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Abstract

Target pests may become resistant to *Bacillus thuringiensis* (Bt) toxins produced by trangenic maize (*Zea mays* L.). Untreated refuge areas are set aside to conserve high frequencies of susceptibility alleles: a delay in resistance evolution is expected if susceptible individuals from refuges mate randomly with resistant individuals from Bt fields. In principle, refuges can be toxin-free maize or any other plant, provided it hosts sufficiently large pest populations mating randomly with populations from Bt-maize fields. Our aim was to examine the suitability of several cultivated or weedy plants [pepper (*Capsicum frutescens* L.), sorghum (*Sorghum* spec.), sunflower (*Helianthus annuus* L.), cocklebur (*Xanthium* spec.), cantaloupe (*Cucumis melo* L.), and hop (*Humulus lupulus* L.)] as refuges for *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) and *Sesamia nonagrioides* Lefebvre (Lepidoptera: Noctuidae), two major maize pests in southern Europe. Larvae of both species were collected on these plants. Their genetic population structure was examined at several allozyme loci. We found little or no evidence for an influence of geographic distance, but detected a significant host-plant effect on the genetic differentiation for both species. *Ostrinia nubilalis* populations from sunflower, pepper, cocklebur, and sorghum appear to belong to the same genetic entity as populations collected on maize, but to differ from populations on hop. Accordingly, females from pepper and cocklebur produced exclusively the 'Z' type sexual pheromone, which, in France, characterizes populations developing on maize. Qualitatively, these plants (except hop) could thus serve as refuges for *O. nubilalis*; however, they may be of little use quantitatively as they were found much less infested than maize. *Sesamia nonagrioides* populations on maize and sorghum reached comparable densities, but a slight genetic differentiation was detected between both. The degree of assortative mating between populations feeding on both hosts must therefore be assessed before sorghum can be considered as a suitable refuge for this species.

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Introduction

The European corn borer (ECB), *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), and the Mediterranean corn borer (MCB), *Sesamia nonagrioides* Lefebvre (Lepidoptera: Noctuidae), are two major polyphagous maize (*Zea mays* L.) pests whose geographic ranges overlap in western Asia

and in Europe south of the 45th parallel (Hudon, 1986; Hudon et al., 1989; Buès et al., 1996). Both are targets of the *Bacillus thuringiensis* (Bt) toxin produced by transgenic Bt-maize varieties. A major concern about such varieties, especially if they are planted densely and over large acreages, is that pest populations may rapidly develop resistance to Bt toxins (Bates et al., 2005). A management strategy aimed at delaying resistance development has therefore been made mandatory in the USA (United States Environmental Protection Agency, 2001) and is under consideration in Europe: the so-called 'high-dose refuge' (HDR) strategy (Alstad & Andow, 1995; Gould, 1998; Bourguet et al., 2005).

Essentially, the HDR strategy consists in setting aside Bt-toxin-free 'refuge' areas, where alleles conferring susceptibility to the Bt toxin would be kept at high frequency in pest populations not exposed to the toxin's selection pressure. It is expected that, provided such refuges are set in close enough vicinity to Bt fields (<800 m; United States Environmental Protection Agency, 2001), random mating among adults will dilute resistance alleles within the global population. As these alleles are assumed to be rare and functionally recessive, it is further expected that most of them will be carried by heterozygotes and eliminated when the latter feed on Bt plants. Allozyme analyses of ECB populations feeding on maize showed a pattern compatible with random mating within fields and a high gene flow between fields across the whole France (Bourguet et al., 2000a), although other scenarios cannot be excluded (Rousset, 2003). In contrast, the possible effect of distance on genetic differentiation between MCB populations has been less extensively studied (Buès et al., 1996), so that empirical bases are lacking to conjecture the efficiency of the HDR strategy in the case of this species.

Refuges can be Bt-toxin-free maize fields. However, in the absence of protection against pest attacks, yields may be expected to be low. Maize refuges thus appear economically unattractive in the short term for individual farmers, which jeopardizes the chances that they actually be implemented (Bourguet et al., 2005). Alternatively or in addition to maize fields, refuges could also consist of areas planted with other plant species, provided they are good hosts for the target pests, and provided they host individuals that mate freely with adults emerging from maize (Gould, 1998; Losey et al., 2001).

In the northern half of Europe, where only ECB is present, mugwort (*Artemisia vulgaris* L.) and hop (*Humulus lupulus* L.), two common ECB host plants, appeared as potential candidates to serve as alternative refuges. However, they were found to host a particular race of the ECB, which interbreeds very little with the race feeding on maize (a finding which has also been discussed in the theoretical framework of sympatric speciation: Bourguet et al., 2000b; Martel et al., 2003; Thomas et al., 2003; Malausa et al., 2005). Moreover, the surface covered by hop and mugwort, compared with that devoted to maize, and the densities reached by the ECB on these three plants, make it unlikely that mugwort/hop refuges would provide the desired ratio of susceptible over resistant ECBs – according to the United States Environmental Protection Agency (2001), this ratio should be ca. 500 susceptible individuals for every resistant moth that might emerge from Bt-maize fields. Finally, the fact that not only ECB, but also MCB extensively infests maize in southern Europe makes it necessary to conceive a global resistance management strategy that takes both lepidopteran pest species into consideration. However, little is known about genetic isolation by distance and/or host plant between MCB populations (Buès et al., 1996). Especially, possible mating barriers between MCBs from populations developing on different host plants have, to our knowledge, never been examined.

The present study was aimed at identifying host plants that would be quantitatively and qualitatively suitable to serve as refuges for Bt-toxin susceptibility alleles in both the MCB and the ECB populations. For this purpose, we examined the densities and genetic variability of ECB and MCB populations collected as larvae in various locations of the French territory from maize and from five other species among their wild or cultivated host plants. We also examined the genetic variability of ECB populations collected on hop – a known host plant for a separate ECB race (Bourguet et al., 2000b; Martel et al., 2003), as a control allowing to test for the presence of the hop host race among the ECB populations collected from the other plants under study. Finally, we identified the sexual pheromone of ECB females collected from two of the newly investigated host plants. The implications of our findings in terms of ECB host race distribution among host plants are discussed.

Materials and methods

Sampling sites

Samples for population genetic analysis. Samples of *O. nubilalis* and *S. nonagrioides* were taken from sites located in central, south-eastern, and south-western France and from one site located near Sevilla in southern Spain (Figure 1). Seven different host plants were considered: maize (*Z. mays*), pepper (*Capsicum frutescens* L.), sorghum (*Sorghum* spec.), sunflower (*Helianthus annuus* L.), cantaloupe (*Cucumis melo* L.), cocklebur (*Xanthium* spec.), and hop (*H. lupulus*). The cocklebur and hop stands were wild, whereas the five other plants were conventional crops – none of the fields sampled in this study was planted with transgenic varieties.

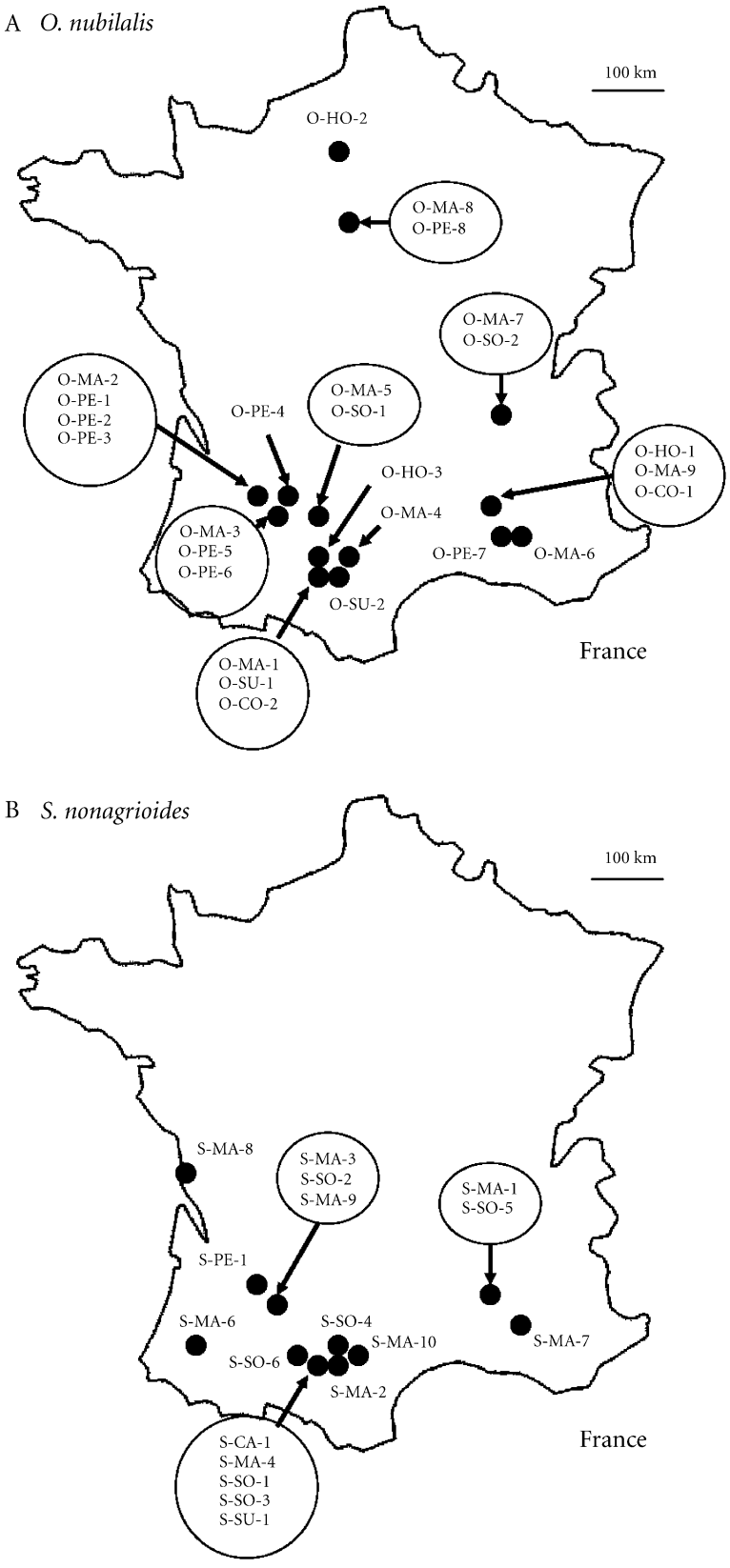

Figure 1 Location of samples of (A) *Ostrinia nubilalis* and (B) *Sesamia nonagrioides*. The sample of *S. nonagrioides* collected at Sevilla, Spain (sample S-MA-5, see Table 1) is not indicated on this map.

Individuals of both species were collected as diapausing larvae, their sex was determined, and they were directly frozen at −20 °C. On each host-plant individual, we did not collect more than a single larva per plant and the plants investigated for the presence of ECB and MCB were randomly chosen in order to reduce the risk of sampling genetically related individuals.

For *O. nubilalis*, we collected samples of 26 populations: nine from maize, three from hop, eight from pepper, two from sorghum, two from cocklebur, and two from sunflower (Table 1 and Figure 1A). For *S. nonagrioides*, we collected samples of 19 populations: 10 from maize, six from pepper, and one from cantaloupe, sorghum, and sunflower each (Table 1 and Figure 1B). Each sample consisted of 16–62 individuals. At least 100 plants were examined in each patch or field of the various host plants we examined. ECB and MCB densities were not formally measured. However, fields were roughly classified while sampling into one of three classes: <0.01 larva per plant, 0.01–0.1 larva per plant, and 0.1–5 larvae per plant.

Samples for sex pheromone analysis. Samples of *O. nubilalis* fifth-instar larvae were collected on pepper at Fongrave (44°53′N, 0°40′E, July 2004) and Orléans (47°55′N, 1°54′E, September 2004), and on cocklebur at Bourg St Andéol (44°23′N, 4°42′E, September 2004). Larvae were reared to adult stage either directly (if collected in July) or after completion of diapause at 4 °C (if collected in September). The sexual pheromones of nine, three, and 11 adult females could be determined for the samples collected at Fongrave, Orléans, and Bourg St Andéol, respectively.

Enzyme electrophoresis

Each ECB or MCB larva was homogenised in 150 µl of Tris-EDTA (pH 6.8). Horizontal starch gel electrophoreses of the homogenates were carried out using a Tris-Borate-EDTA (pH 8.6) buffer system (Pasteur et al., 1987). Seven enzyme systems were revealed as described in Bourguet et al. (2000a) and Buès et al. (1996). These systems were phosphoglucomutase (PGM, EC 5.4.4.2), mannose-6-phosphate isomerase (MPI, EC 5.3.1.8), hydroxybutyrate dehydrogenase (HBDH, EC 1.1.1.30), glucose-phosphate isomerase (GPI, EC 5.3.1.9), aspartate aminotransferase (AAT, EC 2.6.1.1), triose-phosphate isomerase (TPI, EC 5.3.1.1), and creatine kinase (CK, EC 2.7.3.2). These seven systems were revealed for both species except for CK in *O. nubilalis* and except for MPI in *S. nonagrioides*. The *Tpi* locus of *O. nubilalis* is located on the Z chromosome (Glover et al., 1990). In Lepidoptera, females are the heterogametic (WZ) whereas males are the homogametic (ZZ) sex. Thus, at this locus, ECB females are hemiploid whereas ECB males are diploid.

Mpi haplotypes

For *O. nubilalis* larvae, polymerase chain reaction (PCR) amplifications of a fragment of the *Mpi* gene were performed on total DNA extracted as described by Estoup et al. (1996). DNA extractions were performed on the homogenates used for enzyme electrophoresis. The PCR mixtures contained 1 × PCR buffer (Eurogentec, Seraing, Belgium), 0.1 mM dTNPs, 1 unit *Taq* polymerase, and 1 µM of each primer (forward Mpi15: 5′-CACCGTTTGAGGCTCTCTGT-3′, and reverse Mpi16: 5′-TTGGTCTCCTTGGTCTTCTTG-3′). Cycling conditions were 95 °C for 1.5 min, followed by 35 cycles of 95 °C for 30 s, 60 °C (annealing temperature) for 30 s, and a primer extension at 72 °C for 30 s. The *Mpi* fragment amplified by the combination of the Mpi15 and Mpi16 primers encompasses an intron displaying substantial length polymorphism (SF Lee, unpubl.). Four classes of alleles were defined according to the length polymorphism observed on more than 100 sequences of that fragment – these sequences were obtained for another purpose and will be reported elsewhere. The analysis of these sequences showed that the *Mpi* alleles differed either by point mutations or by substantial insertions or deletions (indels). Four discrete classes of alleles – with approximate sizes of 400, 450, 470, and 510 bp – were defined according to the size of this indel. *Mpi* fragments amplified by PCR were therefore assigned unambiguously to one of these four classes of alleles by estimating their length on 3% agarose gels.

Pheromone collection and identification

After emergence, females were placed in individual plastic containers with a water supply and were kept in a rearing room under 24 ± 2 °C, 70 ± 20% r.h., and a L16:D8 reversed photoperiod. Sex pheromone was collected individually from 2- to 5-day-old females sampled during the calling period (the last 3 h of darkness). The pheromone gland was extruded by gentle pressure on the abdomen and kept in this position with metallic forceps. The adsorbent part of an SPME fibre (CW/DVB, 65 µm, Supelco, Bellefonte, PA, USA) was gently rubbed on the gland surface (Frérot et al., 1997). The fibre was then desorbed on the injector (245 °C) of a Varian 3400 CX gas chromatograph (Varian Inc., Palo Alto, CA, USA) equipped with a polar column (Rtx Wax, 30 m, 0.32 id, 0.5 µm, Restek Corp., Bellefonte, PA, USA). The oven temperature was programmed as follows: hold initial temperature (50 °C) for 1 min, increase to 100 °C at 15 °C min^{-1}, hold for 1 min, and increase to 245 °C at 5 °C min^{-1}. The temperature of the FID detector was set at 260 °C. The carrier gas was helium (14 p.s.i.). Natural components were identified by comparison of their retention times with those of a hexane solution of synthetic

Table 1 Characteristics of *Ostrinia nubilalis* and *Sesamia nonagrioides* samples: host plant, location and sample abbreviation, year of sampling, number (n) of individuals analysed, and density class (D): 1 = <0.01 larva per plant, 2 = 0.01–0.1 larva per plant, 3 = 0.1–5 larvae per plant, NA = not available

Species	Host plant	Location	Sample	Latitude	Longitude	Year	n	D
O. nubilalis	Hop	Bourg St Andéol	O-HO-1	44°23′N	4°42′E	2002	44	NA
		Beynes	O-HO-2	48°51′N	1°52′E	2002	42	NA
		Cft Arriège-Garonne	O-HO-3	43°31′N	1°27′E	2003	32	NA
	Maize	Vénerque	O-MA-1	43°26′N	1°27′E	2002	35	3
		Marmande	O-MA-2	44°30′N	0°10′E	2002	45	3
		Agen	O-MA-3	44°12′N	0°38′E	2002	54	3
		Fiac	O-MA-4	43°42′N	1°54′E	2002	45	3
		St Etienne de Tulmont	O-MA-5	44°03′N	1°28′E	2003	30	3
		Mollégès	O-MA-6	43°48′N	4°57′E	2003	29	3
		Côtes d'Arey	O-MA-7	45°28′N	4°51′E	2003	30	3
		Orléans	O-MA-8	47°55′N	1°54′E	2003	29	3
		Pierrelatte	O-MA-9	44°23′N	4°42′E	2003	23	3
	Pepper	Marmande site 1	O-PE-1	44°30′N	0°10′E	2002	41	2
		Marmande site 2	O-PE-2	44°30′N	0°10′E	2002	62	2
		Marmande site 3	O-PE-3	44°30′N	0°10′E	2002	49	2
		St Sylvestre sur Lot	O-PE-4	44°29′N	0°49′E	2002	15	2
		Montesquieu	O-PE-5	44°12′N	0°27′E	2002	58	2
		Petit Guérin	O-PE-6	44°12′N	0°27′E	2003	29	2
		Avignon	O-PE-7	43°53′N	4°49′E	2003	33	2
		Orléans	O-PE-8	47°55′N	1°54′E	2003	29	2
	Sorghum	St Etienne de Tulmont	O-SO-1	46°54′N	1°28′E	2003	16	2
		Côtes d'Arey	O-SO-2	45°28′N	4°51′E	2003	30	2
	Sunflower	Vénerque	O-SU-1	43°26′N	1°27′E	2002	19	2
		Nailloux	O-SU-2	43°22′N	1°38′E	2002	55	2
	Cocklebur	Bourg St Andéol	O-CO-1	44°23′N	4°42′E	2003	35	NA
		Muret	O-CO-2	43°28′N	1°21′E	2003	16	NA
S. nonagroides	Cantaloupe	Saint Léon	S-CA-1	43°24′N	1°34′E	2002	50	1
	Maize	Pierrelatte	S-MA-1	44°23′N	4°42′E	2002	50	3
		Baziège	S-MA-2	43°27′N	1°37′E	2002	50	3
		Agen	S-MA-3	44°12′N	0°38′E	2002	49	3
		Vénerque	S-MA-4	43°26′N	1°27′E	2002	49	3
		Sevilla (Spain)	S-MA-5	37°22′N	5°59′W	2002	31	NA
		Dax	S-MA-6	43°43′N	1°03′W	2002	23	3
		Mollégès	S-MA-7	43°48′N	4°57′E	2002	22	3
		Grues	S-MA-8	46°23′N	1°18′W	2002	29	3
		Port Sainte Marie	S-MA-9	44°35′N	0°12′E	2002	22	3
		Saint Papoul	S-MA-10	44°20′N	2°02′E	2002	50	3
	Pepper	Marmande	S-PE-1	44°30′N	0°10′E	2002	30	1
	Sorghum	Vénerque site 1	S-SO-1	43°26′N	1°27′E	2002	30	3
		Montesquieu	S-SO-2	44°12′N	0°27′E	2002	50	3
		Vénerque site 2	S-SO-3	43°26′N	1°27′E	2002	52	3
		Fiac	S-SO-4	43°42′N	1°54′E	2002	55	3
		Pierrelatte	S-SO-5	44°23′N	4°42′E	2002	55	3
		Saint Hilaire	S-SO-6	43°25′N	1°16′E	2002	43	3
	Sunflower	Vénerque	S-SU-1	43°26′N	1°27′E	2002	30	2

Z and E isomers of the 11-14:OAc, the two known components of the ECB pheromone (Klun et al., 1973). The ratio of both components was calculated as the ratio of the areas of the corresponding peaks.

Analysis of data on population structure

For each sample, we estimated the allelic frequencies, the mean number of alleles (N_{all}), the observed heterozygosity (H_o) and the unbiased expected heterozygosity (H_e) as

described by Nei (1978), and f̂-values [i.e., F_{is} estimates according to Weir & Cokerham (1984)] using GENETIX 4.0 (Belkir et al., 2000). We tested for deviations from Hardy–Weinberg expectations and calculated genotypic linkage disequilibria between loci, within each sample, with GENEPOP 3.2 (Raymond & Rousset, 1995). As *Tpi* is a sex-linked locus for *O. nubilalis*, H_o and f̂-values for samples of this species were estimated and tests for deviations from Hardy–Weinberg expectations were carried out for males only. Finally, the frequency of null alleles at the *Mpi* locus was estimated using the EM algorithm (Dempster et al., 1977) implemented in GENEPOP 3.2.

The genetical structure between samples of a host-plant group (a host-plant group includes all samples collected on a given host plant) was analysed by testing for allelic differentiation using Fisher exact tests and calculating the $\hat{\theta}$ estimator of the F_{st} as described by Weir & Cockerham (1984), using GENEPOP 3.2. The $\hat{\theta}$-values calculated over all samples collected on hop and maize are referred to as $\hat{\theta}$ hop and $\hat{\theta}$ maize, respectively. We also carried out hierarchical analyses of population structure between host-plant groups (Weir & Cockerham, 1984), using TFPGA 1.3 software (Miller, 1997). Finally, isolation-by-distance patterns (Slatkin, 1993) were tested for samples of *S. nonagrioides* by assessing the independence of geographic and genetic distances for various geographical levels. The null hypothesis that geographic and genetic distances were independent was tested against the alternative hypothesis of a positive correlation expected under isolation by distance, estimated as Spearman's rank correlation coefficient. The calculated correlation coefficient was compared with the distribution of correlation coefficients obtained from Mantel-like permutations of the genetic $[\hat{\theta}/(1-\hat{\theta})]$ and geographic [ln (geographical distance)] matrices as described by Rousset (1997) and included in GENEPOP 3.2.

Results

Infestation levels of the various host plants

As shown in Table 1, the highest ECB densities were reached on maize (typically 0.1–5 larvae per plant), followed (at a much lower level) by sorghum, cocklebur, and sunflower (usually 0.01–0.1 larva per plant). Though economically damaging, densities on pepper and on cantaloupe were low (<0.01 larva per plant). MCBs were present at high and comparable densities in maize and sorghum (typically 0.1–5 larvae per plant), and at much lower densities on pepper, sunflower, and cantaloupe (0.01–0.1 larva per plant). It was not found on hop or cocklebur.

Population structure of ECB

All loci – *Tpi*, *Pgm*, *Aat*, *Gpi*, *Hbdh*, and *Mpi* – investigated in this study were polymorphic. However, the *Hbdh* locus displayed polymorphism in only eight (O-HO-1, O-MA-7, O-MA-8, O-PE-1, O-PE-2, O-PE-5, O-PE-6, and O-CO-2) out of the 26 samples. For most individuals, the staining of the MPI system was of insufficient quality for a reliable genotyping. Rather than merely omitting this locus from the analysis, we amplified a fragment of the *Mpi* locus encompassing an intron that displays four different sizes. This enabled us to genotype individuals according to PCR product sizes.

In agreement with our previous results on population genetic studies on ECB (Bourguet et al., 2000a,b; Martel et al., 2003; Bontemps et al., 2004), we found no significant genotypic association between any pair of loci (data not shown). Allele frequencies at each locus and for each sample are given in Supplementary Appendix 1. Within samples, the mean number of alleles was 1.80–3.40 for the polymorphic loci tested, with apparently no particular trend distinguishing samples by host plant. The observed heterozygosity (H_o) was often lower than the expected heterozygosity (H_e). Accordingly, the f̂-values often indicated that heterozygosity tended to be lower than expected under Hardy–Weinberg equilibrium, the deviations from Hardy–Weinberg expectations for the six loci being significant in five of the 26 samples (O-MA-2, O-MA-6, O-MA-9, O-SU-1, and O-CO-2). Except for one sample (O-SU-1), these deviations remained significant even when a Bonferroni correction for multiple tests was applied (Holm, 1979). This result is unexpected as all ECB samples investigated to date were found to be at Hardy–Weinberg equilibrium (Bourguet et al., 2000a,b; Martel et al., 2003; Bontemps et al., 2004). However, a closer examination of the data revealed that these heterozygote deficiencies were mainly due to the *Mpi* locus: indeed, f̂-values at this locus were always positive, and significant deviations from Hardy–Weinberg equilibrium were detected in 10 samples. When this locus was removed from the analysis, none of the samples displayed a significant deviation from Hardy–Weinberg expectations anymore. Hence, the most likely explanation for the deviation found on the complete data set is the presence of undetected 'null' alleles due to a lack of PCR amplifications of one or several *Mpi* haplotypes in these samples. Over all samples, the estimated mean frequency ± SE of this null allele equals 0.399 ± 0.174, ranging from 0.137 to 0.702.

The overall differentiation between samples was highly significant ($P<10^{-5}$) but low ($\hat{\theta}$ overall = 0.016) (Table 2). Similar to what has been previously found by Martel et al. (2003) for ECB populations collected on mugwort, the group of samples collected on hop were significantly differentiated ($\hat{\theta}$ hop = 0.049, $P<10^{-5}$) whereas the group

Table 2 Genetic differentiation within host-plant groups of *Ostrinia nubilalis* and *Sesamia nonagrioides* [$\hat{\theta}$-values estimated according to Weir & Cockerham (1984)] for the various loci and over all loci. NA = not available; NP = no polymorphism

		Locus							
Species	Group of samples	*Tpi*	*Gpi*	*Aat*	*Pgm*	*Hbdh*	*Mpi*	*Ck*	All loci
O. nubilalis	All host plants	0.056***	0.005**	0.035***	0.011	0.004	0.002*	NA	0.016***
	Hop	0.181***	0.054**	0.012	0.012*	0.021	–0.033	NA	0.049***
	Maize	–0.015	–0.003	0.012*	0.018*	0.002	–0.004	NA	–0.001
	Pepper	0.013	0.003	0.011*	–0.003	0.002	0.013	NA	0.019**
	Sorghum	–0.063	0.001	0.038*	0.003	NP	0.001	NA	0.005
	Sunflower	NA	–0.009	–0.006	–0.011	NP	NA	NA	–0.009
	Cocklebur	–0.039	0.002	–0.013	NP	0.028	–0.031	NA	–0.013
S. nonagrioides	All host plants	0.003	0.028***	0.019***	0.108***	NP	NA	0.047***	0.061***
	Maize	0.003	0.050***	0.013**	0.147***	NP	NA	0.045***	0.082***
	Sorghum	NP	0.007	0.025*	0.040***	NP	NA	NP	0.027***
	Pepper + cantaloupe + sunflower	NP	0.006**	0.041*	0.058***	NP	NA	NP	0.039***

*P<0.05, **P<0.01, and ***P<10^{-5}.

of samples collected on maize were not ($\hat{\theta}$ maize = –0.001, P = 0.134) (Table 2). The other groups of samples were weakly (pepper) or not (sorghum, sunflower, and cocklebur) differentiated (Table 2).

We carried out a hierarchical analysis of the distribution of the genetic variability in order to determine the amount of variance explained by differentiation between host-plant groups. As expected, this component of the total variance was significant between the maize and the hop group ($\hat{\theta}$ host plant = 0.018, P<0.01). A significant differentiation between groups ($\hat{\theta}$ host plant = 0.020, P<0.01) was also found when comparing the hop group with the group containing the samples collected on pepper, sorghum, sunflower, and cocklebur. When comparing this latter group with the maize group, the differentiation between host groups dropped to zero ($\hat{\theta}$ host plant = –0.001, P<0.05). We analysed the genetic structure of each pair of samples more precisely. In agreement with the hierarchical analyses, most of the significant exact tests for allelic differentiation between samples involved one of the samples collected on hop (Figure 2). This result holds even when excluding the *Mpi* locus – which displayed heterozygote deficiencies – from the analysis (details not shown).

Sex pheromone of ECB females from pepper and cocklebur

The pheromone glands of 23 females that emerged from larvae collected on pepper and cocklebur were analysed to study the pheromone compositions of the ECB infesting these two host plants. As previously reported in all studies on ECB pheromones (e.g., Klun et al., 1973; Klun & Maini, 1979), the studied components of the sexual pheromones were the E and Z isomers of 11-14:OAc. The experimental GC procedure produced a clear separation of these two isomers and mass spectra confirmed their identification and their purity.

All females either from pepper (n = 12) or from cocklebur (n = 11) displayed the Z11-14:OAc as the main component of their sexual pheromone. Indeed, the E11-14:OAc component represented only 1% on average. According to Klun & Maini (1979), Z and E females produce Z11-14:OAc and E11-14:OAc in ratios of 97 : 3 and 1 : 99, respectively. Consequently, the 20 females analysed in this study were clearly Z females.

Population structure of MCB

Among the six loci examined, three – *Aat*, *Gpi*, and *Pgm* – were polymorphic in all samples. The *Hbdh* locus was monomorphic and the *Tpi* and *Ck* loci were polymorphic in only one (S-MA-5) and three (S-MA-1, S-MA-4, and S-MA-10) samples, respectively. Over all sites, we found no significant genotypic association between any pair of loci (results not shown). Allele frequencies at each locus and for each sample are given in Supplementary Appendix 2. Within samples, the mean number of alleles was 1.83–2.33 for the polymorphic loci tested. The observed heterozygosities (0.18–0.30) were slightly lower than the expected ones (0.23–0.32), and 10 out of the 19 samples displayed an $\hat{f}$-value >0.1. These deviations from Hardy–Weinberg expectations over the loci were significant for five samples (S-CA-1, S-MA-3, S-MA-4, S-MA-5, and S-SO-1) after a Bonferroni correction for multiple tests was applied (Holm, 1979). These deviations were not due to any particular locus, and a significant deficit in heterozygotes was detected over all sites for all three polymorphic loci: *Aat* ($\hat{f}$ = 0.139, P<10^{-3}), *Gpi* ($\hat{f}$ = 0.039, P<10^{-3}), and *Pgm* ($\hat{f}$ = 0.114, P<10^{-5}).

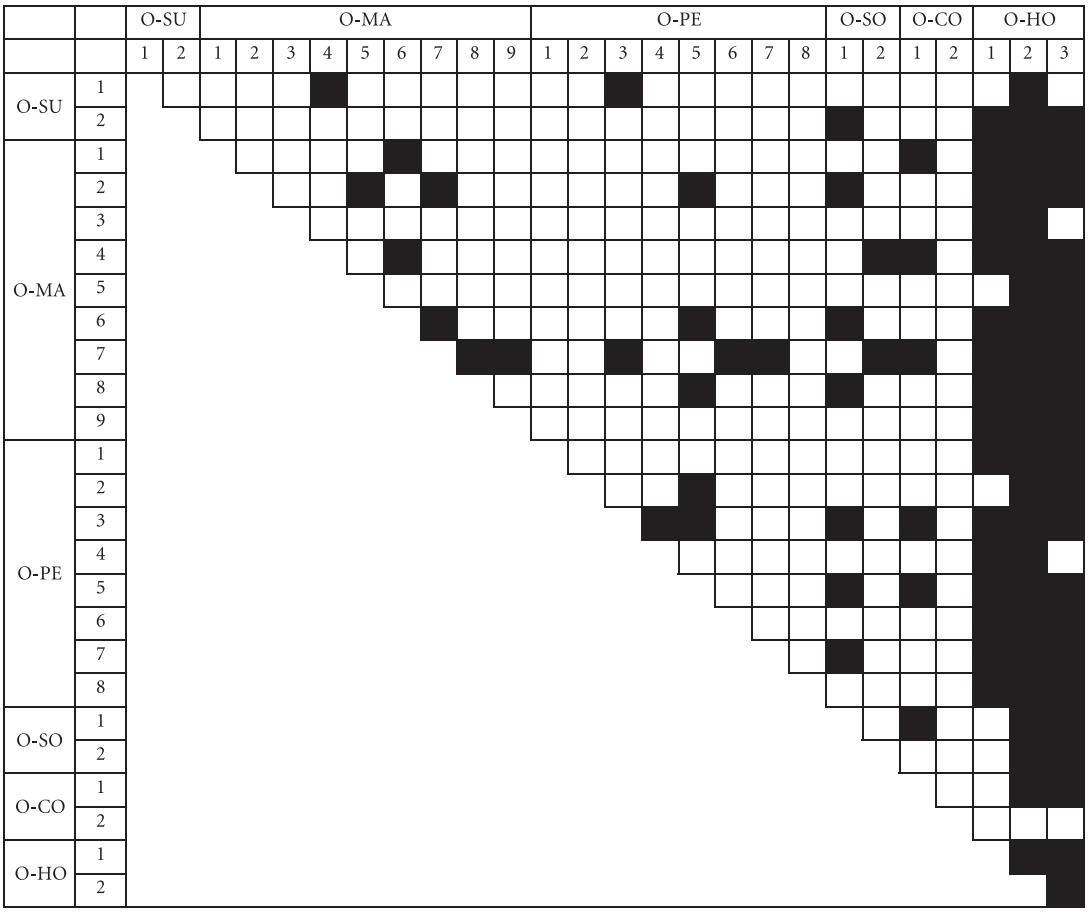

Figure 2 Pairwise exact tests of allelic differentiation for all loci between pairs of samples of *Ostrinia nubilalis*. Black squares indicate pairs of samples for which exact tests were significant ($P<0.05$). Labels O-SU, O-MA, O-PE, O-SO, O-CO, and O-HO correspond to samples collected on sunflower, maize, pepper, sorghum, cocklebur, and hop, respectively.

The overall differentiation between samples was highly significant ($P<10^{-5}$) and the overall $\hat{\theta}$-value equalled 0.061, a value five times higher than that for *O. nubilalis* samples (Table 2). The *Pgm* locus displayed the highest $\hat{\theta}$-value ($\hat{\theta} = 0.108$) and, except at the *Tpi* locus, which was monomorphic in all samples but one, we found a significant differentiation at the four polymorphic loci (Table 2). The groups of samples collected on maize, on sorghum, or on the three other host plants (pepper, cantaloupe, and sunflower) were all differentiated (Table 2).

The hierarchical analysis of the distribution of the genetic variability showed that the maize group was differentiated both from the group of six samples collected on sorghum only ($\hat{\theta}$ host plant = 0.010, $P<0.01$) and from the group of samples collected on all other, non-grass host plants (cantaloupe, pepper, and sunflower, $\hat{\theta}$ host plant = 0.010, $P<0.01$). Conversely, there was no differentiation between the sorghum group and the non-grass host-plant group ($\hat{\theta}$ host plant = –0.008, $P>0.05$).

The genetic differentiation between the maize group and the group containing the samples collected on all the other host plants can be further investigated by comparing within- and between-group isolation by distance patterns (Martel et al., 2003). We found no association between genetic and geographic distances within the group of samples collected on maize or between pairs of samples collected from different plants (maize vs. all other host plants) (Figure 3A; $P>0.800$ in both cases). Conversely, the level of differentiation within the group of samples collected on non-maize host plants was marginally correlated with distance (slope = 0.0158, $R^2 = 0.44$, $P = 0.058$) and was lower than within maize or between pairs of samples collected on different host-plant groups (Figure 3B).

Discussion

Apart from maize, we found both ECB and MCB larvae on pepper, sunflower, sorghum, and cantaloupe, although on

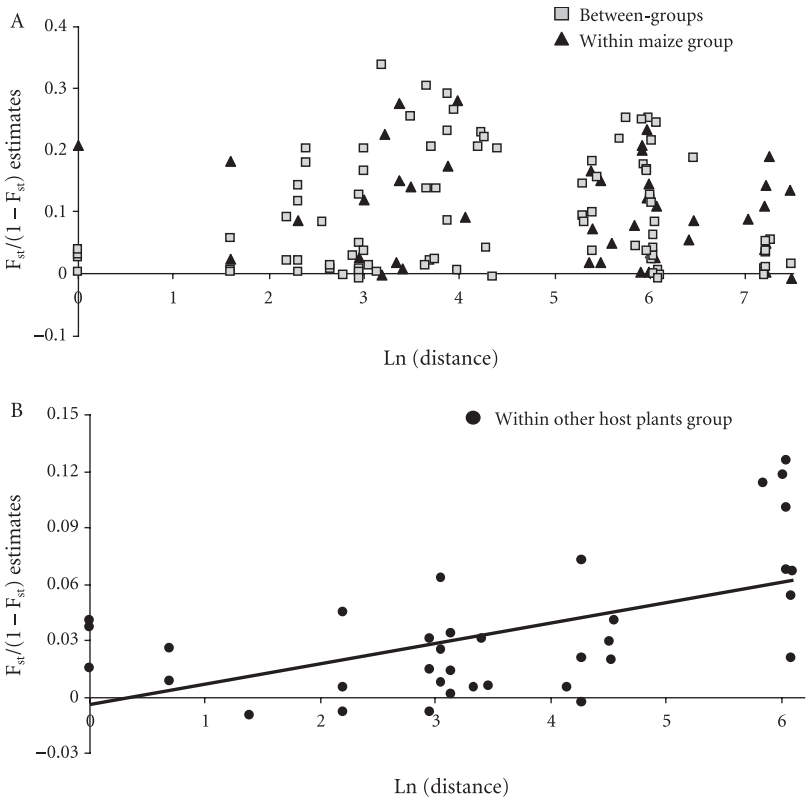

Figure 3 Regressions of $\hat{\theta}/(1-\hat{\theta})$ against log-transformed geographical distances (km) for samples of *Sesamia nonagrioides* collected on maize (within maize) and between pairs of samples collected on (A) the two host-plant groups (between groups) and (B) on other host plants (within other host plants) to test for isolation by distance.

the latter plant we found too few ECB larvae to analyse their genetical population structure. Only ECB was found on cocklebur and hop. This is consistent with previous reports of ECB feeding on a broader range of host plants than MCB: indeed, ECB has been recorded on species belonging to at least 42 different plant families (review in Ponsard et al., 2004), whereas MCB is mainly known to feed on wild and cultivated monocotyledons (rice, sorghum, maize, and *Typha* spec.; Hilal, 1981; Buès et al., 1996). Our results further draw attention to the fact that, within a given area, i.e., for a given global infestation level, not all host plants are infested at equal levels. Indeed, ECB densities were typically >10 times higher on maize than on any other plant we examined. Conversely, MCB was present at comparable densities on maize and on sorghum.

ECB samples showed no deviation from Hardy–Weinberg equilibrium (apart from what is probably an artefact due to the presence of null alleles at *Mpi*). A fraction (ca. $^1/_4$) of the MCB samples did deviate, which might be due to recent demographic bottlenecks due to the high winter mortality in this species (Galichet, 1982; Hassaine et al., 1992), although alternative explanations, such as a Wahlund effect due to the possible accidental presence in our samples of a few individuals of *Sesamia cretica* Led. (Lepidoptera: Noctuidae), cannot be ruled out. Indeed, this species is difficult to distinguish from *S. nonagrioides* at the larval stage and might be present, although at marginal densities, in our sampling area (JF Sylvain, pers. comm.).

The overall genetic differentiation between samples was higher within MCB ($\hat{\theta}$ overall = 0.061, $P<10^{-5}$) than within ECB ($\hat{\theta}$ overall = 0.016, $P<10^{-5}$) samples. As previously found (Martel et al., 2003), we detected no effect of geographic distance on the genetic differentiation between pairs of ECB samples. We also detected no significant effect of geographic distance on the genetic differentiation between pairs of MCB samples collected on maize. A small increase of genetic isolation with increased distance was, however, detected between pairs of MCB samples collected on other host plants. Under the reasonable assumption that both species reach comparable population densities, our results might indicate that MCB tends to be slightly less dispersive than ECB. However, this point would need to be confirmed by a more targeted study.

Our results further show evidence in both species for genetic differentiation between populations of larvae using different host plants. The MCB samples collected on maize showed a significant genetic differentiation from the samples collected on sorghum and from the samples collected on other host plants (the latter group could not be analysed separately for each plant due to limited sample size). Such differentiation may either be due to (1) different selection pressures exerted repeatedly on each

MCB generation by both plant types, or (2) fixed genetic differences maintained from one generation to the next by mechanisms such as assortative mating and oviposition preferences, i.e., to the existence of MCB host races. Further investigation is needed in this respect. Indeed, if the genetic structure we observed results mainly from repeated selection, MCB populations feeding on sorghum could nevertheless contribute to diluting resistance alleles in populations feeding on maize. In this case, sorghum fields could be used to replace some of the 'refuges' – the Bt-toxin-free fields required by the HDR strategy – currently planted with non-Bt maize, or to increase the total amount of such refuges. Conversely, if the genetic structure results mainly from assortative mating, their efficiency in diluting resistance alleles would probably be more limited. In this case, sorghum would be of limited interest for managing the evolution of resistance in MCB populations.

Regarding ECB, our results confirm that populations are split into two genetically differentiated groups that differ by host-plant use, the maize race and the mugwort–hop race. Indeed, the groups of ECB samples collected on maize and hop showed the common and significantly different genetic patterns characterizing the ECB maize race and hop–mugwort race, respectively (Bourguet et al., 2000b; Martel et al., 2003; Thomas et al., 2003; Bontemps et al., 2004; Malausa et al., 2005). Furthermore, our results reveal no evidence suggesting the existence of any additional host race. Indeed, samples collected on pepper, sunflower, sorghum, and cocklebur are genetically distinct from the mugwort–hop race but not significantly different from the maize race: the most parsimonious conclusion is that they consisted mostly – if not entirely – of members of the maize race. This conclusion was reinforced by our results on the sex pheromone produced by females originating from pepper and cocklebur. Indeed, in France, the two ECB host races are known to differ, among other traits, by the sex pheromone produced by females and recognized by males. The maize race and the mugwort–hop race use the so-called Z and E pheromone type, respectively (Thomas et al., 2003; Bontemps et al., 2004; Pélozuelo et al., 2004). Hence, in agreement with the population genetic structure, all females emerging from pepper and cocklebur produced exclusively the Z pheromone type.

The absence or quasi absence of the mugwort–hop race in our samples collected on pepper, sunflower, sorghum, and cocklebur can result from one or a combination of three factors: (1) females of the mugwort–hop race are little attracted to these plant species for oviposition, (2) the mugwort–hop race is largely outnumbered by the maize race in our study areas, or (3) young instars of the mugwort–hop race have a lower survival on these plants than those of the maize race. There are currently no data that would allow us to favor one of these three hypotheses.

Our findings are also interesting in that they contrast somewhat with those of a previous cage experiment (Bethenod et al., 2005) where ECB individuals were offered equal amounts of maize and mugwort to oviposit: the maize race almost exclusively oviposited on maize while the hop–mugwort race showed a less strong preference for mugwort. This result alone could be seen as an indication that the maize race is an oligophagous specialist, and that the wide-spectrum polyphagy of the ECB reported in the literature (Caffrey & Worthley, 1927; Hudon et al., 1989) might mainly resort to observations of polyphagous members of the hop–mugwort race. Our present findings show that the maize race seems also able to oviposit – although to a much lower extent – on a broad range of plant species belonging not only to the Poaceae, such as sorghum and maize, but also to the Solanaceae (pepper) and Compositae (cocklebur).

Studies performed on American and former Soviet Union ECB strains have previously shown that the ECB is able to complete its entire life cycle on sunflower (Legg et al., 1986), sorghum (Atkins et al., 1983; Dyatlova & Frolov, 1990), and pepper (McLeod, 1981). Our results confirm that this is also possible, at least until the fifth instar (the stage at which our samples were collected), for French maize-race populations on pepper, sunflower, cocklebur, and sorghum. However, fitness (estimated as larval weight or developmental time) appeared lower for larvae feeding on sunflower (Legg et al., 1986) or sorghum (Dyatlova & Frolov, 1990) than for the corresponding control individuals feeding on maize. Oviposition was also higher on maize than on sunflower (Legg et al., 1986) and than on sorghum, at least at certain times of the season (Dyatlova & Frolov, 1990).

Our data show that the net effect of possible differences in oviposition choices and larval development results in maize suffering much higher infestations than the other sympatric host plants. Pepper, sunflower, cocklebur, and sorghum thus probably function rather as sink habitats infested by more or less marginal oviposition of maize-race ECB females mainly attracted to nearby maize fields, than as 'true' hosts on which autonomous and dense ECB populations could develop over several generations.

In sum, from a genetic point of view, ECB adults emerged from any of the plants considered in this study – except hop – are probably able to mate with each other. However, sunflower, sorghum, cocklebur, and pepper are planted over smaller acreages and seem to host but low densities of ECB larvae compared with maize. Therefore, they may not replace non-Bt maize refuges in the framework of the HDR resistance-delaying strategy, but merely complement them.

Regarding MCB, the exact origin and strength of the genetic differentiation found between samples collected on maize and those found on sorghum, as well as the effect of geographic isolation on genetic distance between samples, call for closer examination. Indeed, the high MCB densities reached on sorghum could make it a valuable refuge crop for this species, provided neither reproductive nor geographic isolation prevent an efficient mixing between the Bt-maize field and the refuge populations.

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Supplementary material

The following supplementary material is available for this article online at www.blackwellsynergy.com:

Appendix S1. Allele frequencies for each locus and each sample of *Ostrinia nubilalis.* n = number of alleles.

Appendix S2. Allele frequencies for each locus and each sample of *Sesamia nonagrioides.* n = number of alleles.

Supplementary

Appendix 1 Allele frequencies for each locus and each sample of *Ostrinia nubilalis*. n = number of alleles

	Allele	O-SU-1	O-SU-2	O-MA-1	O-MA-2	O-MA-3	O-MA-4	O-MA-5	O-MA-6	O-MA-7	O-MA-8	O-MA-9	O-PE-1	O-PE-2
Tpi	1			0.780	0.609	0.743	0.780	0.828	–	–	–	–	–	–
	2	–	–	0.220	0.239	0.257	0.220	0.172	–	–	–	–	–	–
	3			0.000	0.152	0.000	0.000	0.000	–	–	–	–	–	–
	N			50	46	35	41	29	–	–	–	–	–	–
Aat	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	2	0.111	0.103	0.150	0.100	0.086	0.067	0.065	0.029	0.094	0.067	0.035	0.130	0.054
	3	0.806	0.872	0.800	0.800	0.914	0.933	0.913	0.914	0.891	0.867	0.965	0.852	0.946
	4	0.083	0.026	0.050	0.100	0.000	0.000	0.022	0.057	0.016	0.067	0.000	0.019	0.000
	N	36	78	60	60	58	60	46	70	64	60	86	108	74
Pgm	1	0.000	0.022	0.033	0.054	0.017	0.000	0.000	0.038	0.015	0.065	0.013	0.021	0.026
	2	1.000	0.956	0.933	0.893	0.983	1.000	1.000	0.897	0.882	0.903	0.975	0.979	0.921
	3	0.000	0.022	0.033	0.054	0.000	0.000	0.000	0.064	0.103	0.032	0.013	0.000	0.053
	4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	N	30	90	60	56	58	60	46	78	68	62	80	96	76
Hbdh	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000	0.009	0.022
	2	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.988	1.000	0.978	0.974	0.978
	3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.022	0.018	0.000
	N	28	102	60	60	58	60	46	90	86	70	90	114	90
Gpi	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	2	0.132	0.087	0.133	0.133	0.103	0.050	0.130	0.110	0.076	0.100	0.089	0.100	0.033
	3	0.553	0.644	0.683	0.600	0.724	0.767	0.717	0.671	0.641	0.617	0.644	0.617	0.728
	4	0.316	0.269	0.183	0.267	0.172	0.183	0.152	0.220	0.283	0.283	0.267	0.275	0.239
	5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.008	0.000
	N	38	104	60	60	58	60	46	82	92	60	90	120	92
Mpi	1	–	0.477	–	–	0.446	–	0.525	0.250	–	–	0.500	0.417	0.361
	2	–	0.159	–	–	0.071	–	0.025	0.063	–	–	0.063	0.028	0.083
	3	–	0.318	–	–	0.393	–	0.225	0.438	–	–	0.281	0.278	0.389
	4	–	0.045	–	–	0.089	–	0.225	0.250	–	–	0.156	0.250	0.167
	5	–	0.000	–	–	0.000	–	0.000	0.000	–	–	0.000	0.028	0.000
	6	–	0.000	–	–	0.000	–	0.000	0.000	–	–	0.000	0.000	0.000
	N	–	44	–	–	56	–	40	16	–	–	32	36	36

Appendix 1 (*Continued*)

	Allele	O-PE-3	O-PE-4	O-PE-5	0-PE-6	O-PE-7	O-PE-8	O-SO-1	O-SO-2	O-CO-1	O-CO-2	O-HO-1	O-HO-2	O-HO-3
Tpi	1	–	–	–	0.727	0.776	0.875	0.760	0.829	0.830	0.792	0.457	0.803	0.661
	2	–	–	–	0.273	0.224	0.125	0.240	0.171	0.170	0.208	0.543	0.197	0.194
	3	–	–	–	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.145
	N	–	–	–	44	49	40	25	41	47	24	46	66	62
Aat	1	0.000	0.000	0.000	0.016	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000
	2	0.028	0.135	0.100	0.094	0.061	0.037	0.125	0.133	0.186	0.156	0.188	0.261	0.321
	3	0.847	0.808	0.800	0.875	0.909	0.926	0.719	0.850	0.786	0.844	0.813	0.705	0.679
	4	0.125	0.058	0.100	0.016	0.030	0.037	0.156	0.000	0.029	0.000	0.000	0.034	0.000
	N	72	52	30	64	66	54	32	60	70	32	64	88	84
Pgm	1	0.022	0.013	0.000	0.047	0.000	0.037	0.063	0.033	0.000	0.000	0.016	0.035	0.000
	2	0.957	0.974	0.962	0.938	0.985	0.944	0.906	0.967	1.000	1.000	0.875	0.953	0.925
	3	0.022	0.013	0.038	0.016	0.015	0.019	0.031	0.000	0.000	0.000	0.094	0.012	0.038
	4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.016	0.000	0.038
	N	92	78	26	64	66	54	32	60	70	32	64	86	80
Hbdh	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.031	0.000	0.000
	2	1.000	1.000	0.967	0.984	1.000	1.000	1.000	1.000	1.000	0.969	0.969	1.000	1.000
	3	0.000	0.000	0.033	0.016	0.000	0.000	0.000	0.000	0.000	0.031	0.000	0.000	0.000
	N	104	82	30	64	66	54	32	60	70	32	64	88	84
Gpi	1	0.009	0.000	0.033	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	2	0.160	0.057	0.133	0.172	0.061	0.130	0.125	0.083	0.086	0.063	0.172	0.125	0.202
	3	0.585	0.743	0.700	0.656	0.697	0.648	0.563	0.683	0.643	0.750	0.703	0.466	0.631
	4	0.245	0.200	0.133	0.172	0.242	0.222	0.313	0.233	0.271	0.188	0.125	0.398	0.167
	5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011	0.000
	N	106	70	30	64	66	54	32	60	70	32	64	88	84
Mpi	1	–	–	–	0.423	0.654	0.574	0.250	0.444	0.500	0.750	0.600	0.389	–
	2	–	–	–	0.135	0.038	0.056	0.000	0.000	0.077	0.125	0.000	0.000	–
	3	–	–	–	0.385	0.154	0.278	0.000	0.167	0.250	0.125	0.200	0.222	–
	4	–	–	–	0.058	0.154	0.093	0.750	0.278	0.173	0.000	0.200	0.389	–
	5	–	–	–	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	–
	6	–	–	–	0.000	0.000	0.000	0.000	0.111	0.000	0.000	0.000	0.000	–
	N	–	–	–	52	26	54	4	18	52	8	20	18	–

Appendix 2 Allele frequencies for each locus and each sample of *Sesamia nonagrioides*. n = number of alleles

Locus	Allele	S-CA-1	S-PE-1	S-SU-1	S-SO-1	S-SO-2	S-SO-3	S-SO-4	S-SO-5	S-SO-6	S-MA-1
Ck	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	2	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	N	100	60	60	110	94	78	60	60	72	100
Tpi	1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	N	100	60	60	110	100	60	60	60	60	100
Aat	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	2	0.378	0.625	0.466	0.720	0.454	0.500	0.379	0.440	0.500	0.520
	3	0.622	0.375	0.534	0.280	0.546	0.500	0.621	0.560	0.500	0.480
	N	82	48	58	50	108	74	58	84	104	100
Pgm	1	0.478	0.500	0.260	0.364	0.620	0.350	0.534	0.319	0.394	0.560
	2	0.267	0.346	0.220	0.568	0.231	0.350	0.276	0.372	0.337	0.260
	3	0.256	0.154	0.200	0.068	0.148	0.300	0.138	0.309	0.269	0.180
	4	0.000	0.000	0.320	0.000	0.000	0.000	0.052	0.000	0.000	0.000
	5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	N	90	52	50	44	108	80	58	94	104	100
Hdbh	1	1.000	1.000	1.000	–	1.000	1.000	1.000	1.000	1.000	1.000
	N	100	60	58	0	110	78	56	100	104	100
Gpi	1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	2	0.160	0.155	0.067	0.280	0.163	0.145	0.183	0.167	0.115	0.102
	3	0.777	0.759	0.783	0.660	0.798	0.742	0.717	0.689	0.823	0.837
	4	0.043	0.086	0.017	0.060	0.010	0.065	0.050	0.078	0.042	0.051
	5	0.021	0.000	0.133	0.000	0.029	0.048	0.050	0.067	0.021	0.010
	N	94	58	60	50	104	62	60	90	96	98

Appendix 2. (*continued*)

Locus	Allele	S-MA-2	S-MA-3	S-MA-4	SMA-5	S-MA-6	S-MA-7	S-MA-8	S-MA-9	S-MA-10
Ck	1	0.000	0.000	0.021	0.000	0.000	0.000	0.000	0.000	0.056
	2	0.909	1.000	0.979	1.000	1.000	1.000	1.000	1.000	0.944
	3	0.091	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	N	44	100	94	80	56	42	36	58	36
Tpi	1	1.000	1.000	1.000	0.983	1.000	1.000	1.000	1.000	1.000
	2	0.000	0.000	0.000	0.017	0.000	0.000	0.000	0.000	0.000
	N	96	100	84	60	62	46	44	58	44
Aat	1	0.000	0.000	0.041	0.000	0.000	0.000	0.000	0.000	0.029
	2	0.448	0.550	0.541	0.582	0.617	0.435	0.575	0.431	0.765
	3	0.552	0.450	0.418	0.418	0.383	0.565	0.425	0.569	0.206
	N	96	80	98	98	60	46	40	58	34
Pgm	1	0.511	0.316	0.359	0.314	0.000	0.000	0.000	0.017	0.000
	2	0.256	0.429	0.261	0.407	0.259	0.196	0.136	0.155	0.158
	3	0.233	0.255	0.380	0.279	0.431	0.348	0.364	0.431	0.211
	4	0.000	0.000	0.000	0.000	0.310	0.457	0.500	0.397	0.579
	5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.053
	N	90	98	92	86	58	46	44	58	38
Hdbh	1	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	N	94	100	98	98	62	46	44	58	38
Gpi	1	0.000	0.000	0.000	0.000	0.000	0.000	0.100	0.018	0.029
	2	0.074	0.179	0.156	0.272	0.032	0.087	0.000	0.107	0.000
	3	0.872	0.731	0.708	0.674	0.855	0.565	0.675	0.732	0.765
	4	0.043	0.038	0.073	0.000	0.000	0.000	0.000	0.000	0.000
	5	0.011	0.051	0.063	0.054	0.113	0.348	0.225	0.143	0.206
	N	94	78	96	92	62	46	40	56	34