

# Screening some Common Molecular Markers and a Desaturase Marker, Linked to Sex Pheromone Biosynthesis, in Three Z-Strain and an E-Strain Populations of the European Corn Borer, *Ostrinia nubilalis*, Occurring in Central Europe

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The European corn borer moth, (ECB), *Ostrinia nubilalis* (Lepidoptera: Crambidae, Pyraustinae) is one of the most destructive pests of maize worldwide. ECB has two pheromone-strains, separated by specific ratios of isomers of E- and Z11-tetradecenyl acetates (E11- and Z11-14Ac), but appearing morphologically identical. Accordingly, E- and Z-ECB pheromone traps are available for the respective populations for practical monitoring of the flight, however, traps for Z-strain are unreliable for practical usage in some parts of Central-Europe. E- and Z-ECB populations occur in sympatry in some areas, while in allopatry in other areas. Determining the strains before the flight of adults, when difference in the composition of their respective pheromones is manifested, would be of practical interest for early warning. In addition to the known fatty-acyl-reductase (FAR) marker, further markers would allow more comprehensive studies. We screened the following common markers for mitochondrial and nuclear DNA regions: partial cytochrome c oxidase I (COI), cytochrome B (CytB), the second spacer of the internal transcribed spacer (ITS2), Elongation factor 1a (EF1a) and actin gene (Act). In addition, a marker of the  $\Delta 11$ -desaturase gene (11desat), linked to biosynthesis of female-produced sex pheromone, was also included, because we reported earlier a differential expression for this site. Three Z-ECB populations locating at distant sites within Hungary, an area where only Z-strain occurs, and an E-ECB population in Slovenia, known as the closest-occurring E-strain, were included into the study. Separate laboratory colonies were established from each population, and F<sub>1</sub> generations were sampled to verify the identity of pheromone strains, by analysing the composition of sex pheromone by gas chromatography linked to an electroantennographic detector (GC-EAD). Molecular studies were conducted using specimens taken from the F<sub>2</sub> generations. Results of genetic studies showed that there were no differences between the Z and E populations for the common markers. In contrast to this, several nucleic acid changes (11 nt in 4 positions) were found between the three Z-populations (Hungary) and the E-population (Slovenia) in the desaturase marker. Further study is required to reveal whether the differences found in this study are consistent across E-populations, thus making these markers suitable for diagnostic purposes.

**Keywords:** Sex pheromone strains, cryptic sibling species, maize, pest, moth, diagnostic DNA marker, early warning, larval stage.

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The European corn borer moth (ECB), *Ostrinia nubilalis* Hübner 1796 (Lepidoptera: Crambidae, Pyraustinae) is one of the most important pests of maize and several other crops worldwide (CABI Invasive Species Compendium), with significant impact on maize particularly in Europe (Pronko, 2011). ECB has two cryptic pheromone strains (Kochansky et al., 1975), isolated by specific ratios of isomers of E- and Z11-tetradecenyl acetates (E11-14Ac and Z11-14Ac), respectively. The Z-strain communicates with a mixture of these isomers containing the Z-isomer, as the major constituent of the blend, while the reverse ratio of the same isomers is used by the E-strain. The spread, host plant preferences and economic importance of these respective strains were intensively studied in Europe (Schmitz, 1999; Pelozuelo et al., 2004). The E-strain moved northwards in Europe and reached Scandinavia (Lehmhus et al., 2012). However, little is known about the actual areas of the respective strains. More recent data were needed to track the actual appearance of respective strains for risk assessment in maize. This is because strains are different in their preferences to secondary host plants, especially to some common weed species associated to maize field, which could be potential sources of re-infestations of maize (Schmitz, 1999; Pelozuelo et al., 2004).

However, strains are morphologically identical. Pheromone traps baited with the respective strain-specific lures are expected to attract male moths of the corresponding strains selectively. However, the field performance of these traps is, for unknown reasons, rather poor in most parts of central Europe, therefore pheromone traps are not suitable for practical monitoring (Szócs and Babendreier, 2011). The chemical analysis of the female-produced pheromone is a time-consuming procedure, requires instrumentation and qualified human resources, therefore not feasible for practical application. Moreover, identification of strains would be of much greater importance at larval stage, for early warning preceding the flight period. For this, readily available diagnostic DNA molecular markers could suit well.

In Hungary only Z-ECB strain is known to occur (Peña et al., 1988), however, near to the Southern border of Hungary, for example in Slovenia, E-strains were also found (Rak-Cizej et al., 2014). There is a risk that E-strain populations would migrate towards North and reach Hungary, as well. Therefore, it would be of practical importance to detect the occurrence of E-strains in Hungary, as soon as they arrive.

The search for pheromone strain-specific molecular markers revealed so-far only subtle differences, with the only notable exception being the sex-linked *Tpi* gene (Dopman et al., 2005). Even this difference in the *Tpi* gene needs care in practical exploitation, as although there is 99% uniformity within the E-strain, the similarities within the Z-strain were found to be only 74%.

Comparing some intronic DNA sequences of several olfactory receptor-encoding genes revealed “nearly fixed differences between strains” (Lassance et al., 2011). A further possibility is offered by the allelic variation in a fatty-acyl reductase enzyme gene, expressed in the pheromone gland (pgFAR) (Lassance et al., 2010). In this study Z- and E-ECB populations from several European countries (France, Slovenia and Hungary) were involved, therefore the practical implementation of these findings for recognition of strains across Europe is awaited.

Coates et al. (2013) identified three single nucleotide differences in ECB pgFAR, which difference correlated more than 98% to strain-identity. This difference can obviously be used as a reliable marker, although finding some more pronounced differences would enhance the diagnostic value.

Another approach was offered by the differential expression of a  $\Delta 11$ -desaturase transcript in ECB larvae (Király and Szócs, 2009). Apart from FAR,  $\Delta 11$ -desaturase is the other key enzyme in pheromone biosynthesis of ECB (Roelofs et al., 2002), therefore this approach seems to be also promising. However, further studies are needed to reveal how strongly this differential marker is associated to various E- and Z-populations, occurring across larger areas. If differences responsible for this diagnostic expression were also found at DNA level, this could provide a method more easily applicable in practice. In order to investigate this latter possibility, we sequenced the corresponding part of the genome using the same primers described in Király and Szócs (2009), and looked for selective markers for the Z- and E-strains of ECB. Looking for further possible DNA differences, some common markers, widely used for distinguishing between taxonomically closely related species, or between sibling species, such as COI, CytB, ITS2, EF1a, and an actin-locus for reference, were also included in this study.

## Materials and Methods

### *Insects*

ECB-Z pheromone strain: Three populations, occurring at three geographical regions of Hungary, were sampled in this study. The population at Kéty (Tolna county, SW Hungary) has two flights per season, under that warm and humid climate. The population at Hódmezővásárhely (Csongrád county, SE Hungary) has also two flights per season, since the climate is similarly warm, however, dry. In contrast to these, the population at Romhány (Nógrád county, N Hungary) has only one flight per season under that climate, which is colder than those of the other two regions, while humid.

ECB-E pheromone strain: An E-population at Žalec, Slovenia (courtesy of Dr. M. Rak-Cizej) was included in the study. This population was chosen, as it was the nearest-occurring E-population to Hungary.

Sampling protocol: Mature, overwintering larvae or pupae were collected from stalks of maize during winter of 2010–2012. Collected larvae/pupae were allowed to overwinter under ambient conditions in an outdoor-greenhouse of our institute. In spring, they were transferred into separate rearing rooms, under 26 °C temperature and 18/6 hours light/dark photo period, and adults were let to emerge. F<sub>1</sub> generation was reared on artificial diet (Nagy, 1970) under the above-mentioned long-day laboratory conditions. Emerging adult females were sampled to verify strain-identity (see below), while the rest of the populations were used to establish the next generation. Molecular studies (see below) were conducted on randomly selected specimens, taken from the F<sub>2</sub> generation. Each culture was kept under strict isolation, in well-separated rearing rooms to prevent incidental mixing.

### *Verifying pheromone strains*

Pheromone strains were identified by analysing pooled ovipositor tip extracts of five calling females, extracted in *n*-hexane, by means of gas chromatography coupled to an electroantennographic detector (GC-EAD) (GC: 6890 N, Agilent Technologies Inc.,

Santa Clara, CA, USA, column: DB-Wax 30 m × 0.32 mm × 0.25 μm film thickness, J and W Scientific; EAD: MP15 micromanipulator connected to a IDAC 232 amplifier, Syntech, Kirchzarten, Germany). Injections were made in split/splitless mode, the injector temperature was 220 °C and the temperature program was as follows: 60 °C /1min, then 10 °C /min to 220 °C, 220 °C/5 min. The temperature of the flame ionization detector was set to 225 °C. The transfer line for the antennal detector was set also to 225 °C. The pheromone components in the extracts were identified based on their respective retention times (RT), as compared to corresponding RTs of synthetic E11-14Ac and Z11-14Ac, injected together, along with saturated decyl acetate (S10Ac), as internal standard. The presence of E- and Z11-14Ac in the extracts was further corroborated by the antennal responses (using isolated, strain specific male antennae, as antennal detector). The amounts of E11-14Ac and Z11-14Ac in the extracts were calculated according to the respective peak areas, in the FID-trace.

#### *DNA-based identification*

Total DNA was extracted from a part of abdomen in each individual (minimum number of 15 males and 15 females, per populations, respectively, except “Romhány” population, where altogether 10 specimens were taken) using REDEExtract-N-Ampl™ Seed preparation PCR Kit (Sigma) according to the manufacturer’s instruction with a slight modification. A piece of abdomen was placed in a sterile Eppendorf tube, covered with 100 μl extraction solution and crushed. It was then incubated at 55 °C for 10 minutes, followed by 3 minutes incubation at 95 °C, and then the same quantity (100 μl) of neutralization solution was added to the sample and mixed by vortex mixer. One micro liter from this mixture was used for polymerase chain reaction (PCR) experiments. Amplification was performed in volume of 25 μl of PCR puffer (10 mM Tris-HCl, pH 9.5, 2.5 mM MgCl<sub>2</sub>, 50 mM KCl, 0.1 % Triton X100) containing 100 ng each of dATP, dCTP, dGTP and dTTP and 0.1 nM each sense and antisense primers and 5 U Taq polymerase (Invitrogen). The mitochondrial gene (COI and Cyt B), nuclear protein coding gene Elongation Factor 1 α (EF1α), actin gene, 11-desaturase gene and nuclear ribosomal DNA ITS2 region were amplified (Table 1). A standard thirty-five PCR programme cycles were performed (Eppendorf Mastercycler gradient) with the following parameters: denaturation 95 °C for 4 min, followed 94 °C for 30 s, 50 °C for 20 s and 72 °C for 90 s with final extension at 72 °C for 10 min. The annealing temperatures were 48 °C for amplification of Elongation Factor and 11-desaturase gene, 52 °C for actin gene. After electrophoresis in 1 % agarose gel, PCR products were detected and documented in BioDoc-It™ system (Table 1).

#### *Cloning*

The PCR products were cloned into pJET1.2/blunt cloning vector (Fermentas). The recombinant plasmids were transformed into *Escherichia coli* DH5α competent cells. All cloning steps were based upon standard molecular biology protocols (Sambrook et al., 1989). The recombinant plasmids isolated from selected colonies were sequenced using pJET1.2 forward or reverse sequencing primers by an automated DNA sequencer (Ap-

**Table 1**

Molecular markers tested in this work, name of region targeted, name of primers, sequence of primers and reference

Locus name	Primer name	Primer sequence 5'–3'	Reference
COI	C1-J-1718	ggaggatttgaaattgattagtcc	Loxdale and Lushai, 1998
	C1-N-2191	cccggtaaaatataaaacttc	
	mtD-7.2F	attaggagchchgayatagcatt	Brunner et al., 2002
	mtD-9.2R	caggcaagattaaaaataaaactctg	
CytB	CB-J10747	tgtcgagatgtaaattatgntg	Stewart and Beckenbach, 2005
	CB-N11526	ttcaactggtcgrgtccaatyca	
ITS2	CAS5p8sFc	gcgaacatcgacaagtcgaacgacat	Kim and Lee, 2008
	CAS28sB1d	ttgtttcctccgcttataatagcttaa	
	28Z	agactccttggtccgtgttc	Moritz et al., 2001
	P1	atcactcggctcgtggatcg	
EF1a	M3	cacatyaacattgctgtsatygg	Downie and Gullan, 2004
	RcM44.9	cttgatgaaatcycgtgtgcc	
	M51.9	cargacgtatacaaaatcgg	Downie and Gullan, 2004
	RcM53.2	gcaatgtgrcigtgtggca	
Actin	Act-1	tgggacgatatggaiaiatctggca	Voigt and Wöstemeyer, 2000
	Act-2	gtccciactacgaigg	
	Act-4r	tcitcgtattcttgcttigaiatccacat	
	Act-5r	ttagaagcacttyctgtg	
11desat	11desatforw	cgaggattctctattccacat	Király and Szöcs, 2009
	11desatrev	tccgacctactctttatcattctt	

plied Biosystem Gene Analyzer 3100). Sequence comparisons were performed using Wisconsin Package version 10.0 Genetic Computer Group (GCG) sequence analysis software (Devereux et al., 1984).

## Results

### *Verifying pheromone strains*

Synthetic Z11-14Ac showed baseline separation from E11-14Ac in the gas chromatographic analysis. The ratio of Z- and E11-14Ac, the two pheromone-components in each extracts prepared from Hungarian ECB populations, showed the prevalence of the Z-isomer, typical to Z-ECB strain. In contrast to this, the population from Slovenia showed a reverse ratio, typical to E-strain.

### *Comparing PCR products – similarities between ECB populations*

PCR products of females and males of E- and Z-strains of *Ostrinia nubilalis* showed highly similar patterns, respectively in all primer pairs. Seven of the 10 pairs of

primers tested gave single PCR products, while Act-2 and Act-5r actin gene primers and primers of the EF1a amplified two and four products, respectively. The sizes of the amplicons were: CI-J-1718 – C1-N-2191 (524 bp) and the mtD-7.2F – mtD-9.2R (~500 bp) regions of COI, CAS5p8sFc – CAS-28sB1d (584 bp), the 28-Z - P1 (~1700 bp) regions of ITS2, Act-2 – Act-4r (605 bp) a part of actin gene, 11desatforw and 11desatrev (1472 bp) apart from 11-desaturase gene and 825 bp for CytB region. Five markers (CI-J-1718 – C1-N-2191 COI, CytB, Act-2 and Act-4r actin gene, 11-desaturase and CAS5p8sFc – CAS-28sB1d ITS2) were selected from the initial screening for further sequencing. The PCR products of females and males of E- and Z-strains of *O. nubilalis* were cloned and sequenced except for the actin gene where products only from males of E- and Z-strains were cloned. Sequences of each marker were compared with each other and some available sequences in the GenBank.

**Table 2**

Accession numbers for sequences deposited in the GenBank for markers of sexes and Z- and E-pheromone strains of the European corn borer, ECB, *Ostrinia nubilalis*

Pheromone strain-sex	COI	CytB	ITS2	Desat	Actin
Z-female	MK513456	MK513455	MK480497	MK493122	
E-female	MK513457	MK513454	MK480498	MK493121	
Z-male	MK513459	MK513452	MK480499	MK493120	MK513460
E-male	MK513458	MK513453	MK480500	MK493119	MK513461

Sequences of the products gained in this study were deposited in the GenBank, under the accession numbers shown in (Table 2).

Similarities of sequences of CytB mitochondrial DNA fraction of E- and Z-strains of *O. nubilalis* (compared DNA size 825 nt) were 99.0–100%, and that of sequences in the GenBank 98.9–99.8% (Af442957 – COATES *et al.* 2005, Fj435427 – YANG *et al.* 2008, unpublished).

Similarities of sequences of mitochondrial gene cytochrome oxidase I (COI) of E- and Z-strains of *O. nubilalis* (compared DNA size 524 nt) were 99.6–100%, and that of sequences in the GenBank 98.2–99.6% (Ay649321 – AGUSTI *et al.* 2005; Af442957 – COATES *et al.* 2005; Af321877 – COATES *et al.* 2000, unpublished).

Similarities of ITS2 ribosomal DNA region of E- and Z-strains of *O. nubilalis* (compared DNA size 584 nt) were 98.9–100%, and that of a sequence in the GenBank 99.2–99.7% (Af349036 – COATES *et al.* 2001, unpublished).

Sequence identity of actin gene between the E- and Z-strains was 99.7%. In the GenBank there is no data available on the actin gene of *Ostrinia nubilalis*. The closest identity is to that of *Cydia pomonella* (AY524976) of 92%.

The sequence analysis of tested molecular markers showed high conservation and identity in all cases. The intraspecific variation was no more than 1–2%, regardless the geographical origin. The results indicated that sequence variation of different individuals within the same pheromone strain were higher than between the two pheromone strains in respect of the above common markers.

Similarities of 11 desaturase gene region of E- and Z-strains of *O. nubilalis* (compared DNA size 1472 bp, except ZQ 1541 bp) was also as high as 96.7–99.5, and that of sequences in the GenBank 96.4–99.8% (EF113391 and EF113392 – XUE *et al.* 2007). In this case slightly higher variation was found, as compared to the above common markers, however, these subtle differences on their own would not have any basis for discrimination, especially not, because it could be explained that several cryptic 11-desaturase genes are involved in the pheromone biosynthesis. Indeed, two distinct clades of  $\Delta 11$ -desaturase have been described for *O. nubilalis* which do not sort with pheromone strains (Geiler and Harrison, 2010).

#### *Comparing PCR products – differences between ECB populations*

In contrast to CytB, COI, ITS2 where the subtle differences appeared at various sites, several nucleic acid changes (11 nt in 4 positions) in the desaturase marker were consistent within all of the three Z-strains (Hungary) (both males and females), but different from that of the E-population (Slovenia) (both sexes). The sequence in the three Z-strains (Hungary) was identical to that reported in the Genbank for a Z-strain (USA) (ef113392 – XUE *et al.* 2007).

These differences were as follows (numbered as in NCBI accession number ef113392): GATA and GCAAC nucleotides inserts between 316-317 and 563-564, respectively, at position 332 A changed by G and at 382 C replaced by G.

## Discussion

The widely-used markers (CytB, COI, ITS2, EF1a) tested in this work did not differentiate between the E- and Z-strains of *O. nubilalis*. Also, no diagnostic marker was found using these markers.

In contrast to this, the marker for  $\Delta 11$ -desaturase, originally described as an expression marker (Király and Szöcs, 2009), separated in this study all three Z-strains from the topographically closely occurring E-strain. This indicates that this marker appears not only at expression, but is present in the DNA, as well. However, studies should be extended to more ECB populations, especially to more E-ECB populations, to reveal whether the above-mentioned marker is indeed strain-specific. In this respect, comparing E- and Z-ECB populations, occurring in sympatry could serve pivotal evidence. The 11desat enzyme acts before the bifurcation step leading to strain specific components (Lassance, 2016), which makes unlikely that it were selectively linked to strain-specific pheromone intermediates. However, either one/some of the alleles or cryptic pseudogenes of 11desat should be selectively associated to strains. This could be possible, as several cryptic 11desat and also 14desat were reported in ECB (Xue *et al.*, 2007).

There are only few markers reported in the literature for separating E- and Z-strains. A possibility is offered by specific markers, based on allelic variation in a fatty-acyl reductase, described by Lassance *et al.* (2010, 2011). This method has already been successfully used to determine the first appearance of the Z-strain in Southern-Sweden from a very few larvae (Lehmhus *et al.*, 2012). However, the feasibility of using this method for

various populations, located across the wide distribution range of *O. nubilalis* still has to be verified in more detail. A more recently described method (Coates et al., 2013), based on single nucleotide polymorphism in a fatty-acyl reductase, if applies also to European populations, offers a further possibility.

The diagnostic value of the desaturase marker found in this study needs also further studies. More ECB populations, especially that of the E-strain, should be included in the survey to see how consistent the differences are across various ECB populations. Furthermore, specificity of the desaturase marker has to be checked, as well, as pseudogenes are known to occur. Xue et al. (2007) reported several novel sex pheromone desaturases in ECB, generated through gene duplication and retroposon fusion. In a detailed study Geiler and Harrison (2010) revealed divergent allelic classes for  $\Delta 11$ -desaturase in ECB. They concluded that this enzyme does not contribute to the reproductive isolation between pheromone strains, and may reflect either shared ancestral polymorphism, or recent hybridization between strains. Focusing on host plant races of ECB in relation to four nuclear loci (linked to enzymes of carbohydrate metabolism, pheromone binding protein and flight muscle), Malausa et al. (2007) concluded that reproductive isolation between “maize-Z” and “mugwort-hop-E” race is still incomplete, nevertheless divergence between host races of French ECB populations roots back long before the introduction of corn into Europe. As they pointed out, this slow divergence under high gene flow fits to the speciation of host plant races of the larch budworm, *Zeiraphera diniana* Guenée (Lepidoptera: Tortricidae), described by Emelianov et al. (2004). Not only in desaturase markers, but also in other regions, especially in microsatellite loci of ECB varies the number of alleles. Dalecky et al. (2006) studied 13 microsatellite loci in ECB for polymorphism and found that the number of alleles per locus ranged from one to twelve.

At present, our findings on the representation of the 11desat marker at DNA level may facilitate further studies for a reliable diagnostic method for molecular identification of pheromone strains of ECB.

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