Published in Agricultural and Forest Entomology 10, issue 2, 129-136, 2008
which should be used for any reference to this work

Identification of seven species of hymenopteran parasitoids of *Spodoptera frugiperda*, using polymerase chain reaction amplification and restriction enzyme digestion

Violaine Jourdie, Nadir Alvarez and Ted C. J. Turlings

Laboratoire d'Entomologie Evolutive, Institut de Biologie, Université de Neuchâtel, Case Postale 158, 2009 Neuchâtel, Switzerland

Abstract

1 The fall armyworm *Spodoptera frugiperda* is a voracious pest of numerous crops of economic importance throughout the New World. In its native Mexico, larvae can be attacked by several species of parasitic wasps, which are candidate biological control agents against this and other lepidopteran pests.
2 We attempted to survey the parasitoid fauna on *S. frugiperda* in maize and sorghum fields throughout Mexico. However, our efforts have been hampered by the incomplete development of parasitoid larvae emerging from collected *Spodoptera* caterpillars.
3 This problem was solved by developing a method to identify seven species of parasitic wasps using polymerase chain reaction amplification and restriction enzyme digestion. This enables the precise determination of the species of those parasitoid larvae that are usually not morphologically identifiable.

Keywords Hymenoptera, restriction enzyme digestion, parasitoid, species identification, *Spodoptera frugiperda*.

Introduction

The fall armyworm *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is a devastating pest throughout the Americas (Kranz *et al.*, 1977), inflicting costly damage to several crops of great economic importance, such as corn, rice, sorghum, peanuts, soybeans, alfalfa and forage grasses (Knipling, 1980; Pashley, 1986; Lu & Adang, 1996). Synthetic insecticides are commonly used to control this pest. However, resistance to insecticides has been observed (Yu, 1991, 1992).

As the fall armyworm is attacked by many different species of hymenopteran parasitoids, biological control should be considered as an alternative to insecticides (Gross & Pair, 1986). Parasitoids have long been recognized as excellent candidates for reducing larval populations of this noctuid (Luginbill, 1928; Vickery, 1929). Moreover, it has been documented that parasitization of the caterpillar by a parasitoid reduces its feeding rate and has a direct effect on the damage that it inflicts (Rahman, 1970; Guillot & Vinson, 1973; Brewer & King, 1978; Parkman & Shepard, 1981; Powell, 1989; Fritzsche Hoballah & Turlings, 2001).

Correspondence: Ted C. J. Turlings. Tel: +41 32 718 3158; fax: +41 32 718 3001; e-mail: ted.turlings@unine.ch

Determining which parasitoids are the most successful at attacking a pest species in a particular habitat may help in optimizing biological control. To develop a better understanding of the natural distribution of the fall armyworm parasitoid complex, several surveys have been conducted in different regions of Mexico, where the fall armyworm is likely to have originally evolved on wild crop (Hoballah *et al.*, 2004; Molina-Ochoa *et al.*, 2000, 2004). To date, these surveys have revealed the occurrence of seven species of braconids attacking fall armyworm (*Aleoides laphygmae* Viereck, *Cotesia marginiventris* Cresson, *Chelonus insularis* Cresson, *Chelonus cautus* Cresson, *Homolobus truncator* Say, *Meteorus laphygmae* Viereck and *Glyptapanteles militaris* Walsh), four species of ichneumonids (*Campoletis sonorensis* Cameron, *Ophion flavidus* Brulle, *Pristomerus spinator* Fabricius and *Eiphosoma vitticolle* Cresson), two eulophid species (*Euplectrus plathypenae* Howard and *Aprostocetus* sp. Westwood), and one trichogrammatid species (*Trichogramma atopovirilia* Oatman & Platner). These surveys have been limited to the states of Michoacán, Colima, Jalisco, Nayarit, Sinaloa, Veracruz and Tamaulipas. Additional surveys and ecological studies will be necessary to assess the full potential of the various parasitoids for the control of fall armyworm.

We have been collecting naturally-occurring larvae of fall armyworm throughout Mexico aiming to compare the genetic population structure of several parasitoid species occurring on maize with those on sorghum. Rearing the collected larvae until parasitoid emergence in the laboratory conditions is not always successful and it is not uncommon for the larvae not to spin a cocoon. These individuals cannot complete their life cycle to adulthood. Whereas adult parasitoids can be identified morphologically, this is virtually impossible for the larval stages. Molecular techniques may achieve such identification (Hebert *et al.*, 2003a; Blaxter, 2004; Hebert *et al.*, 2004; Barrett & Hebert, 2005; Hebert & Gregory, 2005; Monaghan *et al.*, 2005; Hajibabaei *et al.*, 2007). Gene sequencing comprises one possibility but it is expensive and time-consuming. As an alternative to sequencing, in the present study, we introduce a fast, easy and relatively cheap molecular diagnosis to identify seven commonly found species of wasp larvae using polymerase chain reaction (PCR) amplification and restriction endonuclease digestion.

Materials and methods

Insects

The species considered were four braconids (*C. marginiventris*, *C. cautus*, *C. insularis* and *M. laphygmae*) and three ichneumonids (*P. spinator*, *C. sonorensis* and *E. vitticolle*). These parasitoids all exit the host as larvae and form cocoons externally. Naturally-occurring *S. frugiperda* larvae were collected from 23 sites in Mexico between June and September 2005 (Table 1). The caterpillars were reared individually on artificial diet in 24-well plates to prevent cannibalism. The parasitoid larvae were placed on a piece of absorbing paper in a plastic container upon emergence from the caterpillars. Parasitoid larvae that spun a cocoon were left in the plastic container until the emergence of the adults. The parasitoid larvae that did not spin a cocoon within 24 h after emergence from the caterpillars were preserved in 100% ethanol. The adult parasitic wasps that emerged from the cocoons were also preserved in 100% ethanol upon emergence. Adults were taxonomically assigned to different species in accordance with Cave (1995).

DNA amplification and sequencing

Total genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen, Germany) in accordance with the manufacturer's instructions. For adult wasps, extraction was conducted on the abdomen whereas, for larvae, half of the body was used. Total DNA was resuspended in 200 μl of elution buffer (two elutions of 100 μl each). The mitochondrial DNA cytochrome *c* oxidase subunit 1 gene (*COI*) was partially amplified using C1-J-1859 (forward) and C1-N-2191 (reverse) primers adapted for the bee (Simon *et al.*, 1994). The final volume was 30 μl, and contained 3 μl of extracted DNA, 1.8 μl of 25 mM $MgCl_2$, 3 μl of 1.5 mM dNTPs, 3 μl of PCR buffer (Promega, Madison, Wisconsin), three units of Taq DNA polymerase (Promega), 1.5 μl of 10 μM forward primer, and 1.5 μl of 10 μM reverse primer. PCR amplification was conducted in a Uno II thermal cycler (Biometra, Germany) using the cycling conditions: initial denaturation at 94 °C (1 min 30 s); 35 cycles of 94 °C for 1 min, 50 °C for 1 min, 70 °C for 1 min; final elongation at 70 °C for 5 min. The PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen) and resuspended in 30 μl.

Table 1 Sampled locations: insects were collected from 23 locations in six mexican states in June, August and September 2005

State	Code	Location	Municipio	GPS coordinates
Colima	C1	Agua Zarca	Coquimatlán	19°13′12.0″N, 103°56′10.1″W
	C2	El Colomo, Coquimatlán	Coquimatlán	19°13′58.3″N, 103°57′17.4″W
	C3	Pueblo Juarez, Coquimatlán	Coquimatlán	19°09′45.9″N, 103°53′44.4″W
	C4	Los Mezcales	Comala	19°19′57.3″N, 103°46′07.6″W
	C6	La Caja	Comala	19°22′31.3″N, 103°48′12.8″W
	C7	Villa de Alvarez	Minatitlán	19°16′57.9″N, 103°46′41.0″W
	C8	Villa de Alvarez	Minatitlán	19°17′01.2″N, 103°46′41.0″W
Chiapas	CH1	Jaritas	Tapachula	14°42′53.3″N, 92°18′24.9″W
	CH2	Jaritas	Tapachula	14°43′01.5″N, 92°18′26.6″W
	CH3	Jaritas	Tapachula	14°44′53.7″N, 92°20′06.2″W
	CH4	Jaritas	Tapachula	14°43′32.3″N, 92°18′58.1″W
	CH5	Jaritas	Tapachula	14°43′20.0″N, 92°19′09.1″W
Jalisco	J1	Usmajac	Sayula	19°52′08.9″N, 103°33′16.7″W
	J2	Usmajac	Sayula	19°52′18.4″N, 103°33′45.6″W
Nayarit	N1	Carretera Jala	Ahuacatlán	21°03′39.6″N, 104°27′09.8″W
	N2	Ejido Mexpan	Ixtlan del Rio	21°02′35.0″N, 104°28′03.1″W
	N3	Ejido Mexpan	Ixtlan del Rio	21°02′38.3″N, 104°27′42.6″W
	N4	El Humedo	Ahuacatlán	21°01′35.9″N, 104°28′17.7″W
Puebla	P1	CIMMYT tropical field station	Agua Fría	20°27′18.3″N, 97°38′28.8″W
	P2	CIMMYT tropical field station	Agua Fría	20°27′10.3″N, 97°38′28.7″W
	P3	CIMMYT tropical field station	Agua Fría	20°27′19.6″N, 97°38′26.3″W
	P4	CIMMYT tropical field station	Agua Fría	20°27′17.4″N, 97°38′26.3″W
Veracruz	V1	Lindero	Lindero	20°29′31.9″N, 97°32′17.2″W

Sequencing was carried out by Macrogen Inc. (South Korea) with the forward primer under BigDye terminator cycling conditions, purifying the reacted products using ethanol precipitation and running them using an Automatic Sequencer 3730xl (Applied Biosystems, Foster City, California). A total of 195 adults and 256 larvae were sequenced.

Sequence analysis

The sequences were manually corrected using Chromas 2.23 (Technelysium Pty Ltd, Australia) and further aligned using CLUSTALW 1.4 (Thompson *et al.*, 1994) implemented in BIOEDIT (Hall, 1999). However, because all sequenced fragments were of the same size, alignment was trivial. BLAST searches were conducted on all sequences to check for possible contamination. Based on sequences obtained in adult wasps, a consensus sequence was generated for each species.

Phylogenetic reconstruction

To confirm that *COI* sequences were variable enough to allow clear distinction between species, a neighbour-joining analysis was performed on adult sequences using the DNADIST program (Phylip 3.5 package; Felsenstein, 1993) implemented in BIOEDIT, with a Kishino–Hasegawa model of substitution (Kishino & Hasegawa, 1989). The genetic distances between and within species were also computed in MEGA (Kumar *et al.*, 2004) with a Tamura–Nei model of substitution (Tamura & Nei, 1993).

Selection of restriction enzymes

A restriction map was created for each consensus sequence using the online restriction map software MOLECULAR TOOLKIT (Colorado State University; http://arbl.cvmbs.colostate.edu/molkit/mapper/index.html). Simulations were run to determine the combination of enzymes that maximizes differences in restriction patterns among species. To test whether this tool would also allow the identification of other closely-related parasitoid species, simulations were run on sequences from *Cotesia plutellae* and *Cotesia flavipes* found on GenBank (accession numbers AM087129 and DQ232330 respectively).

Digestion protocol and electrophoresis

As not all enzymes had a similar optimal temperature, a two-step digestion was performed, in accordance with indications provided by the manufacturers. Enzymatic digestion was performed according to the conditions: 10 μl of Tango buffer 1X (Fermentas, Germany) were added to 10 μl of non-purified PCR product and 2 U of each enzyme active at 37 °C. This mix was incubated overnight at the indicated temperature and 2.5 μl of Tango buffer 10X and 2 U of BclI were then added to the mix and incubated for 2 h at 55 °C. The reaction was stopped by incubating the preparation 15 min at 65 °C. The samples were then loaded on a 2% agarose gel and run at 90 V for 4 h. Due to the long-lasting migration, a large quantity of ethidium bromide had to be added to the gel (15 μl for a 150 ml gel).

Results

Insects

Seven species of parasitoids were reared out of *S. frugiperda* caterpillars. Not all the species were present at all locations and the occurrence of parasitoids and the level of parasitism varied greatly among locations (Table 2). Total of parasitism varied from 2% (location N4) to 58.9% (location CH5). Overall, *C. insularis* was the most abundant parasitoid species (Table 3).

DNA amplification and sequencing

As a first step to molecularly distinguish the seven species of parasitoids collected from *S. frugiperda* throughout Mexico (Table 1), their *COI* gene was successfully amplified using primers compiled by Simon *et al.* (1994). The amplified *COI* fragment was of equal size for all seven species (375 bp) (Fig. 1).

Sequence analysis

After manually correcting the sequences, a neighbour-joining analysis was performed on sequences of adult wasps, and the inter- and intra-specific genetic distances were computed. The phylogenetic analyses showed that the *COI* sequence allows clear distinction between the collected species (Fig. 2). Indeed, genetic distances within species were negligible compared with inter-species distances (Table 4). Sequences of the fifty individuals included in the phylogenetic analysis are available in GENBANK under accession Nos. EF555594 to EF555643.

Selection of restriction enzymes

Restriction maps were generated and different combinations of restriction endonucleases were tested. The combinations providing the clearest species-specific patterns were retained. Sequences were not polymorphic within species at the restriction sites chosen. The four enzymes selected were Bcl I (5′…T$^{\nabla}$GATCA…3′), HpyCH4 V (5′…TG$^{\nabla}$CA…3′), Xho II (5′…R$^{\nabla}$GATCY…3′) and XmnI (5′…GAANN$^{\nabla}$NNTTC…3′). BclI, XhoII and XmnI enzymes were purchased from Fermentas. HpyCH4 V enzyme was purchased from New England Biolabs (Ipswich, Massachusetts).

With this combination of enzymes, a specific pattern was obtained for adult wasps of each species when the digested product was run on an agarose gel (Fig. 3). *Pristomerus spinator* displayed two distinct bands at 230 bp and 110 bp respectively. *Eiphosoma vitticolle* exhibited only one band at 100 bp. One band at 210 bp characterized *C. sonorensis*. *Cotesia marginiventris* was characterized by two close bands at 140 bp and 125 bp. *Chelonus insularis* could be recognized

Table 2 Number of parasitoids collected at each location and relative abundance of each species in observed parasitism

Sampling location	Number of *Spodoptera* collected	Parasitoid stage	*Chelonus insularis*		*Pristomerus spinator*		*Cotesia marginiventris*		*Chelonus cautus*		*Campoletis sonorensis*		*Eiphosoma vitticolle*		*Meteorus laphygmae*		Total number of parasitoids	Total % parasitism
			Number collected	% Parasitism	Number collected	% Parasitism	Number collected	% Parasitism	Number collected	% Parasitism	Number collected	% Parasitism	Number collected	% Parasitism	Number collected	% Parasitism		
C1	119	Adults	2	11.8	3	2.5	1	0.8									18	15.1
		Larvae	12															
C2	120	Adults	1	15.8	5	7.5			2	1.7							30	25.0
		Larvae	18		4													
C3	93	Adults	2	11.8		1.1									1	1.1	13	14.0
		Larvae	9		1													
C4	150	Adults	1	5.3	3	3.3			2	5.3							21	14.0
		Larvae	7		2				6									
C6	120	Adults	2	3.3	1	0.8	2	1.7									7	5.8
		Larvae	2															
C7	150	Adults	7	18.0	1	0.7											28	18.7
		Larvae	20															
C8	150	Adults	3	11.3			1	0.7									18	12.0
		Larvae	14															
CH1	150	Adults		1.3									4	2.7			6	4.0
		Larvae	2															
CH2	150	Adults	3	2.0	13	9.3											17	11.3
		Larvae			1													
CH3	150	Adults	3	3.3	1	0.7									1		7	4.7
		Larvae	2															
CH4	150	Adults	8	9.3	2	2.0							6	4.7			24	16.0
		Larvae	6		1								1					
CH5	56	Adults	12	48.2					1	1.8			5	8.9			33	58.9
		Larvae	15															
J1	125	Adults	10	12.0	2	2.4	4	3.2			2	1.6					24	19.2
		Larvae	5		1													
J2	165	Adults	10	19.4	2	1.2	1	0.6			7	4.2					42	25.5
		Larvae	22															
N1	160	Adults	7	4.4	6	4.4	14	8.8			3	1.9					31	19.4
		Larvae			1													
N2	158	Adults	1	3.2			9	5.7	1	0.6	1	0.6					16	10.1
		Larvae	4															
N3	150	Adults	4	4.7			12	8.0	1	0.7	1	0.7					21	14.0
		Larvae	3															
N4	150	Adults					3	0.0									3	2.0
		Larvae																
P1	70	Adults															1	1.4
		Larvae	1															
P2		Adults					1		1						1		36	
		Larvae	29												4			
P3		Adults	2		8				1								20	
		Larvae	3		6													
P4	80	Adults		17.5					5	6.3							19	23.8
		Larvae	14															
V1	212	Adults		1.4	3				1								11	
		Larvae	3		3	2.8			1	0.9								5.2

Table 3 The proportion of each species and its ranking abundance are given for adults, larvae, and both cumulated (total)

	Adults			Larvae			Total		
Species	Number	%	Rank	Number	%	Rank	Number	%	Rank
Chelonus insularis	71	34.8	1	198	81.8	1	269	60.3	1
Pritomerus spinator	50	24.5	2	20	8.3	2	70	15.7	2
Cotesia marginiventris	47	23.0	3	1	0.4	5	48	10.8	3
Chelonus cautus	9	4.4	6	13	5.4	3	22	4.9	4
Eiphosoma vitticolle	11	5.4	5	5	2.1	4	16	3.6	5
Campoletis sonorensis	14	6.9	4	0	0.0	6	14	3.1	6
Meteorus laphygmae	2	1.0	7	5	2.1	4	7	1.6	7

by one band at 250 bp. *Chelonus cautus* DNA was not digested and therefore exhibited a single band at 400 bp. *Meteorus laphygmae* could be easily identified with its two close bands at 220 bp and 180 bp. Bands shorter than 100 bp were barely distinguishable and were therefore not considered. These patterns observed for each species corresponded to the predictions made by the restriction maps and they were congruent with morphological species classification. The pattern obtained from each larva could be assigned to one of the specific patterns obtained from the adults, thereby allowing us to reliably identify the larvae.

The usefulness of the method was further confirmed with simulations run on *COI* sequences published for *C. plutellae* and *C. flavipes*. The simulations showed distinct patterns for the two species allowing easy discrimination from the other species (Fig. 3).

Discussion

The presented method allows the accurate identification of seven species of parasitoids of *S. frugiperda*. Similar techniques have been employed to identify parasitoid species (Tilmon *et al.*, 2000; Mowry & Barbour, 2004), but never for this many species with only a single test. Moreover, the results from simulations with published sequences of two additional closely-related species of *Cotesia* parasitoids show that the method can be broadly employed.

With *S. frugiperda* being one of the most devastating pests on the American continent, easy and cost effective surveys of parasitoid communities attacking this noctuid may facilitate efforts to understand the distribution and biology of these potential biocontrol agents. Previous surveys conducted by Hoballah *et al.* (2004) and Molina-Ochoa *et al.* (2000, 2004) suggest that the seven species identified in this study represent 60% of the parasitoid species and account for 90% of total detected parasitism of *S. frugiperda* in the sampled locations. Not all parasitoid species are equally successful at parasitizing *S. frugiperda* and there may be considerable differences between seasons and locations. Indeed, in the survey by Hoballah *et al.* (2004), *C. sonorensis* and *C. marginiventris* were responsible for at least 85% of the observed parasitism. According to observations by Molina-Ochoa *et al.* (2000, 2004), *C. insularis*, *C. cautus* and *P. spinator* accounted for over 80% of the observed parasitism at some of the same locations as sampled in the present study. These five dominant species are among the seven that we sampled, which means that the chances of correctly identifying a parasitoid of *S. frugiperda* using this test are very high.

The results of a parasitoid inventory may be biased if only emerging adults are taken into account (Table 2). Our sampling revealed that *C. insularis* accounted for 34.8% of parasitism when only adults were considered. However, almost 82% of the larvae that did not spin a cocoon belonged to this species, implying that *C. insularis* was responsible for 60.3% of total parasitism. Similarly, *C. sonorensis* was ranked fourth (out of seven) in abundance before identifying the larvae, but it moved back to the sixth position when the larvae were included, switching positions with *C. cautus*. The other species remained at the same rank, although the proportion that they represented decreased due to the large number of *C. insularis* present among the larvae. It is important to note that, although *E. vitticolle* accounts for 3.60% and is ranked fifth, it was actually only found in the state of Chiapas. This parasitoid is therefore a relatively

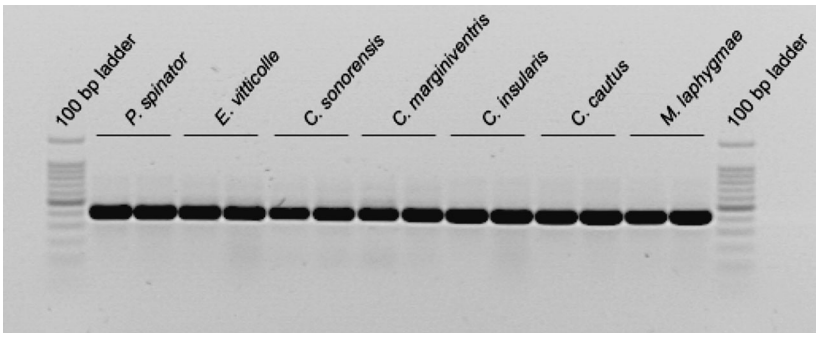

Figure 1 Polymerase chain reaction amplification pattern. Equal size amplification of the cytochrome *c* oxidase subunit 1 fragment was obtained for all seven species.

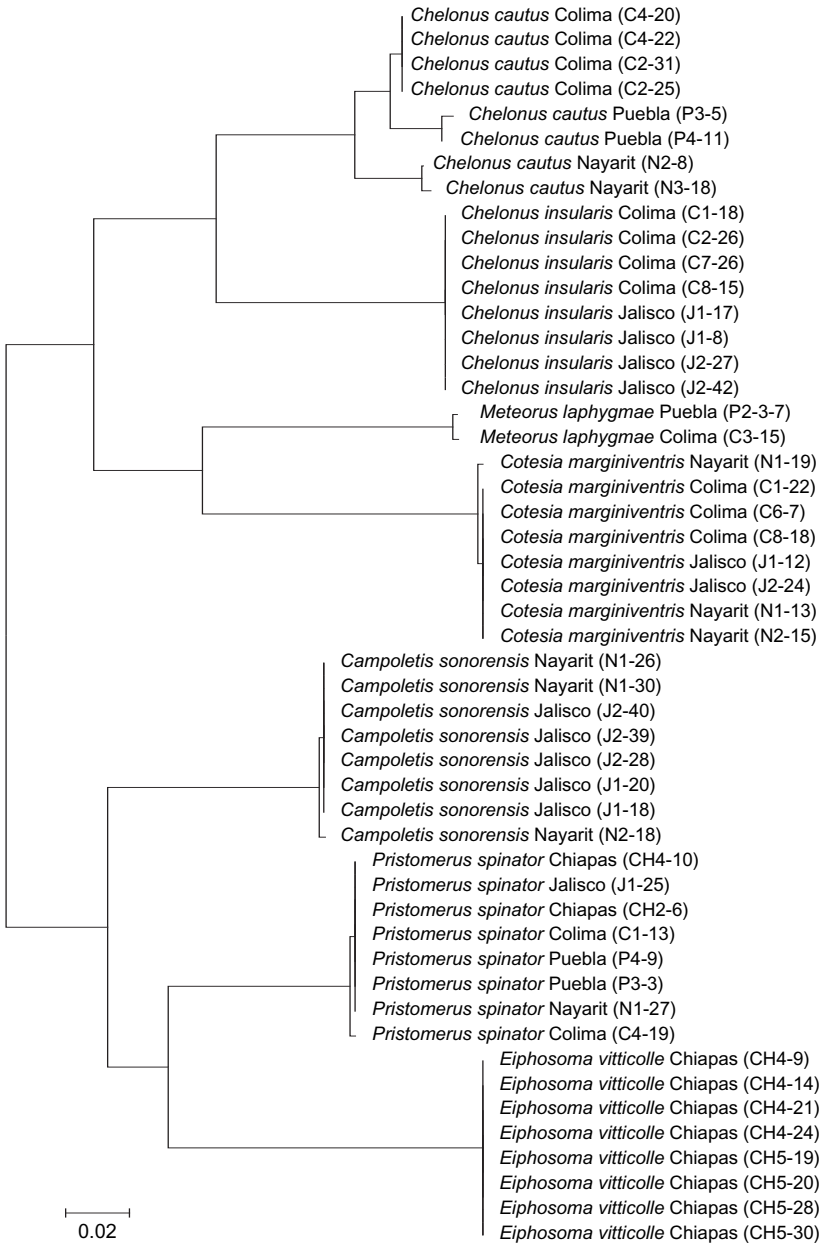

Figure 2 Neighbour-joining Tree. Using the cytochrome *c* oxidase subunit 1 gene sequences with a Kishino–Hasegawa model of substitution, each individual could clearly be assigned to its corresponding species, in the mid-point rooted phylogenetic tree. Eight adult individuals per species from different locations were included in this tree, except for *Meteorus laphygmae* for which only two adults were collected.

abundant species in that area where it was responsible for 18.6% of the parasitism.

Chelonus insularis was the most abundant species in all the states, except for the locations sampled in the state of Nayarit, where *C. marginiventris* represented 53.5% of all parasitoids collected. The collections made in the state of Veracruz yielded only two parasitoid species: *C. insularis* and *C. cautus*. This is very different from the collections made by Hoballah *et al.* (2004). They sampled a very short distance away from our sampling location and found that *C. sonorensis* and *C. marginiventris* accounted for 85% of the observed parasitism. They also observed a total of ten species. This discrepancy appears to indicate that there are strong seasonal variations. They sampled in January and February 2000 and 2001, whereas we sampled in June 2005. The rainy season usually starts in June, however, it was delayed that year. Consequently, few crops had been planted yet and the plants suffered much from drought. This undoubtedly affected the conditions for the insects living on the plants and may have favoured different species.

The present study confirms the efficiency of *COI* as a barcode for animal taxa. Hebert *et al.* (2003a) demonstrated that *COI*-based information was sufficient to assign organisms to higher taxonomic ranks (e.g. genus, family). Moreover, *COI* can also distinguish species at the same time as showing only a low percentage of intraspecific divergence. Mallet and Willmott (2003) objected that DNA sequence differences among closely-related species will be too small to allow their discrimination due to gene introgression. This issue has never been studied comprehensively;

Table 4 Genetic distances within (lower half) and between (diagonal) species. The genetic distances were computed with a Tamura–Nei model of substitution

	Pritomerus spinator	*Meteorus laphygmae*	*Eiphosoma vitticolle*	*Cotesia marginiventris*	*Chelonus insularis*	*Chelonus cautus*	*Campoletis sonorensis*
Pritomerus spinator	0.001						
Meteorus laphygmae	0.276	0.003					
Eiphosoma vitticolle	0.159	0.283	0.000				
Cotesia marginiventris	0.261	0.170	0.278	0.002			
Chelonus insularis	0.246	0.242	0.267	0.191	0.000		
Chelonus cautus	0.227	0.239	0.288	0.213	0.145	0.027	
Campoletis sonorensis	0.146	0.235	0.217	0.255	0.236	0.230	0.001

Intraspecific genetic distances are much lower than interspecific distances, indicating that the cytochrome *c* oxidase subunit 1 fragment allows clear distinction between species.

however, it has been found that closely-related species of vertebrates regularly show more than 2% divergence at another mitochondrial gene, *cytB* (Johns & Avise, 1998). Hebert *et al.* (2003b) further addressed this by examining the extent of sequence diversity at *COI* among congeneric taxa in the major animal phyla. They established that congeneric species of animals regularly possess substantial sequence divergence in their *COI* gene. More than 98% of the 13 320 species pairs analyzed by Hebert *et al.* (2003b) showed sequence divergence greater than 2%, with the mean divergence value being 11.3%, indicating that most pairs were separated by more than 50 diagnostic substitutions in every 500 bp of their *COI* gene. The less than 2% divergence in the remaining species pairs may reflect a short history of reproductive isolation (Hebert *et al.* 2003b). Our results support the notion that *COI* can reliably discriminate species. The inter-specific divergence ranged from 14.5% (between *C. insularis* and *C. cautus*) to 28.8% (between *C. cautus* and *E. vitticolle*) with a mean value of 22.9%, whereas the intra-specific divergence never exceeded 2.7% (*C. cautus*) with a mean value of 0.5%. The high intra-specific distance observed in *C. cautus* may be explained by a strong geographic structure. Indeed, groups of individuals coming from distant geographic regions clearly form distinct clades (Fig. 2). This suggests that *C. cautus* has evolved in Mexico for a very long time and may even have originated in the Mesoamerican region.

It can be concluded that the presented method is reliable for the identification of the studied species. The method should also allow recognition of new species if novel patterns appear, as was confirmed with the simulations made on *C. plutellae* and *C. flavipes* sequences.

Acknowledgements

We are grateful to Trevor Williams, Jaime Molina-Ochoa and David Bergvinson for their assistance in the organization of field work. We also thank the Mexican students who helped us with field collections. Pierre Franck provided insightful comments on an earlier version of the manuscript. This study was supported by the Swiss National Center of Competence in Research 'Plant Survival' and grant 3100A0-107974 from the Swiss National Science Foundation.

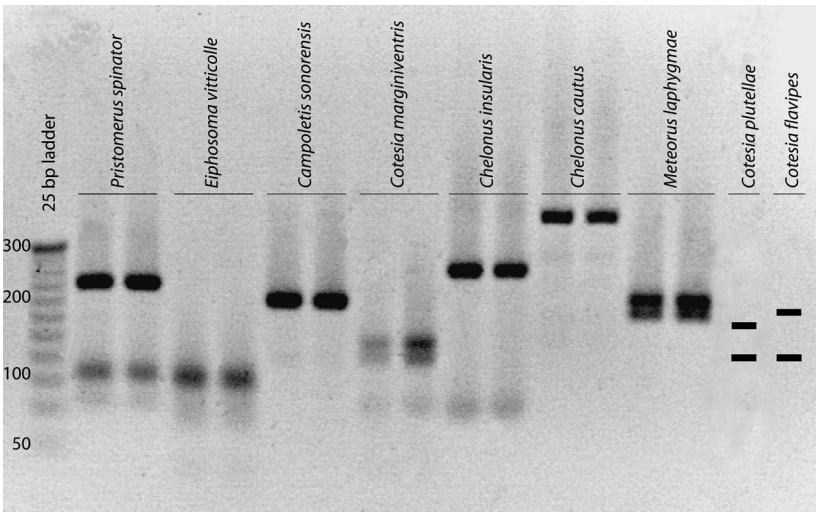

Figure 3 Digestion pattern. A species specific pattern was obtained upon digestion of COI with the combination of restriction endonucleases BclI, XhoII, XmnI and HpyCH4V. Two individuals from different populations were used for each species. For *Cotesia plutellae* and *Cotesia flavipes*, simulated results of digestions are shown at the right of the gel.

References

Barrett, R.D.H. & Hebert, P.D.N. (2005) Identifying spiders through DNA barcodes. *Canadian Journal of Zoology*, **83**, 481–491.

Blaxter, M.L. (2004) The promise of a DNA taxonomy. *Philosophical Transactions of the Royal Society Series B*, **359**, 669–679.

Brewer, F.D. & King, E.G. (1978) Effects of parasitism by a tachinid, *Lixophaga diatraeae*, on growth and food consumption of sugarcane borer larvae. *Annals of the Entomolomological Society of America*, **71**, 19–22.

Cave, R.D. (1995) *Manual para el reconocimiento de Parasitoides de Plagas Agrícolas en America Central*, Primera Edicíon. Zamorano Academic Press, Honduras.

Felsenstein, J. (1993) *PHYLIP (Phylogeny Inference Package)*, Version 3.5c. Department of Genome Sciences, University of Washington, Seattle.

Fritzsche Hoballah, M.E. & Turlings, T.C.J. (2001) Experimental evidence that plants under caterpillar attack may benefit from attracting parasitoids. *Evolutionary Ecology Research*, **3**, 1–13.

Gross, H.R. Jr, & Pair, S.D. (1986) The fall armyworm: status and expectations of biological control with parasitoids and predators. *Florida Entomologist*, **69**, 502–515.

Guillot, F.S. & Vinson, S.B. (1973) Effect of parasitism by *Cardiochiles nigriceps* on food consumption and utilization by *Heliothis virescens*. *Journal of Insect Physiology*, **19**, 2072–2082.

Hajibabaei, M., Singer, G.A.C., Hebert, P.D.N. & Hickey, D.A. (2007) DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics*, **23**, 167–172.

Hall, T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95–98.

Hebert, P.D.N. & Gregory, T.R. (2005) The promise of DNA barcoding for taxonomy. *Systematic Biology*, **54**, 852–859.

Hebert, P.D.N., Cywinska, A., Ball, S.L. & deWaard, J.R. (2003a) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London Series B*, *Biological Sciences* **270**, 313–321.

Hebert, P.D.N., Ratnasingham, S. & deWaard, J.R. (2003b) Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London Series B, Biological Sciences* **270** (Suppl.), S96–S99.

Hebert, P.D.N., Stoeckle, M.Y., Zemlak, T.S. & Francis, C.M. (2004) Identification of birds through DNA barcodes. *PLoS Biology*, **2**, 1657–1663.

Hoballah, M.E., Degen, T., Bergvinson, D., Savidan, A., Tamó, C. & Turlings, T.C.J. (2004) Occurrence and direct control potential of parasitoids and predators of the fall armyworm (Lepidoptera: Noctuidae) on maize in the subtropical lowlands of Mexico. *Agricultural and Forest Entomology*, **6**, 83–88.

Johns, G.C. & Avise, J.C. (1998) A comparative summary of genetic distances in the vertebrates from the mitochondrial cytochrome *b* gene. *Molecular Biology and Evolution*, **15**, 1481–1490.

Kishino, H. & Hasegawa, M. (1989) Evaluation of the maximum-likelihood estimate of the evolutionary tree topologies from DNA-sequence data, and the branching order in hominoidae. *Journal of Molecular Evolution*, **29**, 170–179.

Knipling, E.F. (1980) Regional management of the fall armyworm – a realistic approach? *Florida Entomologist*, **63**, 468–480.

Kranz, J., Schmutterer, H. & Koch, W. (1977) *Diseases, Pests and Weeds in Tropical Crops*. Verlag Paul Parey, Germany.

Kumar, S., Tamura, K. & Nei, M. (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence Alignment. *Briefings in Bioinformatics*, **5**, 150–163.

Lu, Y. & Adang, M.J. (1996) Distiguishing fall armyworm (Lepidoptera: Noctuidae) strains using a diagnostic mitochondrial DNA marker. *Florida Entomologist* **79**, 48–55.

Luginbill, P. (1928) The fall armyworm. *U.S. Department of Agriculture Technical Bulletin* No. 34., pp. 92.

Mallet, J. & Willmott, K. (2003) Taxonomy: renaissance of Tower of Babel? *Trends in Ecology and Evolution* **18**, 57–59.

Molina-Ochoa, J., Hamm, J.J., Lezama-Gutiérrez, R., López-Edwards, M., González-Ramírez, M. & Pescador-Rubio, A. (2000) A survey of fall armyworm (Lepidoptera: Noctuidae) parasitoids in the Mexican States of Michoacán, Colima, Jalisco, and Tamaulipas. *Florida Entomologist* **84**, 31–36.

Molina-Ochoa, J., Carpenter, J.E., Lezama-Gutiérrez, R., Foster, J. E., González-Ramírez, M., Angel-Sahagún, C.A. & Farías-Larios, J. (2004) Natural distribution of hymenopteran parasitoids of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) larvae in Mexico. *Florida Entomologist* **87**, 461–472.

Monaghan, M.T., Blake, M., Gregora, T.R. & Vogler, A.P. (2005) DNA-based species delineation in tropical beetles using mitochondrial and nuclear markers. *Philosophical Transactions of the Royal Society Series B* **360**, 1925–1933.

Mowry, T.M. & Barbour, J.D. (2004) Distinguishing the parasitic wasp, *Peristenus howardi*, from some of its cogeners using polymerase chain reaction and restriction endonuclease digestion. *Journal of Insect Science* **4**, 25.

Parkman, P. & Shepard, M. (1981) Foliage consumption by yellowstriped armyworm larvae after parasitization by *Euplectrus plathypenae* *Florida Entomologist* **64**, 192–194.

Pashley, D.P. (1986) Host-associated genetic differentiation in fall armyworm (Lepidoptera: Noctuidae): a sibling species complex? *Annals of the Entomological Society of America* **79**, 898–904.

Powell, J.E. (1989) Food consumption by tobacco budworm (Lepidoptera: Noctuidae) larvae reduced after parasitization by *Microplitis demolitor* or *M. croceipes* (Hymenoptera: Braconidae). *Journal of Economical Entomology* **82**, 408–411.

Rahman, M. (1970) Effect of parasitism on food consumption of *P. rapae* larvae. *Journal of Economical Entomology* **63**, 820–821.

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H. & Flook, P (1994) Evolution, weighting, 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.

Tamura, K. & Nei, M. (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial-DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**, 512–526.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680.

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.

Vickery, R.A. (1929) Studies of the fall armyworm in the Gulf Coast district of Texas. *U.S. Department of Agriculture Technical Bulletin* No. 138., pp. 64.

Yu, S.J. (1991) Insecticide resistance in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). *Pesticide Biochemistry and Physiology* **39**, 84–91.

Yu, S.J. (1992) Detection and biochemical characterization of insecticide resistance in fall armyworm (Lepidoptera: Noctuidae). *Journal of Economical Entomology* **85**, 675–682.