Bulletin of Entomological Research (2010) **100**, 145–152
doi:10.1017/S0007485309006920
© Cambridge University Press 2009
First published online 8 June 2009

Tracking medfly predation by the wolf spider, *Pardosa cribata* Simon, in citrus orchards using PCR-based gut-content analysis

C. Monzó[1,2], B. Sabater-Muñoz[1]*, A. Urbaneja[1] and P. Castañera[2]

Unidad Asociada de Entomología del Instituto Valenciano de Investigaciones Agrarias y Centro de Investigaciones Biologicas del Consejo Superior de Investigaciones Científicas: [1]Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias, Carretera de Moncada a Náquera km 4,5. Apartado 46113 Moncada, Valencia, Spain: [2]Departamento Biología de Plantas, Centro de Investigaciones Biológicas, C/ Ramiro de Maeztu, 9. 28040 Madrid, Spain

Abstract

The Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), which is often controlled chemically, is a major citrus pest in Spain; however, alternative biological control strategies such as those based on the conservation of polyphagous predators should be developed. The wolf spider, *Pardosa cribata* Simon, is an abundant predator found in citrus orchards in eastern Spain. In this study, we have evaluated polymerase chain reaction (PCR)-based techniques as a means of detecting *C. capitata* DNA remains in *P. cribata* specimens. To do so, two pairs of *C. capitata* species-specific primers were designed and tested. Primer specificity was tested on species closely related to *C. capitata* and with other pests and natural enemies present in citrus orchards. Medfly DNA was detectable in 100% of *P. cribata* from 0 to 12 h post ingestion for both primer pairs, decreasing to 37% at 96 h after prey ingestion for one pair of primers. DNA detectability half-lives were of 78.25 h and 78.08 h for each pair of primers but no statistical differences were found between them. *Pardosa cribata* specimens were field-collected daily after sterile *C. capitata* pupae had been deployed in the citrus orchard. Afterwards, the wolf spiders were analyzed and DNA remains of *C. capitata* were detected in 5% of them, with a peak of 15% coinciding with maximum *C. capitata* emergence. This study is the first to reveal the potential use of DNA markers to track medfly predation by *P. cribata* in citrus orchards and provides a new tool to estimate the potential role of this spider in biological-control conservation programs.

Keywords: prey DNA degradation, diagnostic PCR, wolf spider, medfly

(Accepted 2 March 2009)

*Author for correspondence
Fax: +34 963424001
E-mail: sabater_bea@gva.es

https://doi.org/10.1017/S0007485309006920 Published online by Cambridge University Press

Introduction

The Mediterranean fruit fly or medfly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), is one of the most devastating fruit pests worldwide. In Spain, current medfly control has been based primarily on aerial and ground applications of organophosphate insecticides, especially malathion, mixed with protein baits, although the use of the naturally derived compound spinosad is being promoted as an alternative (Chueca *et al*., 2007). Chemical approaches affect both the food quality and the environment and can induce resistance, as detected recently in Mediterranean populations that have become resistant to malathion (Magaña *et al*., 2007, 2008). Effective control of *C. capitata* requires an area-wide, multitactic pest management program. In recent years, emphasis has been placed on implementing environmentally friendly methods to control medfly in Spain. To this end, different biological control approaches are currently being implemented. One of them involves identifying and conserving polyphagous ground-dwelling predators of the medfly (Urbaneja *et al*., 2006). With respect to *C. capitata*, three developmental stages can be found in the soil of citrus orchards, late third-instars larvae, pupae and teneral adults, which remain on the soil until they are able to fly. All three stages are susceptible to being preyed upon by ground-dwelling predators.

Generalist predators play a major role in biological control of agricultural pests (Legaspi *et al*., 1996; Symondson *et al*., 1996, 2002a,b; Morris *et al*., 1999; Sheppard *et al*., 2004; Foltan *et al*., 2005). Many ground-dwelling predators have been recorded in citrus orchards located in Valencia, Spain. Rove beetles (Coleoptera: Staphylinidae) are the most abundant-active group, representing 38.6% of the total number of predators collected, followed by spiders (Arachnida: Araneae) (28.9%), earwigs (Dermaptera) (18.0%) and ground beetles (Coleoptera: Carabidae) (12.7%) (Monzó *et al*., 2007). Spiders are important generalist predators in agricultural habitats, but their effects on regulating pest populations are poorly known (Greenstone, 1999; Hagen *et al*., 1999; Marc *et al*., 1999; Sunderland, 1999). In a previous study, we found over 50 spider species present in eastern Spanish citrus orchards (Monzó *et al*., 2007), with the most common being the generalist predator *Pardosa cribata* Simon (Araneae: Lycosidae), representing about 19.3%, present throughout the year on the ground in citrus orchards. We have found that *P. cribata* is a highly efficient predator on *C. capitata* under laboratory conditions (Monzó *et al*., 2009). Nevertheless, whether this predator plays a role in regulating *C. capitata* under field conditions is as yet unknown.

Different approaches have been developed to deal with the inherent difficulties of detecting arthropod predation by generalist predators. Monoclonal antibodies and isoenzyme-electrophoresis have been used successfully to detect prey-specific protein within predators under field conditions (Hagler *et al*., 1994, 1997; Agustí *et al*., 1999a; Symondson *et al*., 1999; Traugott, 2003). Nonetheless, these approaches show specific disadvantages and limitations (Juen & Traugott, 2005). Polymerase chain reaction (PCR)-based techniques are increasingly becoming recognized as valuable tools in ecological studies and, recently, have been reviewed for predator/prey identification and detection in the context of their contributions to biological control of arthropods (King *et al*., 2008).

At present, DNA-based techniques are the state-of-the-art for gut content analysis, mainly PCR-based approaches (Agustí *et al*., 1999b; Zaidi *et al*., 1999; Chen *et al*., 2000; Agustí *et al*., 2003a,b; Sheppard *et al*., 2004, 2005; Harper *et al*., 2005). The advantages of this approach are: (i) the techniques to develop molecular probes have become cheaper and more available; (ii) candidate target regions have already been sequenced for a great number of insects, and are increasing; and (iii) once prey specific-primers have been published, they can be used in reproducible protocols worldwide.

Efficient molecular gut content analysis is a powerful tool to establish biological control programs targeting arthropod pests (e.g. Morris *et al*., 1999; Fournier *et al*., 2008). These prey-detection PCR-based techniques have been applied in laboratory conditions to investigate predator-prey trophic interactions; however, applying them to field conditions requires additional work (Harwood & Obrycki, 2005). It appears that, at least in some cases, spiders play an efficient role in limiting pests in agro-ecosystems (King *et al*., 2008).

Here, we report on the development of species-specific PCR primers and protocols, utilizing them to detect *C. capitata* DNA in the guts of *P. cribata* and, thereby, developing a rapid and suitable protocol to track medfly predation by *P. cribata* under field conditions.

Materials and methods

Arthropods

Forty-two arthropod species (phytophagous or predaceous) were chosen to develop *C. capitata* specific primers (table 1). Live arthropods were collected mainly from fields in the Valencia region (Spain). *Bactrocera* species, except *B. oleae* (Gemli), were trapped in sticky traps in Queensland (Australia) by the DPI & F Staff. *Anastrepha suspensa* (Loew) was collected in guava fruits in Florida (USA). A laboratory strain of *C. capitata*, IVIA2000 (San Andrés *et al*., 2007), and the Vienna-8 *tsl* strain from the mass-rearing facility at Caudete de las Fuentes (Valencia, Spain) were used as reference strains and for field assays.

Primer design

Medfly species-specific primers

Internal transcribed spacer 1 (ITS1) sequences from Tephritidae were retrieved from the GenBank database [AF307848 (*C. capitata*), AF189690 (*Ceratitis rosa* Karsch), AF276515 (*Bactrocera cucurbitae* Coquillett) and AF276516 (*Bactrocera dorsalis* (Hendel))] and aligned using GeneDoc software (Nicholas *et al*., 1997), applying an MSA algorithm implemented in the software with blossom 62 as a scoring table, a constant cost length of 20, a gap open cost of 8 and gap extension cost of 4. Three primers (CcITS1-2dir, CcITS1-2rev and CcITS737) were designed on *C. capitata* ITS1 sequence using OLIGO v4 primer analysis software (Rychlik, 1992) (table 2).

Universal primers

A pair of primers (18S_lo1270 and 18S_up1060) was designed on a conserved region of 18S rDNA alignment from sequences AF096450 (*C. capitata*), AF012518 (*Cicindela sedecimpunctata* Klug), Z97594 (*Forficula auricularia* Linneus.),

https://doi.org/10.1017/S0007485309006920 Published online by Cambridge University Press

https://doi.org/10.1017/S0007485309006920 Published online by Cambridge University Press

Table 1. Arthropod species used to screen Cc-specific primers grouped as predator or prey, including order and family level, locality and country of sampling and assigned code.

Group*	Order	Family	Species	Locality	Country	Code	Fig 1 sample	Number of specimens tested
Predators	Acari	Phytoseiidae	*Phytoseiulus persimilis* (Athias-Henriot)	IVIA Lab strain	Spain	Ppe	–	3
			Neoseiulus californicus (McGregor)	IVIA Lab strain	Spain	Nca	–	3
	Araneae	Gnaphosidae	*Trachyzelotes fuscipes* (Koch)	Olocau, VLC	Spain	Tfu	–	3
			Nomisia exornata (Koch)	Bétera, VLC	Spain	Nex	–	1
		Linyphiidae	*Erigone dentipalpis* (Wider)	Bétera, VLC	Spain	Ede	–	2
			Meioneta fuscipalpis (Koch)	Olocau, VLC	Spain	Mfu	–	3
			Pelecopsis inedita (Cambridge)	Olocau, VLC	Spain	Pin	–	2
		Lycosidae	*Pardosa cribata* Simon	Bétera, VLC	Spain	Pc	1	> 50
		Nemesiidae	*Nemesia dubia* Cambridge	Bétera, VLC	Spain	Ndu	–	2
		Thomisidae	*Xysticus bliteus* (Simon)	Bétera, VLC	Spain	Xbl	–	2
		Thomisidae	*Xysticus nubilus* Simon	Bétera, VLC	Spain	Xnu	–	2
		Zodariidae	*Zodarion pusio* Simon	Moncada, VLC	Spain	Zpu	–	3
	Coleoptera	Cicindelidae	*Cicindela campestris* L.	Liria, VLC	Spain	Cca	6	2
		Carabidae	*Pseudophonus rufipes* (Degeer)	Liria, VLC	Spain	Psr	7	20
		Coccinelidae	*Cryptolaemus montrouzieri* Mulsant	Almazora, CS	Spain	Cmo	8	5
	Dermaptera		*Forficula auricularia* L.	Moncada, VLC	Spain	Fau	4	20
			Euborellia moesta (Gené)	Liria, VLC	Spain	Emo	5	2
	Hymenoptera	Formicidae	*Formica rufibarbis* Fabricius	Liria, VLC	Spain	Fru	2	6
			Messor barbarus (L.)	Liria, VLC	Spain	Mba	3	6
Preys	Acari	Tetranychidae	*Tetranychus urticae* Koch	IVIA Lab strain	Spain	Tur	9	10
			Panonychus citri (McGregor)	Almussafes, VLC	Spain	Pci	–	2
	Collembola		*Entomobrya* sp.	Liria, VLC	Spain	Esp	–	2
	Diptera	Agromicidae	*Liriomyza* sp.	Liria, VLC	Spain	Lsp	–	2
		Calliphoridae	*Calliphora* sp.	Liria, VLC	Spain	Cal	–	1
		Cecidomyiidae	*Feltiella acarisuga* (Vallot)	La Mojonera, ALM	Spain	Fac	–	3
		Drosophilidae	*Drosophila melanogaster* Meigen	Yellow strain	Spain	DmY	–	5
				Moncada, VLC	Spain	Dmel	–	3
		Sciaridae	Undetermined species	Liria, VLC	Spain	Sci	–	2
		Syrphidae	*Episyrphus balteatus* (De Geer)	La Mojonera, ALM	Spain	Eba	–	2
		Tephritidae	*Anastrepha suspensa* (Loew)	Immokalee, FL	USA	Asu	13	5
			Bactrocera (D.) oleae (Gemlin)	Almussafes, VLC	Spain	Bol	14	2
			Bactrocera tryoni (Froggatt)	Cairns, QL	Australia	Btr	15	5
			Bactrocera neohumeralis (Hardy)	Cairns, QL	Australia	Bne	16	5
			Bactrocera musae (Tryon)	Cairns, QL	Australia	Bmu	17	5
			Bactrocera frauenfeldi (Schiner)	Cairns, QL	Australia	Bfr	18	5
			Ceratitis rosa Karsch	Hex River Valley	South Africa	Cro	19	5
			Ceratitis capitata Wiedemann	IVIA Lab strain, Vienna-8 strain	Spain	Cc Ccv8#	20, 21	5, 3
	Hemiptera	Aphididae	*Myzus persicae* (Sulzer)	Picassent, VLC	Spain	Mpe	10	8
			Aphis gossypii Glover	Picassent, VLC	Spain	Ago	11	10
			Aphis spireaecola Patch	Picassent, VLC	Spain	Asp	12	7
	Lepidoptera	Gelechiidae	*Tuta absoluta* (Meyrick)	IVIA Lab strain	Spain	Tab	–	3
		Noctuidae	*Spodoptera exigua* (Hübner)	Lab strain, ALM	Spain	Sex	–	2
	Thysanoptera	Thripidae	*Frankliniella occidentalis* (Pergande)	IVIA Lab strain	Spain	Foc	–	4

* , not at taxonomical level; –, not presented in fig. 1. # Cc for the IVIA2000 strain and Ccv8 for the Vienna-8 *tsl* strain. VLC, Valencia; CS, Castellón; ALM, Almeria; FL, Florida; QL, Queensland.

Table 2. Primer sequences (5′–3′) and PCR conditions.

Primer pair name	Forward primer sequence	Reverse primer sequence	Ta (°C)	Amplicon size (bp)
cITS1-2	TAA ATG GAT GAA AGA AGA TGA TG	GCC GAG TGA TCC ACC GCT TAG AG	55	333
CcITS737	TAA ATG GAT GAA AGA AGA TGA TG	TAG TCC AAC AAA AAC AGT AT	50	130
Univ18SrDNA	AGT TAG AGG TTC GAA GGC GAT CAG	TGG TAA GTT TTC CCG TGT TGA GTC	55	233

Ta, annealing temperature.

X89492 (*Leptothorax acervorum* (Fabricius)), EF012870 (*Lasius* (Acanthomyops) *californicus* (Wheeler)), AF487712 (*Myzus persicae* (Sulzer)) and AF062961 (*Tetranychus urticae* Koch) obtained as described above.

Amplification conditions

Each primer pair was used in 20 μl volume reactions, containing 300 nM dNTPs (Eppendorf AG, Hamburg, Deutschland), 1 × DNA pol buffer (Biotools B&M labs S.A., Madrid, Spain), 3 mM $MgCl_2$ (Biotools), 0.75 u DNA polymerase (Biotools), 10 pmol each primer and 10 ng of total DNA. Amplification profile was one denaturation step at 94°C for 2 min, 40 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 15 s, followed by a final extension at 72°C for 150 s. Amplification was performed in a Mastercycler® ep gradient-S thermal cycler (Eppendorf). PCR products were run in a 2% agarose D-1 low EEO (Pronadisa, Sumilab S.L., Madrid, Spain) gel in 0.5 × TBE buffer, stained with ethidium bromide and visualized under UV light.

DNA extraction

All *P. cribata* specimens were frozen (−80°C, 20 min) and dissected, then total DNA was extracted from the opisthosoma following the 'Salting-out' protocol (Sunnucks & Hales, 1996), adding fresh Proteinase-K at 100 μg ml^{-1} after tissue homogenization. The prosoma of each spider was retained in order to compare the results with an immunological assay being developed in parallel (Monzó *et al.*, unpublished data). Other specimens (table 1) were subjected either to the protocol of Latorre *et al.* (1986) or the 'salting-out' as described above for total DNA extraction of complete specimens. Total DNA was finally dissolved in 20–100 μl LTE-R (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0, 6 μg ml^{-1} RNase A), depending on specimen size. DNA integrity was verified by gel electrophoresis in 1% agarose gel and concentration adjusted to 5–10 ng $μl^{-1}$ for PCR amplification.

Test for cross-reactivity and sensitivity

Primer specificity was tested by assaying CcITS primer pairs with total DNA from several individuals of the two strains of *C. capitata*, several closely related species and a wide range of arthropods (prey and predators) potentially present in the same citrus orchards (table 1). Furthermore, we have included other tephritids species, considered exotic in Spain (except *B. oleae*), but potentially present in other regions where *C. capitata* coexist with these tephritids.

Sensitivity was determined by assaying both CcITS primer pairs with ten-fold dilution of *C. capitata* total DNA starting with 10 ng till $1:10^{-10}$ dilution.

Detection period

Live adult specimens of *P. cribata* were collected from citrus orchards close to the Instituto Valenciano de Investigaciones Agrarias (IVIA) in individual 150-ml containers. Spiders were starved (water was supplied daily on soaked cotton) for a seven-day period at 25°C and 16 : 8 h (L : D) photoperiod. After starvation, one medfly adult from the IVIA2000 colony was offered to each spider, and time was set to zero from the moment of medfly capture. Spiders were allowed to feed on medfly over a three-hour period (ingestion period). Afterwards, any remaining prey were withdrawn, and spiders were frozen immediately ($t = 0$) or maintained in starvation (given only water) for 6, 12, 24, 36, 48, 72 or 96 h at 25°C and 16 : 8 h L : D until freezing at −80°C for subsequent molecular assay. Twenty replicates per time were conducted (except for 96 h where 19 spiders were available). Additional spiders ($n = 20$) were starved for seven days and frozen for use as negative controls in the PCR.

DNA from each spider was tested in triplicate by PCR (Agustí *et al.*, 2003a). Each sample scored as zero was tested with the 18S primer pair to assess whether PCR failure was due to a lack of *C. capitata* DNA.

For each pair of primers, data of positive detections were subjected to Probit analysis using Proc Probit in PC SAS version 9.1.3. Chi-square (χ^2) tests were performed to determine the fitting of data to the Probit model. Detectability half-lives (post-ingestion time during which 50% of positives were still detectable) were obtained (Chen *et al.*, 2000). In addition, a χ^2 test of equality was performed to assess whether there were statistical differences between the two primer-pair data sets, by testing whether the slopes and intercepts of each data fitting were the same.

Field assay

The field test was conducted in a 1 ha clementine citrus orchard located in Bétera (UTM X722106 Y4388610; Z30 m altitude) surrounded by other orchards, with spontaneous natural cover crop and drip-irrigation system. High populations of *P. cribata* had previously been documented in this plot (Monzó *et al.*, 2007). About 40,000 Vienna-8 *tsl* sterile male pupae were deployed in the orchard to simulate a high natural medfly infestation (R. Argilés & I. Pla, personal communication). Twenty adult *P. cribata* were randomly captured by hand in the central area of the plot at 24, 48, 72, 96 h and seven days after the release of medfly pupae. All collected individuals were immediately taken to the laboratory and frozen for later DNA extraction. DNA from each spider was tested by PCR using the CcITS737 primer pair in triplicate and scored, as previously mentioned, depending on the number of positives. The percentage of samples with a positive PCR reaction was obtained for the entire assay and for every capturing day. To obtain data about the daily

https://doi.org/10.1017/S0007485309006920 Published online by Cambridge University Press

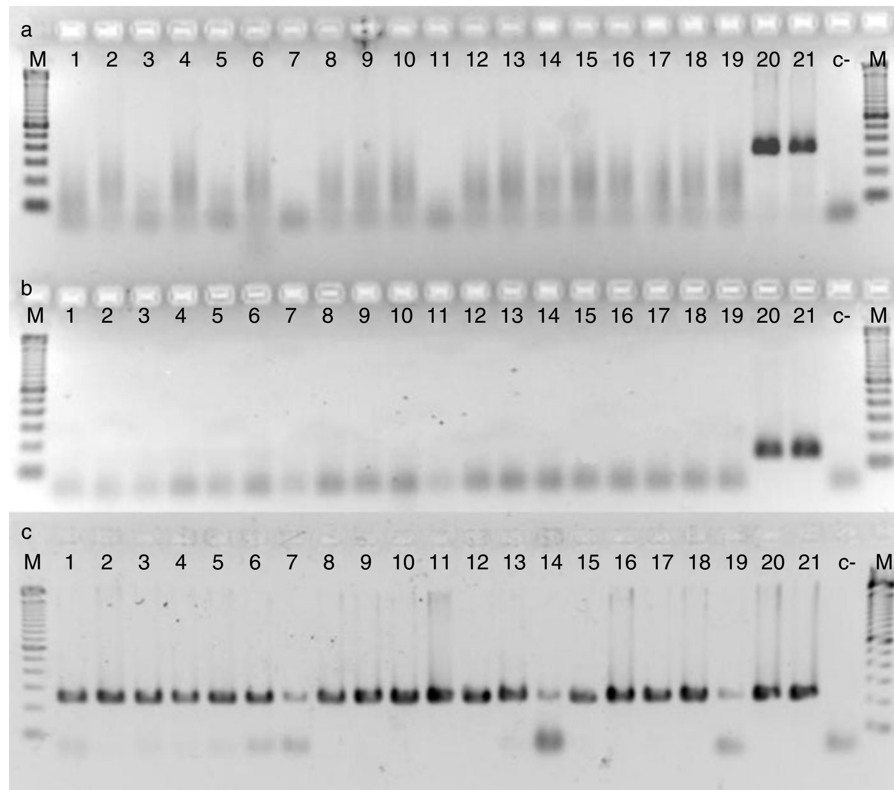

Fig. 1. *Ceratitis capitata* species-specific amplification pattern of ITS primer sets with 18SrDNA control on several arthropods. (a) Specific amplification of ITS1_2 primer pair. (b) Specific amplification of ITS737 primer pair. (c) Control amplification with Univ18SrDNA universal primer pair. M, 100 pb ladder; c-, PCR negative control. Samples 1 to 21 correspond to species coded in the same order in table 1 (Pc, Fru, Mba, Fau, Emo, Cca, Psr, Cmo, Tur, Mpe, Ago, Asp, Asu, Bol, Btr, Bne, Bmu, Bfr, Cro, Cc and Ccv8).

emergence rate (FTD) of Vienna-8 medfly adults, four modified McPhail traps were placed in the orchard, baited with trimedlure® and a tablet of the insecticide dichlorvos® (Biagro, S.L., Valencia, Spain).

Results

Primer design and cross-reactivity

The CcITS1-2 primer pair (CcITS1-2dir and CcITS1-2rev) was first designed on ITS-1 region alignment. CcITS1-2rev was designed on a conserved region of 5.8S rDNA close to ITS-1 end. This primer pair amplified a 330-bp fragment from *C. capitata* DNA, whereas no such product was detected in other tephritids or other arthropods tested (fig. 1a). However, this primer pair produced a smear background in all samples, except in negative controls, suggesting that the conserved CcITS1-2rev primer also binds to *P. cribata* DNA and to other non-target arthropods. A third primer, CcITS737, was then designed on a *C. capitata* specific region, which in combination with CcITS1-2dir primer (CcITS737 primer pair) gives a 130-bp fragment only detectable in *C. capitata* DNA (fig. 1b). The universal primer pair Univ18SrDNA was used to demonstrate *C. capitata* specificity of ITS-1 primers (fig. 1c), as well as to discriminate between unsuccessful CcITS amplification (absence of target-DNA) and a lack of DNA in the PCR reaction (target and non-target DNA).

Both primer pairs were tested for sensitivity, detecting as little as 0.1 pg of *C. capitata* DNA (fig. 2).

Detection period

The proportion of positive detection data fitted the assumptions of the Probit model for both pairs of primers ($\chi^2 = 0.2055$, df = 3, $P = 0.977$ for CcITS1-2 and $\chi^2 = 0.1519$, df = 3, $P = 0.985$ for CcITS737) (fig. 3). Detectability half-lives were of 78.25 h and 78.08 h for CcITS1-2 and CcITS737, respectively. No statistical differences were obtained between both half-lives; indeed, when testing the equality for both sets of data, statistical differences were found for neither ($\chi^2 = 1.9407$, df = 1, $P = 0.1636$).

Field assay

A total of 100 *P. cribata* individuals were analyzed in the field experiment. Five percent of the spiders tested positive for the entire assay. When analyzing the data per sampling day (fig. 4), this rate rose to 15% at 72 h after artificial infestation. The number of medflies captured in the plot gradually increased from 24 h until 96 h after infestation, when the maximum was registered (84.2 ± 18.3 FTD). From here on, the number of medfly captures decreased and leveled off at approximately 25 FTD.

https://doi.org/10.1017/S0007485309006920 Published online by Cambridge University Press

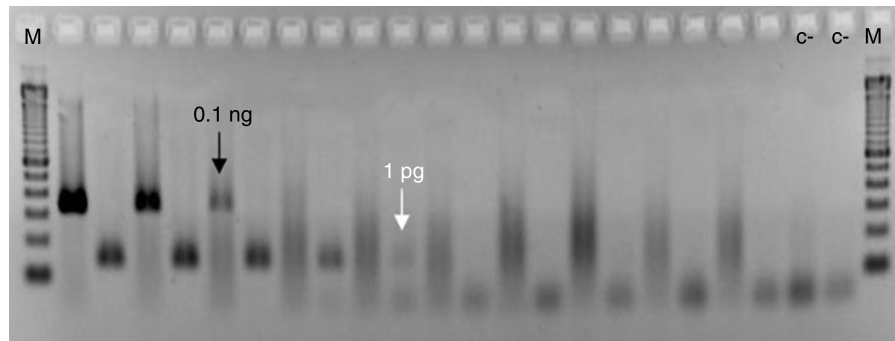

Fig. 2. Sensitivity of CcITS primer sets on *C. capitata DNA* serial dilutions. M, molecular marker 100 pb ladder; c-, PCR negative control.

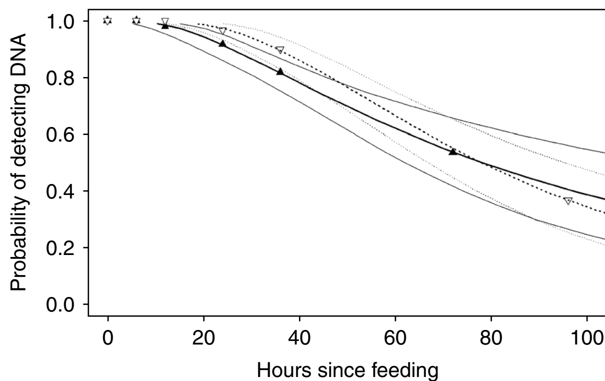

Fig. 3. The *Ceratitis capitata* DNA detection probability curves in *Pardosa cribata* samples after feeding. Lines are fitted Probit model with 95% fiducial limits. Black upright triangles and solid lines, CcITS1-2 primer pair; White upside-down triangles and dotted lines for CcITS737 primer pair.

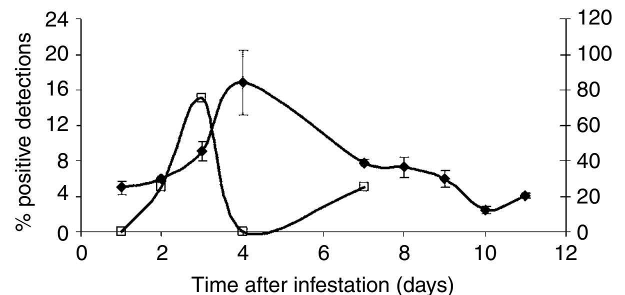

Fig. 4. Percentage of spiders that tested positive with CcITS737 pair of primers and mean (±SE) number of flies captured per trap and day (FTD) in the field test (–□–, % detections; –◆–, FTD).

Discussion

PCR can be used to detect prey within the gut contents of predators, thereby enabling specific trophic interactions to be studied among soil-dwelling arthropods, which cannot be examined by other approaches (reviewed in Symondson, 2002; Juen & Traugott, 2005; King *et al.*, 2008). Accordingly, by using PCR-based prey detection analysis, we were able to track *C. capitata* predation by the wolf spider, *P. cribata*, in citrus orchards, where a wide range of alternative preys were also present, such as aphids, spider mites or collembolan.

Specific primers for *C. capitata* were designed in the ITS-1 rDNA region, due to their presence in multiple copies in each cell, enabling successful detection in predator guts as reported in other arthropod species (Agustí *et al.*, 1999b; Zaidi *et al.*, 1999; Hoogendoorn & Heimpel, 2001) and by the sequence availability in databases. Although this region showed a high level of sequence diversity among tephritids, even at the species level, the *C. capitata* species-specific primers worked successfully with two non-related strains, the laboratory strain (IVIA 2000) and the sterile strain (Vienna-8), rendering them universal for *C. capitata*. The specificity of this primer pair for the tephritid *C. capitata* will allow its use in other regions where it coexists with other tephritid species and on which search of putative predators are planned.

The calculated DNA detectability half-live for both primer pairs is roughly in the range of those reported (96 h to 105.4 h) for other spider species (Harwood *et al.*, 2001; Ma *et al.*, 2005). As reported elsewhere, shorter amplicons are more robust than longer amplicons and can be detected for a longer period. In this study, we were able to detect up to 37% of positives 96 h after prey ingestion with the ITS737 primer pair. Certain traits involving both the molecular markers and the predator-prey interactions may help to explain the long detection times obtained. Our amplified PCR products were short enough for long-term detections, 120 bp and 330 bp for each pair of primers, respectively. The shorter the amplified sequences, the longer the detection time (Agustí *et al.*, 1999b, 2000, 2003b; Zaidi *et al.*, 1999; Hoogendoorn & Heimpel, 2001; de León *et al.*, 2006). However, in this assay, the detection time corresponding to the primer pair amplifying the shortest sequence was nearly the same as the primer pair amplifying the longest sequence. As mentioned before, the primers were designed over a multi-copy DNA region, which also contributes to increasing detection times. There seems to be a correlation between sensitivity and detection efficiency, whereby marker sensitivity increases the detection of the prey in the predator gut contents. Thus, de Leon *et al.* (2006) reported a sensitivity of 6.0 pg for the mitochondrial cytochrome oxidase subunit I (COI) and 25.0 pg for the COII, and detection efficiency was higher for COI regardless of fragment size. When testing the sensitivity of the markers described here, we were able to detect positives even at 0.1 pg. Due to the starvation adaptations, spiders' gut systems are prepared to store ingested food for long-term periods (Harwood *et al.*, 2001). This feature also means DNA

https://doi.org/10.1017/S0007485309006920 Published online by Cambridge University Press

of the prey can be detected for longer (Greenstone *et al*., 2007). The quantity of food ingested could also be a factor influencing detection times. Hagler *et al*. (1997) found a positive effect of meal size on prey detection when carrying out monoclonal-antibody assays on the gut content of three species of insect predators (*Geocoris punctipes* (Say) (Hemiptera: Lygaeidae), *Orius insidiosus* (Say) (Hemiptera: Anthocoridae) and *Hippodamia convergens* Guerin-Maneville) (Coleoptera: Coccinellidae) by indirect ELISA, to detect different ingested quantities of eggs of the pink ballworm *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae). *Pardosa cribata* is similar in size to its *Ceratitis capitata* prey; therefore, one medfly provides it with a large amount of food. This means it is stored in the spider's gut system for longer, thereby making DNA detection easier. By contrast, Zaidi *et al*. (1999) did not find any correlation between quantity of food ingested by the predator and detectability. Our study has also shown that, beyond fragment length, primer design is crucial for amplification success. On average, higher and clearer amplifications were obtained with the shorter primer pair CcITS737 in contrast to Juen & Traugott (2005), who found that their shorter primer pair gave the lower amplification bands.

In the field assay, up to 15% of spiders tested positive 72 h after medfly pupae deployment in a citrus orchard. Most medfly captures were recorded between 48 h and 96 h after pupae release, suggesting that most of the adults emerged in the citrus orchard during this period. The maximum rate of positive detections was just 24 h before the maximum FTD recorded, suggesting that *P. cribata* preyed upon *C. capitata* more actively when more adults were emerging from the pupae deployed. This is consistent with the predatory behavior established under laboratory conditions (Monzó *et al*., 2009), who showed that *P. cribata* was able to prey on teneral adults and larval stages of *C. capitata*, but not on pupae; and predation rate was significantly higher on adults than on larvae.

We would like to highlight that the rates of predation obtained in this work indicate that *P. cribata* is able to play an important role in multi-tactic strategies, currently required to control *C. capitata*. The next step to developing such a strategy should study the conservation of *P. cribata* populations in the citrus ecosystem, such as cover-crop management, thereby improving its potential as a biological control agent.

This is the first study to demonstrate the use of DNA markers to track medfly predation by the wolf spider, *P. cribata*, in citrus agro-ecosystems, providing a new tool to estimate the role of this spider in biological-control conservation strategies.

Acknowledgements

This work was funded by FEOGA Cooperación, the Conselleria d'Agricultura, Pesca i Alimentació de la Generalitat Valenciana and INIA (RTA03-103-C6-01). We are thankful to N. Agustí (IRTA, SP) for assistance at the beginning of this work and to R. Argilés and I. Pla, (TRAGSA, SP) for providing the Vienna-8 pupae. We are also grateful to A. Melic (Araneae, Sociedad Entomológica Aragonesa, Zaragoza SP) for taxonomical advice, E. Carbonell and J. Pérez-Panadés for statistical analysis, and to A. Azanza, H. Montón, P. Vanaclocha, Ó. Mollá (IVIA, SP) and M. Llavador for technical assistance. We also thank DPI & F Staff for *Bactrocera* specimens' collection from Queensland (Australia) and P.A. Stansly (University of Florida, USA) for *A. suspensa* specimens' collection. C.M. was recipient of a PhD grant from the CSIC. B.S.M. is supported by an European Social Fund co-financial contract.

References

Agustí, N., Aramburu, J. & Gabarra, R. (1999a) Immunological detection of *Helicoverpa armigera* (Lepidoptera: Noctuidae) ingested by heteropteran predators: time-related decay and effect of meal size on detection period. *Annals of the Entomological Society of America* **92**, 56–62.

Agustí, N., De Vicente, M.C. & Gabarra, R. (1999b) Development of sequence amplified characterized region (SCAR) markers of *Helicoverpa armigera*: a new polymerase chain reaction-based technique for predator gut analysis. *Molecular Ecology* **8**, 1467–1474.

Agustí, N., De Vicente, M.C. & Gabarra, R. (2000) Developing SCAR markers to study predation on *Trialeurodes vaporariorum*. *Insect Molecular Biology* **9**, 263–268.

Agustí, N., Shayler, S.P., Harwood, J.D., Vaughan, I.P., Sunderland, K.D. & Symondson, W.O.C. (2003a) Collembola as alternative prey sustaining spiders in arable ecosystems: prey detection within predators using molecular markers. *Molecular Ecology* **12**, 3467–3475.

Agustí, N., Unruh, T.R. & Welter, S.C. (2003b) Detecting *Cacopsylla pyricola* (Hemiptera: Psyllidae) in predator guts using COI mitochondrial markers. *Bulletin of Entomological Research* **93**, 179–185.

Chen, Y., Giles, K.L., Payton, M.E. & Greenstone, M.H. (2000) Identifying key cereal aphid predators by molecular gut analysis. *Molecular Ecology* **9**, 1887–1898.

Chueca, P., Montón, H., Ripollés, J.L., Castañera, P., Moltó, E. & Urbaneja, A. (2007) Spinosad bait treatments as an alternative to malathion in controlling the Mediterranean fruit fly, *Ceratitis capitata*, (Diptera: Tephritidae) in the Mediterranean Basin. *Journal of Pesticide Science* **32**, 407–411.

de León, J.H., Fournier, V., Hagler, J.R. & Daane, K.M. (2006) Development of molecular diagnostic markers for sharpshooters *Homalodisca coagulata* and *Homalodisca liturata* for use in predator gut content examinations. *Entomologia Experimentalis et Applicata* **119**, 109–119.

Foltan, P., Sheppard, S., Konvicka, M. & Symondson, W.O.C. (2005) The significance of facultative scavenging in generalist predator nutrition: detecting decayed prey in the guts of predators using PCR. *Molecular Ecology* **14**, 4147–4158.

Fournier, V., Hagler, J., Daane, K., de León J. & Groves, R. (2008) Identifying the predator complex of *Homalodisca vitripennis* (Hemiptera: Cicadellidae): a comparative study of the efficacy of an ELISA and PCR gut content assay. *Oecologia* **157**, 629–640.

Greenstone, M.H. (1999) Spider predation: How and why we study it. *Journal of Arachnology* **27**, 333–342.

Greenstone, M.H., Rowley, D.L., Weber, D.C., Payton, M.E. & Hawthorne, D.J. (2007) Feeding mode and prey detectability half-lives in molecular gut-content analysis: an example with two predators of the Colorado potato beetle. *Bulletin of Entomological Research* **97**, 201–209.

Hagler, J.R., Naranjo, S.E., Bradleydunlop, D., Enriquez, F.J. & Henneberry, T.J. (1994) A monoclonal-antibody to Pink-Bollworm (Lepidoptera, Gelechiidae) egg antigen – A tool

https://doi.org/10.1017/S0007485309006920 Published online by Cambridge University Press

for predator gut analysis. *Annals of the Entomological Society of America* **87**, 85–90.

Hagler, J.R., Naranjo, S.E., Erickson, M.L., Machtley, S.A. & Wright, S.F. (1997) Immunological examinations of species variability in predator gut content assays: Effect of predator:prey protein ratio on immunoassay sensitivity. *Biological Control* **9**, 120–128.

Hagen, K.S., Mills, N.J., Gordh, G. & McMurtry, J.A. (1999) Terrestrial arthropod predators of insects and mite pests. pp. 383–504 *in* Bellows, T.S. & Fisher, T.W. (*Eds*) *Handbook of Biological Control: Principles and Applications of Biological Control*. San Diego, USA, Academic Press.

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.

Harwood, J.D. & Obrycki, J.J. (2005) Quantifying aphid predation rates of generalist predators in the field. *European Journal of Entomology* **102**, 335–350.

Harwood, J.D., Phillips, S.W., Sunderland, K.D. & Symondson, W.O.C. (2001) Secondary predation: quantification of food chain errors in an aphid-spider-carabid system using monoclonal antibodies. *Molecular Ecology* **10**, 2049–2057.

Hoogendoorn, M. & Heimpel, G.E. (2001) PCR-based gut content analysis of insect predators: using ribosomal ITS-1 fragments from prey to estimate predation frequency. *Molecular Ecology* **10**, 2059–2067.

Juen, A. & Traugott, M. (2005) Detecting predation and scavenging by DNA gut-content analysis: a case study using a soil insect predator-prey system. *Oecologia* **142**, 344–352.

King, R.A., Read, D.S., Traugott, M. & Symondson, W.O.C. (2008) Molecular analysis of predation: a review of best practice for DNA-based approaches. *Molecular Ecology* **17**, 947–963.

Latorre, A., Moya, A. & Ayala, F.J. (1986) Evolution of mitochondrial DNA in *Drosophila subobscura*. *Proceedings of the National Academy of Sciences, USA* **83**, 8649–8653.

Legaspi, J.C., Legaspi, B.C., Meagher, R.L. & Ciomperlik, M.A. (1996) Evaluation of *Serangium parcesetosum* (Coleoptera: Coccinellidae) as a biological control agent of the silverleaf whitefly (Homoptera: Aleyrodidae). *Environmental Entomology* **25**, 1421–1427.

Ma, J., Li, D., Keller, M., Schmidt, O. & Feng, X. (2005) A DNA marker to identify predation of *Plutella xylostella* (Lep., Plutellidae) by *Nabis kinbergii* (Hem., Nabidae) and *Lycosa* sp (Aranaea, Lycosidae). *Journal of Applied Entomology* **129**, 330–335.

Magaña, C., Hernández-Crespo, P., Ortego, F. & Castañera, P. (2007) Resistance to Malathion in Field Populations of *Ceratitis capitata*. *Journal of Economic Entomology* **100**, 1836–1843.

Magaña, C., Hernández-Crespo, P., Brun-Barale, A., Couso-Ferrer, F., Bride, J.M., Castañera, P., Feyereisen, R. & Ortego, F. (2008) Mechanisms of resistance to malathion in the medfly *Ceratitis capitata*. *Insect Biochemistry and Molecular Biology* **38**, 756–762.

Marc, P., Canard, A. & Ysnel, F. (1999) Spiders (Araneae) useful for pest limitation and bioindication. *Agriculture, Ecosystems and Environment* **74**, 229–273.

Monzó, C., Sabater-Muñoz, B., Urbaneja, A. & Castañera, P. (2007) Importancia de los depredadores polífagos presentes en suelo de los cítricos en la depredación de *Ceratitis capitata*. *Phytoma España* **194**, 13–14.

Monzó, C., Mollá, O., Castañera, P. & Urbaneja, A. (2009) Activity density of *Pardosa cribata* in Spanish citrus orchards and its predatory capacity on *Ceratitis capitata* and *Myzus persicae*. *BioControl* **54**, 393–402.

Morris, T.I., Campos, M., Kidd, N.A.C. & Symondson, W.O.C. (1999) What is consuming *Prays oleae* (Bernard) (Lep.: Yponomeutidae) and when: a serological solution? *Crop Protection* **18**, 17–22.

Nicholas, K.B., Nicholas Jr, H.B. & Deerfield II, D.W. (1997) GeneDoc: Analysis and Visualization of Genetic Variation. *EMBNET.news* **4**, 1–4.

Rychlik, W. (1992) OLIGO 4.06, Primer Analysis Software. National Biosciences Inc. Publishers, Plymouth, USA.

San Andrés, V., Urbaneja, A., Sabater-Muñoz, B. & Castañera, P. (2007) A novel molecular approach to assess mating success of sterile *Ceratitis capitata* (Diptera: Tephritidae) males in sterile insect technique programs. *Journal of Economic Entomology* **100**, 1444–1449.

Sheppard, S.K., Henneman, M.L., Memmott, J. & Symondson, W.O.C. (2004) Infiltration by alien predators into invertebrate food webs in Hawaii: a molecular approach. *Molecular Ecology* **13**, 2077–2088.

Sheppard, S.K., Bell, J., Sunderland, K.D., Fenlon, J., Skervin, D. & Symondson, W.O.C. (2005) Detection of secondary predation by PCR analyses of the gut contents of invertebrate generalist predators. *Molecular Ecology* **14**, 4461–4468.

Sunderland, K. (1999) Mechanisms underlying the effects of spiders on pest populations. *The Journal of Arachnology* **27**, 308–316.

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

Symondson, W.O.C. (2002) Molecular identification of prey in predator diets. *Molecular Ecology* **11**, 627–641.

Symondson, W.O.C., Glen, D.M., Wiltshire, C.W., Langdon, C.J. & Liddell, J.E. (1996) Effects of cultivation techniques and methods of straw disposal on predation by *Pterostichus melanarius* (Coleoptera: Carabidae) upon slugs (Gastropoda: Pulmonata) in an arable field. *Journal of Applied Ecology* **33**, 741–753.

Symondson, W.O.C., Gasull, T. & Liddell, J.E. (1999) Rapid identification of adult whiteflies in plant consignments using monoclonal antibodies. *Annals of Applied Biology* **134**, 271–276.

Symondson, W.O.C., Glen, D.M., Ives, A.R., Langdon, C.J. & Wiltshire, C.W. (2002a) Dynamics of the relationship between a generalist predator and slugs over five years. *Ecology* **83**, 137–147.

Symondson, W.O.C., Sunderland, K.D. & Greenstone, M.H. (2002b) Can generalist predators be effective biocontrol agents? *Annual Review of Entomology* **47**, 561–594.

Traugott, M. (2003) The prey spectrum of larval and adult *Cantharis* species in arable land: An electrophoretic approach. *Pedobiologia* **47**, 161–169.

Urbaneja, A., Mari, F.G., Tortosa, D., Navarro, C., Vanaclocha, P., Bargues, L. & Castañera, P. (2006) Influence of ground predators on the survival of the mediterranean fruit fly pupae, *Ceratitis capitata*, in Spanish citrus orchards. *Biocontrol* **51**, 611–626.

Zaidi, R.H., Jaal, Z., Hawkes, N.J., Hemingway, J. & Symondson, W.O.C. (1999) Can multiple-copy sequences of prey DNA be detected amongst the gut contents of invertebrate predators? *Molecular Ecology* **8**, 2081–2087.

https://doi.org/10.1017/S0007485309006920 Published online by Cambridge University Press