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Did the introduction of maize into Europe provide enemy-free space to *Ostrinia nubilalis*? Parasitism differences between two sibling species of the genus *Ostrinia*

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Pseudoperichaeta nigrolineata.

Abstract

We examined whether maize offers enemy-free space (EFS) to its pest *Ostrinia nubilalis*, and may thereby have contributed to its divergence from the sibling species, *Ostrinia scapulalis*, feeding mainly on mugwort, when introduced into Europe five centuries ago. We collected *Ostrinia* larvae on maize (70 populations, 8425 individuals) and mugwort (10 populations, 1184 individuals) and recorded parasitism using both traditional (counting emerging parasitoids) and molecular methods (detection by specific polymerase chain reaction). The main parasitoid was *Macrocentrus cingulum* (Braconidae). On mugwort, parasitism was twice that on maize, and parasitoid-related mortality was 8 times higher. This suggests that maize affords substantial EFS to *Ostrinia* feeding on it. The lower Mortality:Infestation ratio in maize suggests that *O. nubilalis*' immune response might be stronger than that of *O. scapulalis*. If so, adapting to maize and diverging from *O. scapulalis* would decrease the impact of parasitism on *O. nubilalis* at both ecological and evolutionary levels.

Introduction

Acquiring the ability to feed on a new host may be a first step towards ecological speciation (Schluter, 2001; Rundle & Nosil, 2005) in phytophagous insects (Howard & Berlocher, 1998; Drès & Mallet, 2002). Evolving such ability can be facilitated if the new host offers enemy-free space (EFS; Jeffries & Lawton, 1984; Ode, 2006), i.e. if a fitness loss experienced by the insect when feeding on the new host is offset by a fitness gain because of a lower level of parasitism and/or predation by natural enemies. Such situation may for instance arise when the new host is a recently introduced plant species that is little protected from phytophagous insects by co-adapted natural enemies (review in Price *et al.*, 1980; Gratton & Welter, 1999; Chen & Welter, 2007).

Examples where EFS may have not only facilitated the colonization of a new host but also promoted host-associated differentiation between closely related taxa are scarce. They include the fruit fly *Rhagoletis pomonella* Walsh (Feder, 1995), several sister-taxa of the

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goldenrod-associated insect community (Stireman *et al.*, 2005), and species of the lepidopteran genera *Pieris* (Ohsaki & Sato, 1990, 1994) and *Heliothis* (Sisterson & Gould, 1999; Oppenheim & Gould, 2002).

The present work looks for evidence that maize (*Zea mays* L.), which was introduced into Europe *ca*. 500 years ago (Gay, 1999), offers EFS to the maize pest *Ostrinia nubilalis* Hübner (Lepidoptera: Crambidae) *sensu* Frolov *et al.* (2007), and may thereby contribute to its divergence from the sibling species *Ostrinia scapulalis sensu* Frolov *et al.* (2007). Both species are believed to be of Eurasian origin and currently occur in sympatry over a large part of their respective ranges in Europe. They form genetically (Bourguet *et al.*, 2000; Martel *et al.*, 2003; Leniaud *et al.*, 2006; Malausa *et al.*, 2007a,b) and ecologically (Thomas *et al.*, 2003; Bethenod *et al.*, 2005; Calcagno *et al.*, 2007; Malausa *et al.*, 2008) differentiated, but weakly interfertile (Malausa *et al.*, 2005) taxa that feed on different host plants: *O. nubilalis* feeds mainly on maize, whereas *O. scapulalis* is thought to feed mainly on mugwort (*Artemisia vulgaris* L.) and hop (*Humulus lupulus* L.), both native to Eurasia. One scenario that might explain the co-existence of these two differentiated taxa (but see Malausa *et al.*, 2007a) is that the introduction of maize into Europe triggered ecological speciation within an ancestral species close to the current *O. scapulalis*. As for most candidate cases of ecological speciation [e.g. *Rhagoletis pomonella* (Feder *et al.*, 2003)], it is difficult if not impossible to ensure that the impact of geographic isolation on genetic divergence was minor. Nevertheless, such candidate cases provide insight into the mechanism(s) that may have contributed or still contribute to genetic isolation driven by divergent adaptation, i.e. to the kind of process that must be hypothesized to account for ecological speciation.

Berdegue *et al.* (1996) clarified that EFS can facilitate a shift from an original to an alternative host plant in a phytophagous taxon if two conditions are met: (1) on the original host plant, the fitness must be higher in the absence than in the presence of natural enemies and (2) in the presence of natural enemies the fitness must be higher on the alternative than on the original host plant.

Several natural enemies have been reported to cause substantial mortality to natural *Ostrinia* sp. populations across Europe, so that Berdegue *et al.*'s (1996) condition (1) is likely to be met. They include three main species of larval parasitoids (Thompson & Parker, 1928; Baker *et al.*, 1949; Grenier *et al.*, 1990; Monetti *et al.*, 2003; Thomas *et al.*, 2003; Agustì *et al.*, 2005): *Lydella thompsoni* Herting, *Pseudoperichaeta nigrolineata* Walker (both Diptera: Tachinidae) and *Macrocentrus cingulum* Reinhard [Hymenoptera: Braconidae, also referred to as *M. abdominalis* Fab., *M. grandii* Goidanich or *M. gifuensis* Ashmead (Thompson & Parker, 1928; Parker, 1931; van Achterberg & Haeselbarth, 1983)]. They also include *Trichogramma brassicae*, an egg parasitoid used for biological control (Smith, 1996). *Lydella thompsoni* (Hsiao *et al.*, 1966 and included references, Galichet *et al.*, 1985; Cagáň *et al.*, 1999), *P. nigrolineata* (Martinez & Reymonet, 1991) and *Trichogramma* sp. (Smith, 1996; Kuske *et al.*, 2004) also parasitize other hosts, whereas reports of *M. cingulum* on hosts outside the genus *Ostrinia* are anecdotal (van Achterberg, 1993; De Nardo & Hopper, 2004). Finally, *Ostrinia* sp. can be infected by intracellular parasites known as microsporidia (the main species infesting *Ostrinia nubilalis* being *Nosema pyrausta* sometimes referred to as *Perezia pyraustae* Paillot, Microsporida: Nosematidae; Kramer, 1959; Lewis *et al.*, 2006).

Thomas *et al.* (2003) found that *Ostrinia* larvae collected on mugwort showed a higher mortality because of larval insect parasitoids than sympatric larvae collected from maize in the vicinity of Paris, France. This suggests that Berdegue *et al.*'s (1996) condition (2) could be fulfilled as well, although no general conclusion can be reached from this study alone, as it was performed at a single site and during a single year (Heard *et al.*, 2006).

The present study extends Thomas *et al.*'s (2003) study to *Ostrinia* populations collected across France on mugwort and hop (*O. scapulalis sensu* Frolov *et al.*, 2007), and maize (*O. nubilalis sensu* Frolov *et al.*, 2007), and uses methods providing complementary information. We assessed the levels of parasitism with the traditional method of rearing larvae after they have completed their diapause, counting those yielding parasitoid pupae and identifying emerging parasitoids. In addition, we used molecular methods (Tilmon *et al.*, 2000; Agustì *et al.*, 2005) to specifically test for the presence of DNA of a particular parasite (*M. cingulum*, *L. thompsoni*, *P. nigrolineata*, microsporidia) in bulk DNA extracted from diapausing larvae, and thus detect the presence of parasites before diapause, independently of the mortality they might cause (Day, 1994).

Materials and methods

Insect samples

Sample A – Early in the winters 2002–2003, 2003–2004 and 2004–2005, we received samples of 64 populations of diapausing *Ostrinia* larvae collected in maize (*Zea mays*) fields across France by extension services of the French Ministry of Agriculture (Services Régionaux de Protection des Végétaux; Fig. 1, Table 1). Samples were preferentially taken from maize fields free of any insecticide treatments. At the beginning of the winter 2002–2003, we also collected samples of seven populations of diapausing larvae in mugwort stands (*Artemisia vulgaris*) located in central and northern France (Fig. 1, Table 1).

Sample B – We also used a sample set collected by Malausa *et al.* (2007b) in the winters 2004–2005 and 2005–2006 (Table 2). In addition to maize and mugwort, these samples include populations from a third host plant: hop (*Humulus lupulus*). They came from six sites

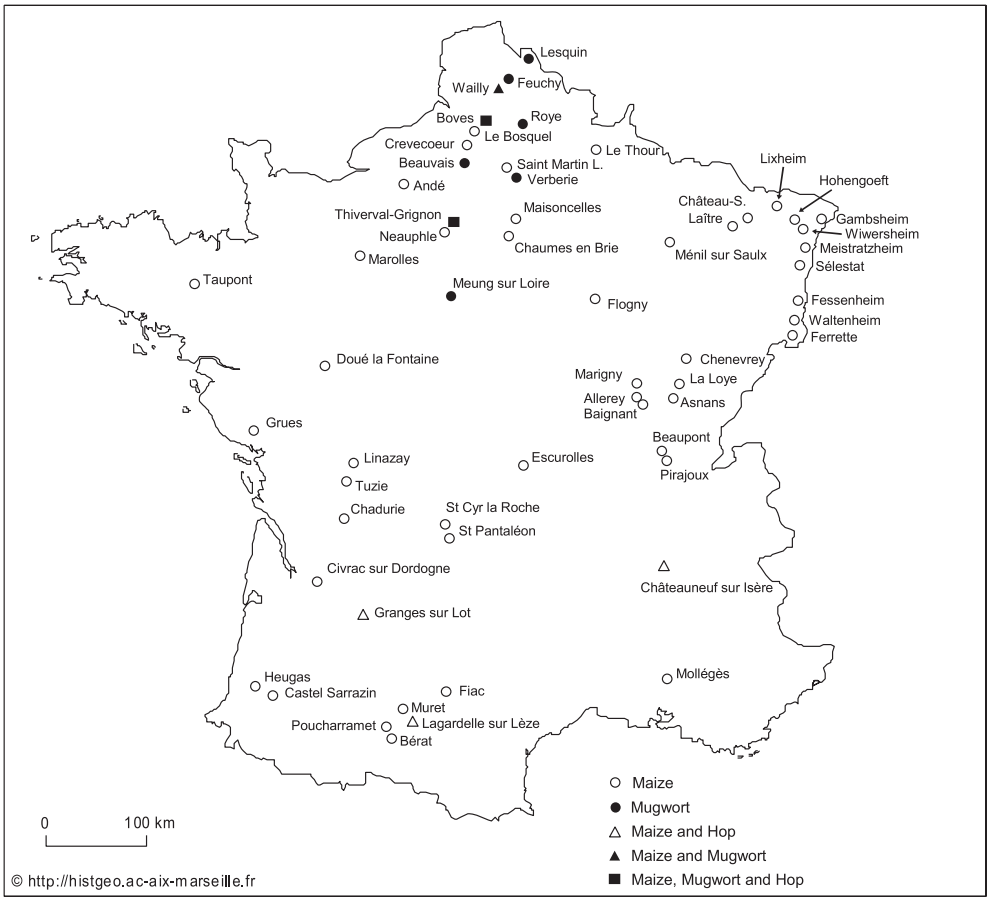

Fig. 1 Map of the sampling sites.

(Fig. 1) where maize and at least one of the two other plant species – mugwort or hop – could be found in close sympatry, i.e. at a distance < 2 km. Within any given site (except Ile de France), samples from the two or three host species were collected within the same week. In all cases, they were frozen immediately upon returning to the laboratory.

We used specimens of the parasitoids *M. cingulum*, *P. nigrolineata* and *L. thompsoni* from various origins (Table 3) to design specific primers and to assess polymerase chain reaction (PCR) quality (positive controls).

DNA extractions

We extracted total genomic DNA from parasites and *Ostrinia* larvae from the whole body, except for the head of certain *Ostrinia* larvae, by using either the DNeasy Blood & Tissue (Qiagen, Venlo, the Netherlands) or the PureGene Extraction kit (Gentra Systems, Minneapolis, MN, USA). We resuspended all pellets in 100 μL of TE (10 mM Tris–HCl pH 8.0, 1 mM ethylenediaminetetraacetic acid) and stored extracts at −20 °C until PCR amplification.

Molecular detection of *Lydella thompsoni* and *Pseudoperichaeta nigrolineata*

To detect parasitism by one of the two tachinid species, we used the two pairs of specific primers designed in the Cytochrome oxydase I (*COI*) gene by Agustì *et al.* (2005): FL3/RL8 for *L. thompsoni* and FP3/RP6 for *P. nigrolineata*. For all samples listed in Tables 2 and 4 (see below), we conducted PCR amplifications in 25 μL containing 2 μL DNA, 1.25 μL 10× buffer, 0.5 μL dNTP, 0.5 μM of each primer, 1 U Taq polymerase (Qiagen) and 6.15 μL of H_2O. PCR comprised an initial denaturation at 95 °C for 2 min, followed by 35 cycles of: 95 °C, 30 s; 58 °C (for *L. thompsoni*) or 62 °C (for *P. nigrolineata*), 30 s; 72 °C, 40 s, with a final elongation step at 72 °C for 5 min. We separated PCR products by electrophoresis in 3% agarose gels stained with ethidium bromide. All PCR amplifications included a negative and a positive control.

Molecular detection of *Macrocentrus cingulum*

To design specific primers to detect *M. cingulum*, we amplified a fragment of the *COI* gene in DNA extracts of

Table 1 'Traditional' parasitism rates (%) in samples of *Ostrinia* sp. populations collected on maize (*Zea mays*) and mugwort (*Artemisia vulgaris*) during sampling A.

					'Traditional' parasitism (%)	
Host plant	Region	Location	Winter	*n*	Tachinids	*M. cingulum*
Z. mays	Midi-Pyrénées	Muret*	2002–03	117	3.42	0
		Fiac	2003–04	97	4.12	0
		Bérat	2004–05	148	0	0
		Poucharramet	2004–05	124	3.23	0
	Aquitaine	Castel Sarrazin*	2002–03	77	5.19	0
		Heugas Eple	2003–04	140	2.86	0
			2004–05	159	0.63	0
		Civrac sur Dordogne	2004–05	196	10.71	0
	Poitou-Charentes	Tuzie*	2002–03	100	6.00	0
		Chadurie	2003–04	156	0.64	0
		Linazay	2004–05	100	4.00	0
	Rhône-Alpes	Beaupont*	2002–03	216	2.78	0
			2003–04	118	0	0
		Pirajoux	2004–05	162	4.94	0
	Pays de la Loire	Marolle les Brault et Monhoudou*	2002–03	124	0	0
		Grues	2003–04	123	4.07	0
		Doué la Fontaine	2004–05	152	7.24	0
	Auvergne	Escurolles*	2002–03	71	0	0
			2003–04	134	0.75	0
			2004–05	137	0	0
	Île-de-France	Neauphle le vieux	2002–03	19	0	0
		Maisoncelles en Brie	2003–04	94	0	0
		Chaumes en Brie	2004–05	126	0	0
	Bourgogne	Baignant	2002–03	31	0	0
		Allerey sur Saône	2002–03	139	0	0
			2003–04	91	0	0
			2004–05	177	3.95	0
		Flogny La Chapelle	2002–03	33	0	0
			2004–05	47	0	0
		Marigny les Reullee	2003–04	121	0	0
	Franche-Comté	Chenevrey*	2002–03	26	0	0
		La Loye	2003–04	152	0	0
			2004–05	132	1.52	0
		Asnans	2003–04	159	1.26	0
			2004–05	100	2.00	0
	Lorraine	Château-Salins*	2002–03	129	3.88	0
		Laître-sous-Amance*	2002–03	118	4.24	0
		Ménil sur Saulx*	2002–03	107	0	0
		Lixheim	2004–05	138	0	0
	Champagne-Ardenne	Le Thour	2003–04	118	0.85	0
			2004–05	129	2.33	0
	Picardie	Saint Martin Longueau*	2002–03	69	1.45	0
		Le Bosquel-Conty	2003–04	108	0	0
		Crèvecoeur le Grand	2004–05	62	0	0
	Haute-Normandie	Andé*	2002–03	111	0	0
			2003–04	112	3.57	0
	Alsace	Waltenheim	2002–03	77	0	0
		Hohengoeft	2002–03	79	0	0
			2004–05	118	0	0
		Wiwersheim	2002–03	74	0	0
			2003–04	122	0	0
		Fessenheim	2002–03	108	0	0
			2003–04	138	0	0
			2004–05	168	0	0
		Ferrette	2002–03	99	0	0

Table 1 (*Continued*).

Host plant	Region	Location	Winter	*n*	'Traditional' parasitism (%)	
					Tachinids	*M. cingulum*
		Meistratzheim	2003–04	102	0	0
		Gambsheim	2003–04	134	5.22	0
			2004–05	167	4.79	0
		Sélestat	2004–05	138	11.59	0
	PACA	Mollégès	2003–04	101	2.97	0
			2004–05	146	8.90	0
	Limousin	St Pantaléon de Larche	2003–04	174	0	0
		St Cyr la Roche	2004–05	142	11.27	0
	Bretagne	Taupont	2004–05	73	1.37	0
A. vulgaris	Nord-Pas-de-Calais	Lesquin	2002–03	99	1.01	29.29
		Feuchy	2002–03	100	0	0
	Picardie	Roye	2002–03	100	0	7.00
		Beauvais	2002–03	142	0	0
		Verberie	2002–03	23	0	34.78
	Île-de-France	Grignon	2002–03	149	0	19.46
	Centre	Meung sur Loire	2002–03	114	0	22.81

These rates were estimated by counting the number of tachinid pupae (*Lydella thompsoni* and *Pseudoperichaeta nigrolineata* were pooled because their pupal stages could not be distinguished) and of cocoons of *Macrocentrus cingulum* among the total number of living larvae (*n*) placed under diapause-breaking conditions after completion of diapause in the laboratory.
*Data for *L. thompsoni* and *P. nigrolineata* parasitism in these populations have been previously reported in Agustì *et al.* (2005). A subsample of these populations has also been analysed using the molecular method (Table 4).

Table 2 Parasitism rates in *Ostrinia* sp. populations collected on maize, mugwort and hop (sample B), as estimated by molecular detection (*n*, number of diapausing larvae analysed).

Region	Location	Winter	Host-plant	*n*	'Molecular' parasitism rate (%)			
					L. thompsoni	*P. nigrolineata*	*M. cingulum*	Microsporidia
Nord-Pas-	Wailly lès	2005–2006	*Artemisia vulgaris*	59	0	0	20.34	0
de-Calais	Arras	2005–2006	*Zea mays*	46	0	0	0	2.17
Picardie	Boves	2005–2006	*A. vulgaris*	56	23.21	0	50.00	75.0
		2005–2006	*Humulus lupulus*	45	0	0	11.11	2.22
		2005–2006	*Z. mays*	52	3.85	0	0	82.69
Île-de-France	Thiverval – Grignon	2004–2005	*A. vulgaris*	56	0	0	25.00	1.79
	– Gambais	2005–2006	*H. lupulus*	45	0	0	13.33	0
	– Jouars – St Quentin	2004–2006	*Z. mays*	91	3.30	0	1.10	2.20
Rhône-Alpes	Châteauneuf sur Isère	2005–2006	*H. lupulus*	35	0	0	5.71	0
		2005–2006	*Z. mays*	69	2.90	0	0	15.94
Aquitaine	Granges sur Lot	2005–2006	*H. lupulus*	58	1.72	0	0	5.17
		2005–2006	*Z. mays*	55	3.70 (*n* = 54)	5.56 (*n* = 54)	1.82	35.19 (*n* = 54)
Midi-Pyrénées	Lagardelle sur Lèze	2005–2006	*H. lupulus*	54	0	1.85	0	0
		2005–2006	*Z. mays*	53	0	1.89	0	3.77

35 individuals of this species collected across its range (Table 3), using the universal primers Lco1490 and Hco2198 (Folmer *et al.*, 1994). We conducted PCR in 25 μL containing 12.5 μL of Taq PCR Master Mix (Qiagen), 11 μL of H_2O, 1 μL of DNA, and a final concentration of 0.5 μM of each primer. The PCR included a denaturation step at 95 °C for 2 min followed by 35 cycles of: 95 °C, 1 min; 40 °C, 1 min and 72 °C, 1.5 min, and a final elongation at 72 °C for 7 min.

Sequencing of PCR products (Genome Express, Meylan, France) yielded eight haplotypes (Genbank, FJ617011 to FJ617018), based on which we designed a pair of specific forward (FM1: 5′-CCTCCGTTATCATTAAATATTAGAC) and reverse (RM1: 5′-AAAATAGCAGTAATTAAAATA GATCA) primers amplifying a 184-bp fragment. These primers differ from *O. nubilalis* (Genbank: AY649321), *P. nigrolineata* (Genbank: AY649320) and *L. thompsoni* (Genbank: AY649319) COI sequences and we checked that there was no cross amplification with these species.

Table 3 Origin of the parasite specimens used for designing the molecular markers and as positive polymerase chain reaction controls.

	Characteristics of the *Ostrinia* samples from which the parasites were recovered				
Parasite	*n*	Country	Location	Host-plant	Collector
Lydella thompsoni	One adult	France	Castillon Savès, Midi-Pyrénées	*Z. mays*	Service Régional de Protection des Végétaux, Haute-Garonne, France
	Two adults	France	Pamiers, Midi-Pyrénées	*Z. mays*	The authors
	One adult	France	Bérat, Midi-Pyrénées	*Z. mays*	The authors
	Two adults	France	Chis, Aquitaine	*Z. mays*	The authors
Pseudoperichaeta nigrolineata	One adult	France	Saint Clar, Midi-Pyrénées	*Z. mays*	Service Régional de Protection des Végétaux, Haute-Garonne, France
Macrocentrus cingulum	Five cocoons	France	Paris, Ile de France	*A. vulgaris*	The authors
	Six cocoons	France	Lille, Nord Pas de Calais	*A. vulgaris*	The authors
	One cocoon	France	Amiens, Picardie	*A. vulgaris*	The authors
	Five cocoons	Germany	Kropp, Schleswig-Holstein	*A. vulgaris*	V. Calcagno, T. Malausa and the authors
	Six cocoons	Japan	Kannondai, Ibaraki	*S. italica*	L. Pélozuelo
	Five adults	China	Beijing, Hebei	*Z. mays*	K. He and Z. Wang, Chinese Academy of Agricultural Sciences (Beijing, China)
	Seven adults	United States	Rosemount, Minnesota	*Z. mays*	C. Hsu and G. Heimpel, U. Minnesota (St. Paul, USA)
Microsporidia	17 Larvae	France	Poucharramet, Midi-Pyrénées	*Z. mays*	Service Régional de Protection des Végétaux, Haute-Garonne, France

n, number of individuals. *Ostrinia* host plants were maize (*Z. Mays*), mugwort (*A. vulgaris*) and foxtail millet (*Setaria italica* L. P. Beauv.).

Table 4 Parasitism, as estimated by molecular detection, in a subsample of diapausing larvae collected in winter 2002–2003 and frozen directly after collection (i.e. without completion of diapause) of a subset of the *Ostrinia* populations listed in Table 1.

				'Molecular' parasitism rate (%)			
Host-plant	Region	Location	*n*	*L. thompsoni*	*P. nigrolineata*	*Macrocentrus cingulum*	Microsporidia
Zea mays	Midi-Pyrénées	Muret*	50	0	0	30.00	20.40 (*n* = 49)
	Aquitaine	Castel Sarrazin*	50	4.00	8.00	18.00	2.00
	Poitou-Charentes	Tuzie*	50	4.00	0	26.00	2.00
	Rhône-Alpes	Beaupont*	50	6.00	10.00	0	0
	Pays de la Loire	Marolles les Brault et Monhoudou*	50	0	0	0	4.00
	Auvergne	Escurolles*	50	0	0	28.00	0
	Lorraine	Château-Salins*	50	4.00	4.00	0	2.08 (*n* = 48)
		Laître-sous-Amance*	50	4.00	8.00	0	0
		Ménil sur Saulx*	50	0	4.00	0	0
	Picardie	Saint-Martin Longueau*	50	0	0	0	2.00
	Haute-Normandie	Andé*	50	4.00	0	2.00	2.00
	Franche-Comté	Chenevrey*	50	0	0	0	2.00
Artemisia vulgaris	Nord-Pas-de-Calais	Lesquin	50	2.00	0	70.0	8.00
		Feuchy	49	0	0	0	0
	Picardie	Roye	49	0	0	30.61	0
		Beauvais	50	4.00	0	22.00	0
	Île-de-France	Grignon	48	4.17	0	58.33	0
	Centre	Meung sur Loire	40	0	0	85.00	0

n, Number of diapausing larvae analysed (unless stated otherwise for one particular parasite in the relevant column: indeed, it happened occasionally that the amount of DNA was not sufficient to conduct analyses for all four parasites).
*Data for *Lydella thompsoni* and *Pseudoperichaeta nigrolineata* parasitism in these populations have been previously reported in Agustì *et al.* (2005).

To evaluate our ability to accurately assess parasitism rates by *M. cingulum* with FM1 and RM1, we artificially 'contaminated' *O. nubilalis* DNA extracts with *M. cingulum* DNA extracts at various ratios (1/10, 1/100 or 1/1000) to mimic what is to be expected in a DNA extract of a parasitized larva.

The PCR conditions were as with Lco1490/Hco2198, except for annealing temperature (50 °C). PCR products were separated by electrophoresis on a 2.5% agarose gel stained with ethidium bromide. Those PCR yielded positive results for *M. cingulum* and 'artificially contaminated' *O. nubilalis* extracts at all ratios, and gave negative results for *O. nubilalis*, *P. nigrolineata* and *L. thompsoni* extracts. We thus subsequently used FM1/RM1 for molecular detection of *M. cingulum* in *Ostrinia* larvae (Tables 2 and 4). All PCR amplifications included a negative and a positive control.

Molecular detection of microsporidia

To detect possible microsporidia infections, we carried out PCR on DNA extracts of *Ostrinia* larvae using a primer set (V1f: forward, 5′-CACCAGGTTGATTCTGCCTGAC, Weiss *et al.* (1994) and 530r: reverse, 5′-CCGCGG CTGCTGGCAC, Ironside *et al.* (2003) adapted from Baker *et al.* (1995)) amplifying a *c.* 440-bp region of the small subunit (SSU) rDNA which is extremely conserved among microsporidia species.

We conducted PCR in 10 μL containing 1× PCR buffer, dNTP (0.25 mM each), 1 U Taq-polymerase (Qiagen), and 1 μM of each primer. PCR comprised 30 cycles of: 92 °C, 30 s; 65 °C, 30 s and 72 °C, 1 min. Conditions for initial denaturation were 92 °C for 3 min, and were 72 °C for 10 min for the final extension step. We separated PCR products by electrophoresis in 2% agarose gels stained with ethidium bromide.

Levels of parasitism

For a 40–50 individual subsample of certain populations of sample A (Table 4) and for all populations of sample B (Table 2) – all frozen at −20 °C directly after collection or reception – we carried out PCR to detect the presence of *L. thompsoni*, *P. nigrolineata*, *M. cingulum* and microsporidia. For each parasite and *Ostrinia* population, we calculated the percentage of parasitism at the beginning of the winter diapause as the ratio of *Ostrinia* larvae that gave a 'positive' amplification signal over the total number of larvae screened by PCR.

All remaining larvae of sample A (Table 1) were used to estimate the mean percentage of parasitism after diapause completion, i.e. the 'traditional' method for studying parasitism. To allow completion of diapause, we maintained larvae at 8 °C in complete darkness for 3 months. We then counted and discarded dead larvae (mean ± SE: 44.6 ± 16.6% of each population). Between 19 and 216 larvae per population were still alive, or 8186 individuals. To break diapause, we placed survivors at 25 °C, under a 16:8 h light:dark (L:D) photoperiod, with a moistened piece of cotton (Grenier *et al.*, 1990). Within 3 weeks, all *Ostrinia* larvae reached pupation, turned into a tachinid pupa or a braconid cocoon, or died for an unknown reason. For each population and each parasitoid, we calculated percentages of parasitism at the end of the winter diapause as the ratio of pupae (for Tachinids) or cocoons (for *M. cingulum*) over the total number of *Ostrinia* larvae placed in diapause-breaking conditions, i.e. by the number of larvae that had survived the winter.

Statistical analysis

Within each of the two datasets obtained by the molecular (Tables 2 and 4) and by the traditional (Table 1) method, we compared the percentages of parasitism between host plants by fitting a logistic regression model by maximum likelihood with 'host plant' as a fixed effect and 'locality' as a random effect, using the lmer package in R (Bates & Maechler, 2009) and 'probit' as a link function. We considered each parasite separately, as well as the overall level of parasitism. In order to test whether the traditional and molecular methods measured the same or different phenomena, i.e. whether lower emergence of parasitoids was due to lack of parasitism or larval mortality of parasitoids on one or both hosts, we tested for differences between the two methods for populations listed in Table 4 by fitting a logistic regression model by maximum likelihood with 'host plant', 'method' and their interaction as fixed effects, using the lmer package in R (Bates & Maechler, 2009) and 'probit' as a link function. As sample sizes were usually unbalanced, we tested each effect by controlling for all other factors (Type II ANOVA). We conducted all statistical analyses with R software (R Development Core Team, 2007).

Results

Levels of parasitism in sample A

The two tachinid flies *L. thompsoni* and *P. nigrolineata*, and the braconid wasp *M. cingulum* were, as expected (Thompson & Parker, 1928), by far the major parasitoids infesting diapausing *Ostrinia* larvae. The traditional method revealed additional hymenopteran parasitoids: the ichneumonids *Sinophorus turionus* Ratzeburg, *Diadegma fenestralis* Holmgren, *Eriborus terebrans* Gravenhorst (also known as *Diadegma terebrans* Momoi) and *Pristomerus* sp., the braconids *Microgaster tibialis* Nees, *Bracon brevicornis* Wesmaet, *Apanteles* sp., and the pteromalid *Trichomalopsis* sp. However, they were always extremely rare (< 1% in all populations). Thompson & Parker's (1928) comprehensive study on European *Ostrinia* also indicates that egg and pupal parasitoids are few

and typically cause but low rates of parasitism. Nevertheless, as our study was conducted on larvae collected in autumn, it must be acknowledged that it excludes parasites of earlier or later stages, as well as those possibly infesting only the first annual *Ostrinia* generation in bivoltine zones.

Across years and space, *Ostrinia* populations of sample A showed clear, host-plant-related differences in the rate of parasitism by the three predominant parasitoids. Indeed, whereas mortality rates from unknown reasons were similar (mean ± SE: 42.6 ± 5.3% in mugwort-collected populations vs. 35.8 ± 1.6% in maize-collected ones; $\chi^2 = 1.54$, d.f. = 1, $P = 0.214$), parasitism rates at the end of the winter diapause estimated by the traditional method were *c.* 8 times higher in mugwort-collected than in maize-collected populations (Table 1, mean ± SE: 16.3 ± 5.4% vs. 2.1 ± 0.4%; $\chi^2 = 9.51$, d.f. = 1, $P = 0.002$). Parasitism rates at the beginning of the winter diapause estimated by the molecular method showed the same trend, though they differed only by a factor 3 (Table 4, mean ± SE: 45.7 ± 13.1% vs. 15.7 ± 4.1%; $\chi^2 = 83.22$, d.f. = 1, $P < 0.0001$).

This difference in parasitism rates was mostly because of *M. cingulum*. Indeed, over all years and locations, not a single *M. cingulum* was found emerging from 7459 larvae collected from 64 populations on maize, whereas a substantial number of cocoons of this species were recovered from five of the seven populations collected in mugwort (Table 1). Hence, the traditional method revealed a significant difference in the rate of *M. cingulum* emerging after diapause from *Ostrinia* collected on the two host plants (Table 1, mean ± SE: 16.2 ± 5.3% vs. 0%; $\chi^2 = 9.53$, d.f. = 1, $P = 0.002$). The molecular method confirmed that *M. cingulum* DNA was detected much more frequently in mugwort-collected than in maize-collected populations at the beginning of diapause (Table 4, mean ± SE: 44.3 ± 13.1% vs. 8.7 ± 3.7%; $\chi^2 = 88.49$, d.f. = 1, $P < 0.0001$).

In contrast to *M. cingulum*, parasitism by tachinids was higher on maize than on mugwort (Table 1, mean ± SE: 2.1 ± 0.4% vs. 0.1 ± 0.1%; $\chi^2 = 7.35$, d.f. = 1, $P = 0.006632$). The molecular method showed a complete absence of *P. nigrolineata* on mugwort but not on maize (Table 4, mean ± SE: 2.8 ± 1.1% vs. 0%; $\chi^2 = 2.38$, d.f. = 1, $P = 0.305$) and a higher *L. thompsoni* infestation rates on maize than on mugwort (Table 4, mean ± SE: 2.2 ± 0.7% vs. 1.7 ± 0.8%; $\chi^2 = 15.21$, d.f. = 1, $P < 0.001$). Regardless of method, the estimate of overall parasitism by Tachinids was usually low (< 10% for *P. nigrolineata* and < 6% for *L. thompsoni*, Table 4), so that they contributed less to total parasitism than *M. cingulum*.

Finally, microsporidia – which were studied only by means of the molecular method – were also more abundant in populations collected on maize than on mugwort (Table 4, mean ± SE: 3.0 ± 1.6% vs. 1.3 ± 1.3%; $\chi^2 = 109.56$, d.f. = 1, $P < 0.0001$). However, they were absent in 50% of the population samples and occurred at low rates (< 8% except in one population where it reached 20%) in the remaining population samples.

Noteworthy, *M. cingulum* parasitism was much higher when estimated by the molecular than by the traditional method, and the size of this effect was significantly higher on mugwort than on maize (method: $\chi^2 = 263.32$, d.f. = 1, $P < 0.0001$; plant:method interaction: $\chi^2 = 20.40$, d.f. = 1, $P < 0.0001$, Fig. 2). The molecular method also yielded higher estimates than the traditional method for Tachinids, but in this case the size of the effect did not differ according to host plant (method: $\chi^2 = 24.13$, d.f. = 1, $P < 0.0001$; plant:method interaction: $\chi^2 < 0.0001$, d.f. = 1, $P = 0.999$; Fig. 2).

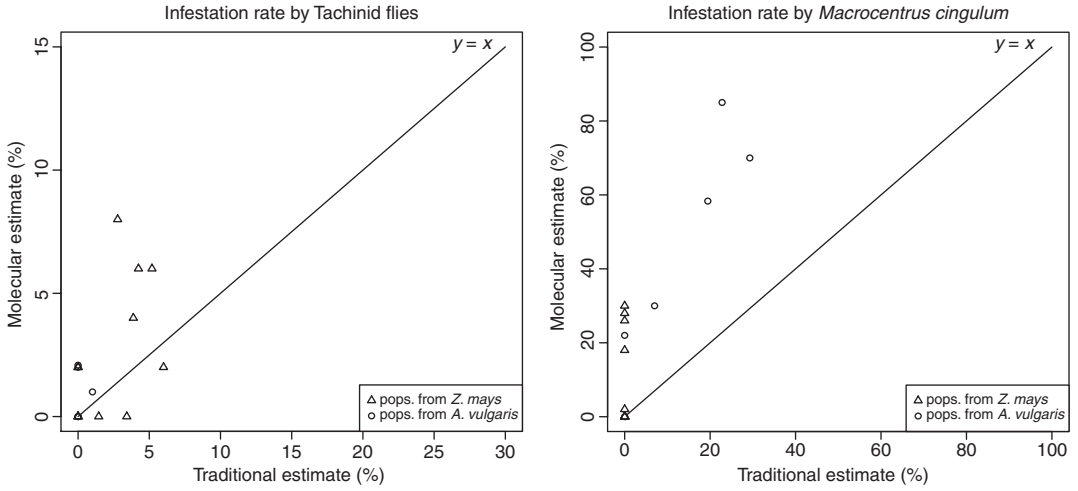

Fig. 2 Traditional vs. molecular estimates of parasitism rates by Tachinids and by *Macrocentrus cingulum*. Data points situated above the $y = x$ line correspond to molecular estimates that were larger than traditional ones.

Levels of parasitism in sample B

The differences we found in sample A between *Ostrinia* populations collected from maize and mugwort, notably with respect to *M. cingulum* and *P. nigrolineata* infestations, could be due to differences in host plant, but also to differences in adult parasitoid density between locations and years. Therefore, we decided to examine pairs or triplets of *Ostrinia* populations collected on different host plants in the same location (< 2 km apart) and at the same time (sample B; Table 2).

Consistently with results obtained for sample A, *M. cingulum* DNA was again detected in populations collected on mugwort much more frequently (20–50%) than in sympatric populations collected on maize (only one infested larva in samples of three populations with *c.* 50 individuals each). *P. nigrolineata* was absent in most locations and *L. thompsoni* infestation rates remained low in all populations (Table 2). Like sample A, sample B showed higher microsporidia infestation rates in maize-collected than in mugwort-collected population samples (Table 2). An influence of locality on microsporida infection rates cannot be excluded, considering the fact that in Boves, *Ostrinia* larvae were highly infested by microsporidia on both maize (*c.* 83%) and mugwort (75%).

Parasitism rates on hop were estimated in too few populations to draw any firm conclusions. However, *M. cingulum* infestation rates were lower in hop than in mugwort in both places where sympatric populations were sampled on the two plants, and higher in hop than in maize in three of four places where it was present in sympatric populations sampled on the two plants.

Discussion

Ecological speciation by host shift in phytophagous insects (Howard & Berlocher, 1998; Drès & Mallet, 2002) involves adaptation to a new host plant and genetic differentiation between an ancestral taxon feeding on the original host plant(s) and a derived taxon feeding on the new one. The initial fitness cost of shifting to a new plant species is reduced – and thus the shift is facilitated – if the new plant offers EFS (Jeffries & Lawton, 1984; Ode, 2006), i.e. if natural enemies cause a significantly stronger fitness reduction to individuals feeding on the original host plant(s) [condition (1) in Berdegue *et al.* (1996)] than to those feeding on the new plant [condition (2) in Berdegue *et al.* (1996)]. Such a situation can notably arise when the new host is a recently introduced plant species – such as maize in Europe – as it may then be less protected from phytophagous insects by co-adapted natural enemies (review in Price *et al.*, 1980; Gratton & Welter, 1999; Chen & Welter, 2007).

In the present study, *Ostrinia* larvae entering diapause were highly parasitized when collected on mugwort, and 2–3 times less so when collected on maize (molecular estimates: 46% vs. 16% when considering sample A alone and 45% vs. 21% when pooling samples A and B). In addition, the maize-collected larvae showed an 8 times lower post-diapause mortality because of parasitoid emergence than the mugwort-collected ones (sample A, 16% vs. 2%).

The higher parasitism and parasitoid-related mortality in mugwort-collected *Ostrinia* larvae were mainly because of *M. cingulum*, which caused the largest part of infestation (up to 85%) and of post-diapause mortality (up to 35%) in these populations. In contrast, *M. cingulum* was absent (according to the traditional method) or present at lower rates (according to the molecular method) in populations feeding on maize. The higher infestation of populations collected on maize by tachinids and microsporidia was not sufficient to offset the higher *M. cingulum* infestation of populations collected on mugwort.

Altogether, our data therefore show that *M. cingulum* causes a substantial mortality on mugwort and hop (which are believed to be *O. scapulalis*' 'original' host plants), and that both parasitism and post-diapause parasitoid-caused mortality are significantly lower in *O. nubilalis* populations collected on maize, the recently introduced host. Several proximal mechanisms by which a plant can protect insects against parasites – and hence provide EFS – have been documented (Price *et al.*, 1980).

Firstly, they include mechanisms causing lower oviposition. Indeed, (i) maize stalks have a larger diameter than most mugwort stalks. Although apparently *M. cingulum* does not sting through the stalk (Parker, 1931; Edwards & Hopper, 1998), this difference in host plant structure could prevent oviposition, as shown for apple vs. hawthorn fruit in the case of the fly *R. pomonella* (Feder, 1995), wild vs. cultivated sunflower seeds for the sunflower moth *Homoeosoma electellum* (Chen & Welter, 2007), or *Physalis* fruit for two heliothine moth species (Sisterson & Gould, 1999; Oppenheim & Gould, 2002). (ii) Mugwort plants grow in denser stands than maize and White & Andow (2005) found evidence that *M. cingulum* infestation may increase with plant density. (iii) Maize (Lupolli *et al.*, 1990; Schnee *et al.*, 2006) and mugwort (Barney *et al.*, 2005) emit distinct volatiles whereas female *M. cingulum* are attracted to certain plant volatiles more than others (Ding *et al.*, 1989a,b; Udayagiri & Jones, 1993; Jones, 1996).

Secondly, after oviposition, the parasitoid's development success may vary with host plant. Indeed, *M. cingulum* DNA was detected in a significantly higher fraction of *Ostrinia* larvae than the fraction that died and turned into a *M. cingulum* cocoon after diapause. This discrepancy was observed in samples collected from mugwort (44% vs. 16%) but it was particularly striking in those collected from maize (9% vs. 0%). Over all years and locations, not a single *M. cingulum* adult was

observed emerging from 7459 larvae collected from maize in 3 years and 64 populations, whereas five in 12 of these populations showed positive PCR signals corresponding to 2–30% infestation by *M. cingulum*. Plant chemicals may affect the parasitoid *via* the insect host (Denno *et al.*, 1990; Turlings & Benrey, 1998; Singer *et al.*, 2004). For instance, DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoaxin-3-one), which is produced by maize, has been shown to influence the development of the parasitoid *D. terebrans* (Hymenoptera: Ichneumonidae) in *O. nubilalis* (Campos *et al.*, 1990). Although this influence was positive for DIMBOA and *D. terebrans*, it may be negative for other plant-produced chemical substances and/or for *M. cingulum*.

However, differences in *M. cingulum* oviposition and/or survival could also result from genetic differences between *O. scapulalis* and *O. nubilalis*, rather than or in addition to resulting directly from the host plants on which they feed. For instance, maize may have shifted the phenology of *O. nubilalis* (Thomas *et al.*, 2003 but see Malausa *et al.*, 2005) to a time window that for some reason is less favourable for oviposition by *M. cingulum*. Similarly, *O. nubilalis* may be better protected than *O. scapulalis* by its immune system. The closely related *O. furnacalis*, which is also under attack by *M. cingulum* in Asia, is, to some extent, able to prevent the parasitoid's development by encapsulating the embryo in a layer of specialized hemocytes (Hu *et al.*, 2003). This immune defence mechanism may perform better in one than in the other European *Ostrinia* species. At least one other set of closely related but genetically differentiated taxa displays such genetically based difference in immune defences [i.e. *Pieris rapae* vs. *P. melete* and *P. napi* (Ohsaki & Sato, 1990)]. In our case, this would however raise the question of how low resistance is maintained in *O. scapulalis* despite ongoing gene flow (Malausa *et al.*, 2005) between the two *Ostrinia* species and strong selection pressure exerted by *M. cingulum*.

In sum, whereas our data clearly show that parasitism by *M. cingulum* is lower in *Ostrinia* larvae feeding on maize than in those feeding on mugwort, the mechanistic explanation of this difference is an open question. We see no strong evidence in our data or elsewhere indicating which is(are) the best candidate(s), so that only empirical studies can tell. From an evolutionary perspective, it will be interesting to focus specifically on assessing the relative importance of factors resulting directly from the plant vs. factors resulting from genetic differences between the two insect hosts. Indeed, the possibility that genetic differences involved in divergent adaptation to different host plants and reduction of gene flow are also involved – be it directly, by drift, genetic linkage or pleiotropy – in differences in resistance to parasitoids offers an interesting perspective on how protection from parasites may result from EFS on an ecological timescale and from ecological speciation on an evolutionary timescale.

Whether the divergence between the two sibling species *O. scapulalis* and *O. nubilalis* in Europe is really due to ecological speciation on distinct host plants and, if so, whether the introduction of maize triggered this speciation is still under debate (Malausa *et al.*, 2007b). However, within such a scenario, how important may EFS have been for the adaptation of *O. nubilalis* to maize and its divergence from *O. scapulalis*? Berdegue *et al.* (1996) argue that EFS is crucial when fitness is higher on the original than on the alternative host plant in the absence of natural enemies [condition (3) in Berdegue *et al.* (1996)]: in such case, EFS may make the difference by offsetting this initial fitness cost. Calcagno *et al.* (2007) found contrasting results with respect to condition (3) in *Ostrinia*: in a greenhouse experiment, *O. scapulalis* survived better on maize than on mugwort, but in a more realistic field experiment protected by an insect-proof net, it was the opposite. Thus, at least under certain circumstances, maize is, *per se*, a poorer resource than mugwort for *O. scapulalis*, so that the presence of natural enemies and maize providing EFS may have been key to permitting the host shift. By protecting *Ostrinia* larvae from their main parasitoid, *M. cingulum*, maize may thus have favoured the evolution of a species that then became one of its worst enemies around the world.

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