichogrammatidae) in the vineyards of Alsace (France). *Journal of Applied Entomology* **125**, 469–477.

Benasson, D., Zhang, D.X., Hartl, D.L. & Hewitt, G.M. (2001) Mitochondrial pseudogenes: evolution's misplaced witnesses. *Trends in Ecology and Evolution* **16**, 314–321.

Biever, K.D. & Boldt, P.E. (1970) Utilization of soft X-rays for determining pupal parasitism of *Pieris rapae*. *Annals of the Entomological Society of America* **63**, 1482–1483.

Black, W.C. (1993) PCR with arbitrary primers: approach with care. *Insect Molecular Biology* **2**, 1–6.

Black, W.C., IV, DuTeau, N.M., Puterka, G.J., Nechols, J.R. & Pettorini, J.M. (1992) Use of the random amplified polymorphic DNA polymerase chain reaction (RAPD–PCR) to detect DNA polymorphisms in aphids. *Bulletin of Entomological Research* **82**, 151–159.

Borghuis, A., Pinto, J.D., Platner, G.R. & Stouthamer, R. (2004) Partial cytochrome oxidase II sequences distinguish the sibling species *Trichogramma minutum* Riley and *Trichogramma platneri* Nagarkatti. *Biological Control* **30**, 90–94.

Burks, R.A. & Pinto, J.D. (2002) Reproductive and electrophoretic comparisons of *Trichogramma californicum* Nagaraja and Nagarkatti with the *T. minutum* complex. *Proceedings of the Entomological Society of Washington* **104**, 33–40.

Cameron, P.J., Powell, W. & Loxdale, H.D. (1984) Reservoirs for *Aphidius ervi* (Hymenoptera: Aphidiidae), a polyphagous parasitoid of cereal aphids (Hemiptera: Aphididae). *Bulletin of Entomological Research* **74**, 647–656.

Campbell, B.C., Stefen-Campbell, J.D. & Werren, J.H. (1993) Phylogeny of the *Nasonia* species complex (Hymenoptera: Pteromalidae) inferred from an internal transcribed space (ITS2) and 28s rDNA sequences. *Insect Molecular Biology* **2**, 225–237.

Castañera, P., Loxdale, H.D. & Nowak, K. (1983) Electrophoretic study of enzymes from cereal aphid populations. II. Use of electrophoresis for identifying aphid parasitoids (Hymenoptera) of *Sitobion avenae* (F.) (Hemiptera: Aphididae). *Bulletin of Entomological Research* **73**, 659–665.

Castrovillo, P.J. & Stock, M.W. (1981) Electrophoretic techniques for detection of *Glypta fumiferanae*, an endoparasitoid of Western spruce budworm. *Entomologia Experimentalis et Applicata* **30**, 176–180.

Chang, S.-C., Hu, N.-T., Hsin, C.-Y. & Sun, C.-N. (2001) Characterization of differences between two *Trichogramma* wasps by molecular markers. *Biological Control* **21**, 75–78.

Chatterjee, S.N., Mohandas, T.P. & Taraphdar, T. (2003) Molecular characterization of the gene pool of *Exorista sorbillans* (Diptera: Tachinidae), a parasitoid of silkworm, *Bombyx mori*, in India. *European Journal of Entomology* **100**, 195–200.

Chen, Y., Giles, K.L. & Greenstone, M.H. (2002) Molecular evidence for a species complex in the genus *Aphelinus* (Hymenoptera: Aphenlinidae), with additional data on aphidiine phylogeny. *Annals of the Entomological Society of America* **95**, 29–34.

Chen, Y., Pike, K.S., Greenstone, M.H. & Shufran, K.A. Molecular markers for identification of the hyperparasitoids *Dendrocerus carpenteri* and *Alloxysta xanthopsis* in *Lysiphlebus testaceipes* parasitizing cereal aphids. *BioControl*, in press.

Cole, T.J., Ram, M.S., Dowell, F.E., Omwega, C.O., Overholt, W.A. & Ramaswamy, S.B. (2003) Near-infrared spectroscopic method to identify *Cotesia flavipes* and *Cotesia sesamiae* (Hymenoptera: Braconidae). *Annals of the Entomological Society of America* **96**, 865–869.

Daza-Bustamante, P., Fuentes-Contreras, E., Rodriguez, L.C., Figueroa, C.C. & Niemeyer, H.M. (2002) Behavioural differences between *Aphidius ervi* populations from two tritrophic systems are due to phenotypic plasticity. *Entomologia Experimentalis et Applicata* **104**, 321–328.

Dawah, H.A. (1989) Separation of *Chlorocytus* species (Hymenoptera: Pteromalidae), parasitoids of stem-boring Hymenoptera (Eurytomidae and Cephidae) using enzyme electrophoresis. *Entomologist* **108**, 216–222.

Dawah, H.A., Al-Haddad, F.H. & Jervis, A. (2002) Morphological and biological characterization of three closely related species of *Pediobius* Walker (Hymenoptera: Eulophidae). *Journal of Natural History* **36**, 423–433.

Demichelis, S. & Manino, A. (1998) Electrophoretic detection of parasitism by Dryindae in Typhlobcybinae leafhoppers (Homoptera: Auchenorrhyncha). *Canadian Entomologist* **130**, 407–414.

Edwards, O.R. & Hopper, K.R. (1999) Using superparasitism by a stem borer parasitoid to infer a host refuge. *Ecological Entomology* **24**, 7–12.

Edwards, O.R. & Hoy, M.A. (1993) Polymorphism in two parasitoids detected using random amplified polymorphic DNA polymerase chain reaction. *Biological Control* **3**, 243–257.

Edwards, O.R. & Hoy, M.A. (1995) Random amplified polymorphic DNA markers to monitor laboratory-selected, pesticide-resistant *Trioxys pallidus* (Hymenoptera: Aphidiidae) after release into three California walnut orchards. *Environmental Entomology* **24**, 487–496.

Elder, J.F. & Turner, B.J. (1995) Concerted evolution of repetitive DNA sequences in eukaryotes. *Quarterly Review of Biology* **70**, 297–320.

Ehrlich, H.A. (1989) *PCR technology: principles and applications for DNA amplification*. New York, Stockton Press.

Elder, J.F., Jr. & Turner, B.J. (1995) Concerted evolution of repetitive DNA sequences in eukaryotes. *Quarterly Review of Biology* **70**, 297–320.

Erlandson, M., Braun, L., Baldwin, D., Soroka, J., Ashfaq, M. & Hegedus, D. (2003) Molecular markers for *Peristenus* spp. (Hymenoptera: Braconidae), parasitoids associated with *Lygus* spp. (Hemiptera: Miridae). *Canadian Entomologist* **135**, 71–83.

Escribano, A., Williams, T., Goulson, D., Cave, R.D., Chapman, J.W. & Caballero, P. (2000) Effect of parasitism on a nucleopolyhedrovirus amplified in *Spodopera frugiperda* larvae parasitized by *Campoletis sonorensis*. *Entomologia Experimentalis et Applicata* **97**, 257–264.

Gariepy, T., Kuhlmann, U., Haye, T., Gillott, C. & Erlandson, M. (2005) A single-step multiplex PCR assay for the detection of European *Peristenus* spp., parasitoids of *Lygus* spp. *Biocontrol Science and Technology* **15**, 481–495.

Geogehegan, J.E., Chudek, J.A., MacKay, R.L., Lowe, C., Moritz, S., McNicol, R.J., Birch, A.N.E., Hunter, G. & Majerus, M.E.N. (2000) Study of the anatomical changes in *Coccinella septempunctata* (Coleoptera: Coccinellidae) induced by diet and by infection with the larvae of *Dinocampus coccinellae* (Hymenoptera: Braconidae) using magnetic resonance imaging. *European Journal of Entomology* **97**, 457–461.

Greenstone, M.H. (1996) Serological analysis of arthropod predation: past, present and future, pp. 265–300 *in* Symondson, W.O.C. & Liddell, J.E. (*Eds*) *The ecology of agricultural pests – biochemical approaches*. London, Chapman and Hall.

Greenstone, M.H. & Edwards, M.J. (1998) DNA hybridization probe for endoparasitism by *Microplitis croceipes* (Hymenoptera: Braconidae). *Annals of the Entomological Society of America* **91**, 415–421.

Hagler, J.R. & Jackson, C.G. (1998) An immunomarking technique for labeling minute parasitoids. *Environmental Entomology* **27**, 1010–1016.

Harper, G.L., King, R.A., Dodd, C.S., Harwood, J.D., Glen, D.M., Bruford, M.W. & Symondson, W.O.C. (2005) Rapid screening of invertebrate predators for multiple prey DNA targets. *Molecular Ecology* **14**, 819–827.

Hart, A.G., Bowtell, R.W., Köckenberger, W., Wenseleers, T. & Ratneks, F.L.W. (2003) Magnetic resonance imaging in entomology: a critical review. *Journal of Insect Science* **3**, 1–9.

Hillis, D.M. & Dixon, M.T. (1991) Ribosomal DNA: molecular evolution and phylogenetic inference. *Quarterly Review of Biology* **66**, 411–453.

Hochuli, A. & Lanzrein, B. (2001) Characterization of a 212 kD protein, released into the host by the larva of the endoparasitoid *Chelonus inanitus* (Hymenoptera: Braconidae). *Journal of Insect Physiology* **47**, 1313–1319.

Hoy, M.A. (2003) *Insect molecular genetics*. 2nd edn. San Diego, California, Academic Press.

Hoy, M.A, Jeyaprakash, A., Morakote, R., Lo, P.K. & Nguyen, R. (2000) Genomic analyses of two populations of *Ageniaspis citricola* (Hymenoptera: Encyrtidae) suggest that a cryptic species may exist. *Biological Control* **17**, 1–10.

Hufbauer, R., Bogdanowicz, S.M. & Perez, L. (2001) Isolation and characterization of microsatellites in *Aphidius ervi* (Hymenoptera: Braconidae) and their applicability to related species. *Molecular Ecology Notes* **1**, 197–199.

Hufbauer, R.A., Bogdanowicz, S.M. & Harrison, R.G. (2004) The population genetics of a biological introduction: mitochondrial DNA and microsatellite variation in native and introduced populations of *Aphidius ervi*, a parasitoid wasp. *Molecular Ecology* **13**, 337–348.

Hung, A.C. & Huo, S. (1985) Malic enzyme, phosphoglucomutase and phosphoglucose isomerase enzymes in *Trichogramma* [Hym: Trichogrammatidae]. *Entomophaga* **30**, 143–149.

Iline, I.I. & Phillips, C.B. (2004) Allozyme markers to help define the South American origins of *Microctonus hyperodae* (Hymenoptera: Braconidae) established in New Zealand for biological control of Argentine stem weevil. *Bulletin of Entomological Research* **94**, 229–234.

Jeffries, M.J. & Lawton, J.H. (1984) Enemy free space and the structure of ecological communities. *Biological Journal of the Linnean Society* **23**, 269–286.

Jensen, M.K., Kester, M.K., Kankare, M. & Brown, B.L. (2002) Characterization of microsatellite loci in the parasitoid, *Cotesia congregate* (Say) (Hymenoptera: Braconidae). *Molecular Ecology Notes* **2**, 346–348.

Jones, D.B., Giles, K.L., Chen, Y. & Shufran, K.A. (2005) Estimation of hymenopteran parasitism in cereal aphids by using molecular markers. *Journal of Economic Entomology* **98**, 217–221.

Kazmer, D.J., Hopper, K.R., Coutinot, D.M. & Heckel, D.G. (1995) Suitability of random amplified polymorphic DNA for genetic markers in the aphid parasitoid, *Aphelinus asychis* Walker. *Biological Control* **5**, 503–512.

Keen, D.P., Keen, J.E., He, Y. & Jones, C.J. (2001) Development of an enzyme-linked immunosorbent assay for detection of the gregarious hymenopteran parasitoid *Muscidifurax raptorellus* in house fly pupae. *Biological Control* **21**, 140–151.

Kimani-Njogu, S.K., Overholt, W.A., Woolley, J.B. & Omwega, C.O. (1998) Electrophoretic and phylogenetic analyses of selected allopatric populations of the *Cotesia flavipes* complex (Hymenoptera: Braconidae), parasitoids of cereal stem borers. *Biochemical Systematics and Ecology* **26**, 285–296.

Kirk, A.A., Lacey, L.A., Brown, J.K., Ciomperlik, M.A., Goolsby, J.A., Vacek, D.C., Wendel, L.E. & Napompeth, B. (2000) Variation the *Bemisia tabaci s.l.* species complex (Hemiptera: Aleyrodidae) and its natural enemies leading to successful biological control of *Bemisia* biotype B in the USA. *Bulletin of Entomological Research* **90**, 317–327.

Kitthawee, S., Julsilikul, D., Sharpe, R.G. & Baimai, V. (1999) Protein polymorphism in natural populations of *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae) in Thailand. *Genetica* **105**, 125–131.

Kühn, S. & Hoffmann, M. (1999) Infection of *Coscinodiscus* spp. by the parasitoid nanoflagellate *Pirsonia diadema*: II. Selective infection behaviour for host species and individual host cells. *Journal of Plankton Research* **21**, 443–454.

Kuris, A.M., Torchin, M.E. & Lafferty, K.D. (2002) *Fecampia erythrocephala* rediscovered: prevalence and distribution of a parasitoid of the European shore crab, *Carcinus maenas*. *Journal of the Marine Biological Association of the United Kingdom* **82**, 955–960.

Lafferty, K.D. & Kuris, A.M. (2002) Trophic strategies, animal diversity and body size. *Trends in Ecology and Evolution* **17**, 507–513.

Landais, I., Chavigny, P., Castagnone, C., Pizzol, J., Abad, P. & Vanlerberghe-Masutti, F. (2000) Characterization of a highly conserved satellite DNA from the parasitoid wasp *Trichogramma brassicae* **255**, 65–73.

Landry, B.S., Dextraze, L. & Boivin, G. (1993) Random amplified polymorphic DNA markers for DNA fingerprinting and genetic variability of minute parasitic wasp species (Hymenoptera: Mymaridae and Trichogrammatidae) used in biological control programs of phytophagous insects. *Genome* **36**, 580–587.

Li, Y.-F., Korol, A.B., Fahima, T., Beiles, A. & Nevo, E. (2002) Microsatellites: genomic distribution, putative functions and mutational mechanisms: a review. *Molecular Ecology* **11**, 2453–2465.

Li, Z.-X. & Shen, Z.-R. (2002) PCR-based technique for identification and detection of *Trichogramma* spp. (Hymenoptera: Trichogrammatidae) with specific primers. *Entomologia Sinica* **9**, 9–16.

Liu, C.-L., You, L.-S., Luo, Q.-H., Ruan, Y., Huang, A.-P. & Guo, C. (2003) Random amplified polymorphic DNA of the similar braconid wasps in cotton field and paddy field from China (Hymenoptera, Braconidae). *Acta Zootaxonomica Sinica* **28**, 568–572.

Loxdale, H.D. & Lushai, G. (1999) Slaves of the environment: The movement of herbivorous insects in relation to their ecology and genotype. *Philosophical Transactions of the Royal Society of London B* **354**, 1479–1495.

Loxdale, H.D. & MacDonald, C. (2004) Tracking parasitoids at the farmland field scale using microsatellite markers, pp. 107–126 *in* Werner, D. (*Ed.*) *Biological resources and migration*. Proceedings of the international conference and OECD workshop, Phillips University, Marburg, Germany. Berlin, Springer-Verlag.

MacDonald, C. & Loxdale, H.D. (2004) Molecular markers to study population structure and dynamics in beneficial insects (predators and parasitoids). *International Journal of Pest Management* **50**, 215–224.

MacDonald, C., Brookes, C.P., Edwards, K.J., Baker, D.A., Lockton, S. & Loxdale, H.D. (2003) Microsatellite isolation and characterization in the beneficial parasitoid wasp *Diaretiella rapae* (M'Intosh) (Hymenoptera: Braconidae: Aphidiinae). *Molecular Ecology Notes* **3**, 601–603.

May, B., Leonard, D.E. & Vadas, R.L. (1977) Electrophoretic variation and sex linkage in spruce budworm. *Journal of Heredity* **68**, 355–359.

Morgan, P.B., Jones, C.J., Patterson, R.S. & Milne, D. (1988) Use of electrophoresis for monitoring purity of laboratory colonies of exotic parasitoids (Hymenoptera: Pteromalidae). *Advances in Parasitic Hymenoptera Research*, **1988**, 525–531.

Orrego, C. & Agudelo-Silva, F. (1993) Genetic variation in the parasitoid wasp *Trichogramma* (Hymenoptera: Trichogrammatidae) revealed by DNA amplification of a section of the nuclear ribosomal repeat. *Florida Entomologist* **76**, 519–524.

Paran, I. & Michelmore, R.W. (1993) Development of reliable PCR-based markers linked to downy mildew resistance genes in lettuce. *Theoretical and Applied Genetics* **85**, 985–993.

Parks, S.-J., Taton, T.A. & Mirkin, C.A. (2002) Array-based electrical detection of DNA with nanonparticle probes. *Science* **295**, 1503–1506.

Persad, A.B., Jeyaprakash, A. & Hoy, M.A. (2004) High-fidelity PCR assay discriminates between immature *Lipolexis oregmae* and *Lysiphlebus testaceipes* (Hymenoptera: Aphidiidae) within their aphid hosts. *Florida Entomologist* **87**, 18–24.

Pinto, J.D., Kazmer, D.J., Planter, G.R. & Sassaman, C.A. (1992) Taxonomy of the *Trichogramma minutum* complex (Hymenoptera: Trichogrammatidae): allozyme variation and its relationship to reproductive and geographic data. *Annals of the Entomological Society of America* **85**, 413–422.

Pinto, J.D., Platner, G.R. & Sassaman, C.A. (1993) Electrophoretic study of two closely related species of North American *Trichogramma: T. pretiosum* and *T. deion* (Hymenoptera: Trichogrammatidae). *Annals of the Entomological Society of America* **86**, 702–709.

Pinto, J.D., Platner, G.R. & Stouthamer, R. (2003) The systematics of the *Trichogramma minutum* species complex (Hymenoptera: Trichogrammatidae), a group of important North American biological control agents: the evidence from reproductive compatibility and allozymes. *Biological Control* **27**, 167–180.

Pintureau, B. (1993) Enzyme polymorphism in some African, American, and Asiatic *Trichogramma* and *Trichogrammatoidea* species (Hymenoptera: Trichogrammatidae). *Biochemical Systematics and Ecology* **21**, 557–573.

Prinsloo, G., Chen, Y., Giles, L. & Greenstone, M.H. (2002) Release and recovery in South Africa of the exotic aphid parasitoid *Aphelinus hordei* verified by the polymerase chain reaction. *BioControl* **47**, 127–136.

Pungerl, N.B. (1986) Morphometric and electrophoretic study of *Aphidius* species (Hymenoptera: Aphidiidae) reared from a variety of aphid hosts. *Systematic Entomology* **11**, 327–354.

Ram, P., Tshernyshev, W.B., Afonina, V.M., Greenberg, S.M. & Ageeva, L.I. (1995) Studies on strains of *Trichogramma evanescens* Westwood from different regions of Eurasia. *Biocontrol Science and Technology* **5**, 329–338.

Ratcliffe, S.T., Robertson, H.M., Jones, C.J., Bollero, G.A. & Weinzerl, R.A. (2002) Assessment of parasitism of house fly and stable fly (Diptera: Muscidae) pupae by pteromalid (Hymenoptera: Pteromalidae) parasitoids using a polymerase chain reaction. *Journal of Medical Entomology* **39**, 52–60.

Richardson, B.J., Baverstock, P.R. & Adams, M. (1986) *Allozyme electrophoresis. A handbook for animal systematics and population studies*. New York, Academic Press.

Roehrdanz, R.L., Reed, D.K. & Burton, R.L. (1993) Use of polymerase chain reaction and arbitrary primers to distinguish laboratory-raised colonies of parasitic Hymenoptera. *Biological Control* **3**, 199–206.

Sappal, N.P., Jeng, R.S., Hubbes, M. & Liu, F. (1995) Restriction fragment length polymorphisms in polymerase chain reaction amplified ribosomal DNAs of three *Trichogramma* species (Hymenoptera: Trichogrammatidae). *Genome* **38**, 419–425.

Schneider, M.V., Driessen, G., Beukeboom, L.W., Boll, R., van Eunen, K., Selzner, A., Talsma, J. & Lapchin, L. (2003) Gene flow between arrhenotokous and thelytokous populations of *Venturia canescens* (Hymenoptera). *Heredity* **90**, 260–267.

Silva, I.M.M.S., Honda, J., van Kan, F., Hu, J., Neto, L., Pintureau, B. & Stouthamer, R. (1999) Molecular differentiation of five *Trichogramma* species occurring in Portugal. *Biological Control* **16**, 177–184.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P. (1994) Evolution, weightings, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America* **87**, 651–701.

Soldevila, A.I. & Jones, D. (1991) Immunoanalysis of unique protein in *Trichoplusia ni* parasitized by the braconid wasp *Chelonus* near *curvimaculatus*. *Insect Biochemistry* **21**, 845–856.

Stouthamer, R., Hu, J., van Kan, F.J.P.M., Platner, G.R. & Pinto, J.D. (1999) The utility of internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of *Trichogramma*. *BioControl* **43**, 421–440.

Stouthamer, R., Gai, Y., Koopmanschap, A.B., Platner, G.R. & Pinto, J.D. (2000) ITS-2 sequences do not differ for the closely related species *Trichogramma minutum* and *T. platneri*. *Entomologia Experimentalis et Applicata* **95**, 105–111.

Stuart, M.K. (1998) An antibody diagnostic for hymenopteran parasitism is specific for a homologue of elongation factor-1α. *Archives of Insect Biochemistry and Physiology* **39**, 1–8.

Stuart, M.K. & Burkholder, W.E. (1991) Monoclonal antibodies specific for *Laelius pedatus* (Bethylidae) and *Bracon hebetor* (Braconidae), two hymenopterous parasitoids of stored-product pests. *Biological Control* **1**, 302–308.

Stuart, M.K. & Greenstone, M.H. (1996) Serological diagnosis of parasitism: a monoclonal antibody-based immunodot assay for *Microplitis croceipes* (Hymenoptera: Braconidae). pp. 301–321 *in* Symondson, W.O.C. & Liddell, J.E. (*Eds*) *The ecology of agricultural pests*. London, Chapman & Hall.

Stuart, M.K. & Greenstone, M.H. (1997) Immunological detection of hymenopteran parasitism in *Helicoverpa zea* and *Heliothis virescens*. *Biological Control* **8**, 197–202.

Suckling, D.M., Burnip, G.M., Gibb, A.R., Daly, J.M. & Armstrong, K.F. (2001) Plant and host effects on the leafroller parasitoid *Dolichogenidia tasmanica*. *Entomologia Experimentalis et Applicata* **100**, 253–260.

Sunnucks, P. & Hales, D.F. (1996) Numerous transposed sequences of mitochondrial cytochrome oxidase I-II in aphids of the genus *Sitobion* (Hemiptera: Aphidiae). *Molecular Biology and Evolution* **13**, 510–524.

Sunnucks, P., Wilson, A.C.C., Beheregaray, L.B., Zenger, K., French, J. & Taylor, A.C. (2000) SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and ecology. *Molecular Ecology* **9**, 1699–1710.

Takada, H. (1998) A review of *Aphidius colemani* (Hymenoptera: Braconidae; Aphidiinae) and closely related species

indigenous to Japan. *Applied Entomology and Zoology* **33**, 59–66.

Taton, T.A., Mirkin, C.A. & Letsinger, R.L. (2000) Scanometric DNA array detection with nanoparticle probes. *Science* **289**, 1757–1760.

Taylor, D.B. & Szalanski, A.L. (1999) Identification of *Muscidifurax* spp. by polymerase chain reaction-restriction fragment length polymorphism. *Biological Control* **15**, 270–273.

Tilmon, K.J., Danforth, B.N., Day, W.H. & Hoffmann, M.P. (2000) Determining parasitoid species composition in a host population: a molecular approach. *Annals of the Entomological Society of America* **93**, 640–647.

Tomiuk, J., Wöhrmann, K. & Eggers-Schumacher, H.A. (1979) Enzyme patterns as a characteristic for the identification of aphids. *Zeitschrift für Angewandte Entomologie* **88**, 440–446.

Unruh, T.R., White, W., Gonzales, D., Gordh, G. & Luck, R.F. (1983) Heterozygosity and effective population size in laboratory populations of *Aphidius ervi* (Hym.: Aphidiidae). *Entomophaga* **28**, 245–258.

Vanlerberghe-Masutti, F. & Chavigny, P. (1997) Characterization of a microsatellite locus in the parasitoid wasp *Aphelinus abdominalis* (Hymenoptera: Aphelinidae). *Bulletin of Entomological Research* **87**, 313–318.

Vaughn, T.T. & Antolin, M.F. (1998) Population genetics of an opportunistic parasitoid in an agricultural landscape. *Heredity* **80**, 152–162.

Walton, M.P., Loxdale, H.D. & Allen-Williams, L. (1990a) Electrophoretic 'keys' for the identification of parasitoids (Hymenoptera: Braconidae: Aphelinidae) attacking *Sitobion avenae* (F.) (Hemiptera: Aphididae). *Biological Journal of the Linnean Society* **40**, 333–346.

Walton, M.P., Powell, W., Loxdale, H.D. & Allen-Williams, L. (1990b) Electrophoresis as a tool for estimating levels of hymenopterous parasitism in field populations of the cereal aphid, *Sitobion avenae*. *Entomologia Experimentalis et Applicata* **54**, 271–279.

Weathersbee, A.A., III, Shufran, K.A., Panchal, T.D., Dang, P.M. & Evans, G.A. (2004) Detection and differentiation of parasitoids (Hymenoptera: Aphidiidae and Aphelinidae) of the brown citrus aphid (Homoptera: Aphididae): Species-specific polymerase chain reaction amplification of 18S rDNA. *Annals of the Entomological Society of America* **97**, 286–292.

Wool, W., van Emden, H.F. & Bunting, S.W. (1978) Electrophoretic detection of the internal parasite, *Aphidius matricariae* in *Myzus persicae*. *Annals of Applied Biology* **90**, 21–26.

Wynne, I.R., Loxdale, H.D. & Brookes, C.P. (1992) Use of a cellulose acetate system for allozyme electrophoresis. pp. 494–499 *in* Berry, R.J., Crawford, T.J. & Hewitt, G.M. (*Eds*) *Genes in ecology*. Oxford, Blackwells Scientific Publications.

Yazlovetskii, I.G., Sumenkova, V.V., Ageeva, L.I. & Dyurich, G.F. (1981) Species identification of *Trichogramma* by electrophoresis in polyacrylamide gel. *Soviet Agricultural Science* **9**, 40–43.

Zhu, Y.-C. & Greenstone, M.H. (1999) Polymerase chain reaction techniques for distinguishing three species and two strains of *Aphelinus* (Hymenoptera: Aphelinidae) from *Diuraphis noxia* and *Schizaphis graminum* (Homoptera: Aphididae). *Annals of the Entomological Society of America* **92**, 71–79.

Zhu, Y.-C. & Williams, L., III (2002) Detecting the egg parasitoid *Anaphes iole* (Hymenoptera: Mymaridae) in tarnished plant bug (Heteroptera: Miridae) eggs by using a molecular approach. *Annals of the Entomological Society of America* **95**, 359–365.

Zhu, Y.-C., Burd, J.D., Elliott, N.C. & Greenstone, M.H. (2000) Specific ribosomal DNA marker for early polymerase chain reaction detection of *Aphelinus hordei* (Hymenoptera: Aphelinidae) and *Aphidius colemani* (Hymenoptera: Aphidiidae) from *Diuraphis noxia* (Homoptera: Aphididae). *Annals of the Entomological Society of America* **93**, 486–491.

Zhu, Y.-C., Dowdy, A.K. & Baker, J.E. (1999) Detection of single-base substitution in an esterase gene and its linkage to malathion resistance in the parasitoid *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae). *Pesticide Science* **55**, 398–404.

Zhu, Y.-C., Dowdy, A.K. & Baker, J.E. (2004a) Molecular differentiation of two strains of the parasitoid *Anisopteromalus calandrae* (Hymenoptera: Pteromalidae) using specific PCR primers. *Journal of Entomological Science* **39**, 1–10.

Zhu, Y.C., Riddick, E.W., Williams, L., Schotzko, D.J., Logarzo, G.A & Jackson, C.G. (2004b) Potential of detection and identification of nymphal parasitoids (Hymenoptera: Braconidae) of *Lygus* bugs (Heteroptera: Miridae) by using polymerase chain reaction and ITS2 sequence analysis techniques. *Annals of the Entomological Society of America* **97**, 743–752.

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