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Cadherin Characterization and Cytochrome Oxidase (COI) HRM Analysis in Different Geographical Populations of the Mediterranean Corn Borer, *Sesamia nonagrioides*

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Abstract

The risk of insects evolving resistance to Bt transgenic crops is a major concern in agriculture. In Lepidoptera, one of the factors associated with resistance to Bt toxin is the cadherin Cry1A toxin receptor in the membranes of larval midgut cells. A cDNA encoding a putative Cry1A toxin receptor with high similarity to cadherin sequences identified in other Lepidoptera, was isolated from *Sesamia nonagrioides* (Lepidoptera: Noctuidae). Polymorphisms were identified in cadherin sequences from *S. nonagrioides* populations from three geographic regions in Greece. In addition, Barcoding-High Resolution Melting (HRM) analysis using mitochondrial cytochrome oxidase (COI) sequences were implemented to distinguish the different geographical populations of *S. nonagrioides.* The characterization of *S. nonagrioides* cadherin sequences in combination with an HRM-COI assay may serve as an efficient tool for distinguishing different *Sesamia* populations and potentially Bt resistant vs susceptible individuals aiming at rapid monitoring of Bt resistance in the field.

Keywords: *Bacillus thuringiensis,* Bt-resistance, cadherin-like protein, Cry1A receptor, barcoding, HRM, COI, lepidoptera

Abbreviations: Bt-R, *Bacillus thuringiensis* resistance; RH, relative humidity; LD, light/dark, *CAD*, cadherin; *SnCAD*, *S. nonagrioides* cadherin; SNP, single nucleotide polymorphisms

1. Introduction

Bacillus thuringiensis (Bt) is a spore-forming bacterium found naturally in the soil. During sporulation Bt produces crystalline inclusion bodies containing insecticidal δ-endotoxins, the Cry proteins. The Cry toxins are lethal to insects and nematodes but nontoxic to vertebrates. Because of these properties Bt has been employed extensively in the field as an environmentally friendly pesticide, and Bt transgenic plants have been cultivated globally in the past decade (Sanahuja et al., 2011).

More than 170 million hectares were planted to Bt transgenic crops worldwide in 2012, with Bt transgenic corn being the second most important Bt transgenic crop after transgenic soybean (James, 2012). Such widespread use of Bt crops has raised the concern that due to the high selection pressure, the insects would soon develop resistance to the Bt toxin and the beneficial effects of Bt plants would be compromised. For that reason management strategies have been employed in order to delay resistance development in insect populations (Andow & Alstad 1998; Bates et al., 2005; Ferry et al., 2006; Tabashnik et al., 2008a; Tabashnik, 2008b; Tabashnik et al., 2009). Understanding the molecular basis of resistance is critical for sustaining the present effect of resistance surveillance and for developing new management practices to prevent potential resistance outbreaks in the future.

The largest acreage planted to transgenic Bt corn in the European Union is in Spain (~ 117,000 ha in 2012) (James, 2012). The main lepidopteran pests of corn in Southern Europe are the Mediterranean corn borer *Sesamia nonagrioides* Lefèbvre (Lepidoptera: Noctuidae) and the European corn borer *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae). In Greece and Spain, *S. nonagrioides* is usually more abundant than *O. nubilalis* and causes greater crop damage, resulting in severe yield loss (Castanera, 1986; Andreadis et al., 2007; Zanakis et al., 2009; Andreadis et al., 2011).

Lepidopteran species are highly susceptible to Cry1 toxins which bind to the membrane of midgut epithelial cells of larvae and lead to cell lysis. The mode of action of Cry toxins is a multi-step process. Solubilization of the crystal in the gut lumen releases the protoxin which is then proteolytically processed to produce the active form. The active toxin binds to specific receptors on the brush border membrane of larval midgut cells, and subsequent conformational change of the toxin and oligomerization allows it to insert into the membrane. This leads to cytolytic pore formation and insect death (Griffitts & Aroian, 2005; Pigott & Ellar, 2007; Bravo et al., 2011). An alternative model has been also proposed whereby binding of the toxin on the receptor triggers a signal pathway involving a G protein, adenyl cyclase and protein kinase A, which leads to cell death (Zhang et al., 2006).

Resistance to Bt at the molecular level may operate at any stage during this entire process, from toxin ingestion to cell lysis. Therefore, it is important to understand the molecular mechanism of this process in order to get insights into the molecular basis of resistance.

Biochemical and genetic studies have implicated a number of molecules as potential membrane toxin receptors such as cadherins, aminopeptidases, alkaline phosphatases and glycolipids (Griffitts & Aroian, 2005; Pigott & Ellar, 2007; Bravo et al., 2011). Recently, ABC transporter molecules also have been implicated as novel Cry1 A toxin receptors in the later stage of oligomer-mediated membrane insertion and associated with resistance (Gahan et al., 2010).

Cadherins are a large family of glycoproteins that are present in vertebrates and invertebrates and are involved in many different processes such as cell adhesion and cell signaling. Cadherins are typified by a long extracellular domain containing cadherin repeats (CR), a membrane proximal region, a transmembrane region, and a short intracellular domain (Angst et al., 2001; Wheelock & Johnson, 2003). Cadherin cDNAs have been isolated from a number of lepidopteran species such as *Manduca sexta* (L.) (Lepidoptera: Sphingidae) (Vadlamudi et al., 1995), *Bombyx mori* (L.) (Lepidoptera: Bombycidae) (Nagamatsu et al., 1998a), *Heliothis virescen*s (F.) (Lepidoptera: Noctuidae) (Gahan et al., 2001), *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) (Morin et al., 2003) and *O. nubilalis* (Flannagan et al., 2005). Although their physiological role in Lepidoptera has not been elucidated so far, cadherin-like proteins seem to be important in the binding of Cry1A toxins, in different species (Bravo et al., 2011).

A 210-kd cadherin-like glycoprotein was first identified as a Cry1Ab binding molecule in *M. sexta* (Vadlamudi et al., 1993, 1995). Subsequent binding studies showed that Cry1Aa and Cry1Ac toxins also bind cadherin-like proteins, in *B. mori* and *H. virescens*, respectively (Nagamatsu et al., 1998b, 1999; Gahan et al., 2001). A cadherin-like protein from *O. nubilalis* was shown to bind the Cry1Ab protein (Flannagan et al., 2005). A toxin binding region, TBR1, corresponding to amino acids 865-875 (NITIHITDTNN) in the cadherin sequence of *M. sexta* was predicted to interact with loop 2 of the Cry1A domain II (Gomez et al., 2001, 2002). A second binding region was identified in the cadherin repeats CR9 and CR11 of *B. mori* and *M. sexta*, respectively (Nagamatsu et al., 1999; Dorsch et al., 2002). This region, TBR2, was narrowed down to amino acids 1331-1342 (IPLPASILTVTV) in the *M. sexta* cadherin sequence and predicted to bind loop α-8 on the Cry1A domain II (Gomez et al., 2003). In addition, in *M. sexta* the extracellular cadherin repeat 12 was shown to be important for mediating Cry1Ab binding (Hua et al., 2004). An overlapping site critical for Cry1Ac binding was also identified in *H. virescens* and it was shown that point mutations in this region, specifically in Leu^{1425} and Phe^{1429}, could lead to substantial decrease in toxin binding (Xie et al., 2005).

The first conclusive evidence that cadherin is associated with resistance to the Bt toxin was reported by Gahan et al. (2001). By genetic analysis these authors demonstrated that in the tobacco budworm, *H. virescens*, resistance to Cry1Ac was tightly linked to a cadherin encoding gene. Similarly, genetic analysis in the pink bollworm, *P. gossypiella*, showed that three different cadherin deletion alleles were linked to Cry1Ac resistance in lab-selected strains (Morin et al., 2003; Tabashnik et al., 2004, 2005; Fabrick & Tabashnik, 2007). Interestingly the third allele is interrupted by an intact and active non-LTR retrotransposon designated *CR1-1_Pg* (Fabrick et al., 2011). In addition, a disruption in the *H. armigera* cadherin gene was shown to be associated with resistance to the Cry1Ac toxin (Xu et al., 2005). Finally, a recent report demonstrated that a novel allele with a deletion in the intracellular

domain of cadherin is linked to Cry1Ac resistance in field-selected populations of *H. armigera* (Zhang et al., 2012).

Barcoding is a method of identifying species using short DNA sequences (Kress et al., 2005; Hollingsworth et al., 2009; Chen et al., 2010). Barcoding has been widely used in species identification, cryptic species identification, biodiversity studies, forensic analysis, and phylogenetics (Ronning et al., 2005; Ward et al., 2005). The preferred sequence used in animal barcoding is the mitochondrial cytochrome oxidase 1 gene (COI) (Hebert et al., 2003; Hebert et al., 2004).

Molecular studies on candidate Cry1A toxin receptor genes from *S. nonagrioides* have not been conducted so far. In the present study we report the isolation and characterization of a full length cDNA from larval midgut tissue of *S. nonagrioides* that encodes a cadherin-like protein. We have also performed a preliminary analysis of cadherin-like sequences from different *S. nonagrioides* populations from three geographic regions in Greece in order to identify useful polymorphisms. Molecular analysis of candidate toxin receptor genes that may be associated with resistance in *S. nonagrioides* will provide the tools for rapid DNA-based screens which could easily detect resistant alleles in the field and lead to efficient monitoring and management of resistance to the Bt toxin. Additionally, we conducted Barcode-HRM analysis based on the mitochondrial COI gene in an effort to distinguish *S. nonagrioides* populations and set a basis for rapid genotype identification of the Mediterranean corn borer.

2. Materials and Methods

2.1 Insect Culture

The experimental population of *S. nonagrioides* came from a laboratory colony, which was established by adults collected from the region of Thessaloniki (40°38′N, 22°45′E) with the use of a blacklight trap. Immediately after their capture adults were transferred to the laboratory and were caged in a plastic cylinder (13 cm in diameter, 30 cm in height) with one corn plant and sucrose solution 20% (w:w). Egg masses were collected daily, by removing corn leaves or entire plants with eggs, and were placed in small plastic boxes (4.5 cm in diameter, 3 cm in height) with tight-fitting lids on moistened filter paper with propionic acid (1:1000 dilution) (Fantinou et al., 2003). Newly hatched neonates were placed in plastic Petri dishes (9 cm in diameter, 1.5 cm in height) and were reared on an artificial diet (Poitout & Bues 1970, González-Nunez et al., 2000) at 25 ± 1 °C, 70 ± 5 % RH, and a photoperiod of 16:8 (LD) h.

2.2 Field Collected Insects

Early (2nd - 3rd) and late instars (4th - 5th) of *S. nonagrioides* were collected from the region of Serres (41°05′N, 23°24′E), Larissa (39°34′N, 22°27′E) and Thessaloniki (40°38′ N, 22°45′E) by dissecting corn stalks. Each time only one larva per corn plant was taken from plants at least 1.5 m apart to minimize the possibility that sibs were collected. After their collection larvae were transferred to the laboratory and were directly frozen in liquid nitrogen. Then they were stored at -80 ^{o}C until analysis.

2.3 RNA Isolation, cDNA Synthesis and Cloning

Total RNA was extracted from fourth instar midgut tissue of *S. nonagrioides* laboratory culture (geographical area: Thessaloniki) using the RNeasy midi kit (Qiagen). First strand cDNA synthesis was performed using 2.5 μg total RNA, 0.5 μg 3' RACE Adapter primer 5'-GGCCACGCGTCGACTAGTAC $(T)_{17}$-3', 1 mM dNTPs and 200U M-MuLV reverse transcriptase (New England Biolabs, Beverly, USA) in 50 μl total volume. 1/10 of the synthesized cDNA was used as template for each of two PCR reactions with degenerate primer pairs CADF1/CADR3 and CADF4/CADR, respectively. The PCR reactions were performed in 1X Reaction Buffer, 0.2 mM dNTPs, 0.2 μM forward primer, 0.2 μM reverse primer and 1U of the DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) (Tsaftaris et al., 2006). The thermocycler programm was: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 48 °C for 1 min, 72 °C for 1 min, and final extension 72 °C for 15 min. The PCR products were separated on a 1% agarose gel and amplification products of the expected size could be observed (1.5 kb and 0.45 kb). These products were cloned into the pGEM T easy vector, sequenced, and found to contain sequence homologous to cadherin by BLAST similarity search analysis. Specific primers were generated based on the sequence of these fragments, specifically the forward primer CADF3SP and reverse primers CAD450R1SP, and nested reverse CAD450R2SP. Two PCR reactions were performed using 1/10 of the cDNA described above as template with primer pairs CAD-F3SP/CAD-450 R1SP and CAD-F3SP/ CAD-450R2SP. The reactions were performed as above and the thermocycler conditions were: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 70 °C for 1 min, 72 °C for 2 min, and final extension 72 °C for 15 min. Two products were amplified of approximately 2.5 kb which was the expected size, and with the expected distance between them. These products

were cloned into pGEM T easy vector and sequenced. BLAST analysis revealed homology to cadherin. To obtain the 3'end of the *SnCAD* cDNA, 3'RACE -PCR was performed with forward primers CAD3'F1 and nested CAD3'F2 and the Abridged Universal Amplification Primer (AUAP) (Invitrogen, Paisley, UK) and 1/10 of the cDNA described above as template. The PCR reactions were performed as above and the reaction conditions were: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 65 °C for 1 min, 72 °C for 2 min, and final extension 72 °C for 15 min. The 5'end of the *SnCAD* cDNA was obtained using the 5'RACE System for Rapid Amplification of cDNA Ends, Version 2.0, Kit (Invitrogen, Paisley, UK). The primers used were reverse primer CAD5'N3 which was used to synthesize cDNA as before, and two reverse primers CAD5'N2 and nested CAD5'N1 which were used for the PCR reactions according to the recommendations of the manufacturer.

For comparing cadherin-like sequences of different populations from different geographical areas, RNA was extracted from field collected late instars of *S. nonagrioides* from the regions of Serres, Larissa and Thessaloniki. cDNA was synthesized as above, and PCR products were generated using primers CADINT5 and CAD450R2SP. PCR products were extracted from agarose gels, purified (Macherey and Nagel) cloned and sequenced (Macrogen, Korea). All primers used in the above experiments are listed in Supplementary material Table 1.

Table 1. Cadherin proteins from different lepidopteran species and insect classification

Protein name	Accession no	Species	Family	Superfamily
SnCAD	EU025853	*Sesamia nonagrioides*	Noctuidae (subfamily: Hadeninae)	Noctuoidea
SfCAD	CAC41167	*Spodoptera frugiperda*	Noctuidae (subfamily: Hadeninae)	Noctuoidea
HvCAD	AAK85198	*Heliothis virescens*	Noctuidae (subfamily: Heliothinae)	Noctuoidea
HaCAD	AAQ54935	*Helicoverpa armigera*	Noctuidae (subfamily: Heliothinae)	Noctuoidea
LdCAD	AAL29896	*Lymantria dispar*	Lymantriidae	Noctuoidea
PgCAD	AAP30715	*Pectinophora gossypiella*	Gelechiidae	Gelechioidea
CsCAD	ABG91735	*Chillo suppressalis*	Crambidae	Pyraloidea
OnCAD	AAY44392	*Ostrinia nubilalis*	Crambidae	Pyraloidea
MsCAD	AAM21151	*Manduca sexta*	Sphingidae	Sphingioidea
BmCAD	AB026260	*Bombyx mori*	Bombycidae	Bombycoidea

2.4 Phylogenetic Analysis

Homology searches were performed with a BLAST program against the DDBJ/EMBL/GenBank database (http:// www.ncbi. nlm.nih.gov/). The protein sequence alignment was generated with the ClustalW method (Thompson et al., 1994). The phylogenetic tree was calculated using the *MEGA 3* software (Kumar et al., 2004) using the Neighbour-Joining algorithm and p-distance correction (Saitou & Nei, 1987). Bootstrap values were derived from 1000 replicate runs.

2.5 Southern Hybridization

30 μg genomic DNA isolated from 4th instar *S. nonagrioides* (laboratory culture, Thessaloniki) using the DNeasy tissue kit (Qiagen) were digested with *Dr*aI, *Eco*RI, *Eco*RV, and *Hind*III (TaKaRa, Otsu, Japan), electrophoresed through a 0.9 % (w/v) agarose gel and transferred to a positively charged Nylon membrane (Tsaftaris et al., 2006). The digoxigenin labeled *SnCAD* probe was prepared with PCR using the PCR DIG Probe Synthesis Kit (Rôche, Mannheim, Germany) with primers CADF1SP and CAD5'N1. The cycling conditions were: 94 °C for 3min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and then 72 °C for 15 min. Hybridization was performed with DIG Easy Hyb buffer at 38 °C according to the manufacturer and stringent washes were at 68 °C in 0.5X SSC containing 0.1 % (w/v) SDS (twice). Detection was performed using the DIG Luminescent Detection Kit according the instructions and chemiluminescence was detected using the GeneGenome Bio Imaging System (Syngene, Cambridge, U.K.).

2.6 Expression Analysis

Total RNA was extracted from *S. nonagrioides* midguts from field-collected 2nd , 3rd 4th and 5th instars using the RNeasy midi kit (Qiagen). First strand cDNA synthesis was performed using 1.0 μg total RNA, 0.5 μg 3' RACE Adapter primer, 5'-GGCCACGCGTCGACTAGTAC $(T)_{17}$-3' (Invitrogen), 1 mM dNTPs and 200U of

Superscript II (Invitrogen) in 20 µl total volume, according to the specifications of the manufacturer. Quantitative real-time PCR, was performed in a PCR reaction mix (20 µl) containing 5 µl of 1:20 diluted cDNA, 0.25 µM of each primer and 1X Platinum SYBR Green qPCR Supermix-UDG (Invitrogen, Paisley, UK) and using the Corbett Rotor Gene 6000. Each reaction was performed in triplicates. General thermocycler conditions were 50 °C for 2 min, 95 °C for 2 min, then 40 cycles at 95 °C for 20 s, annealing at 58 °C for 25 s, 72 °C for 30 s. To identify the PCR products a melting curve was performed from 65 to 95 °C with observations every 0.2 °C and a 10-s hold between observations. A final step was performed at 72 °C for 5 min. The *SnCADF* and *SnCADR* primers were used to amplify a 450 nt fragment of *SnCAD* and the Elongation Factor 1-alpha *(EF1-a)* was used as the reference gene with primers *SnEF1aF* and *SnEF1aR* which amplify a fragment of 311 bp. Relative quantification and statistical analysis were performed with the REST software (Pfaffl et al., 2002). All primers used in expression analysis are shown in Supplementary material (Table 1). A two-sample unpaired *t*-test was used to investigate the difference in the relative expression ratio between the control group of each strain and the larval instars, using Minitab 16 Statistical Software (Minitab Inc., State College, PA). Prior to the analysis, the corrected relative expression ratio data were subjected to the Anderson-Darling (A-D) goodness-of-fit test for normalization of the variables (D'Agostina & Stephens 1987).

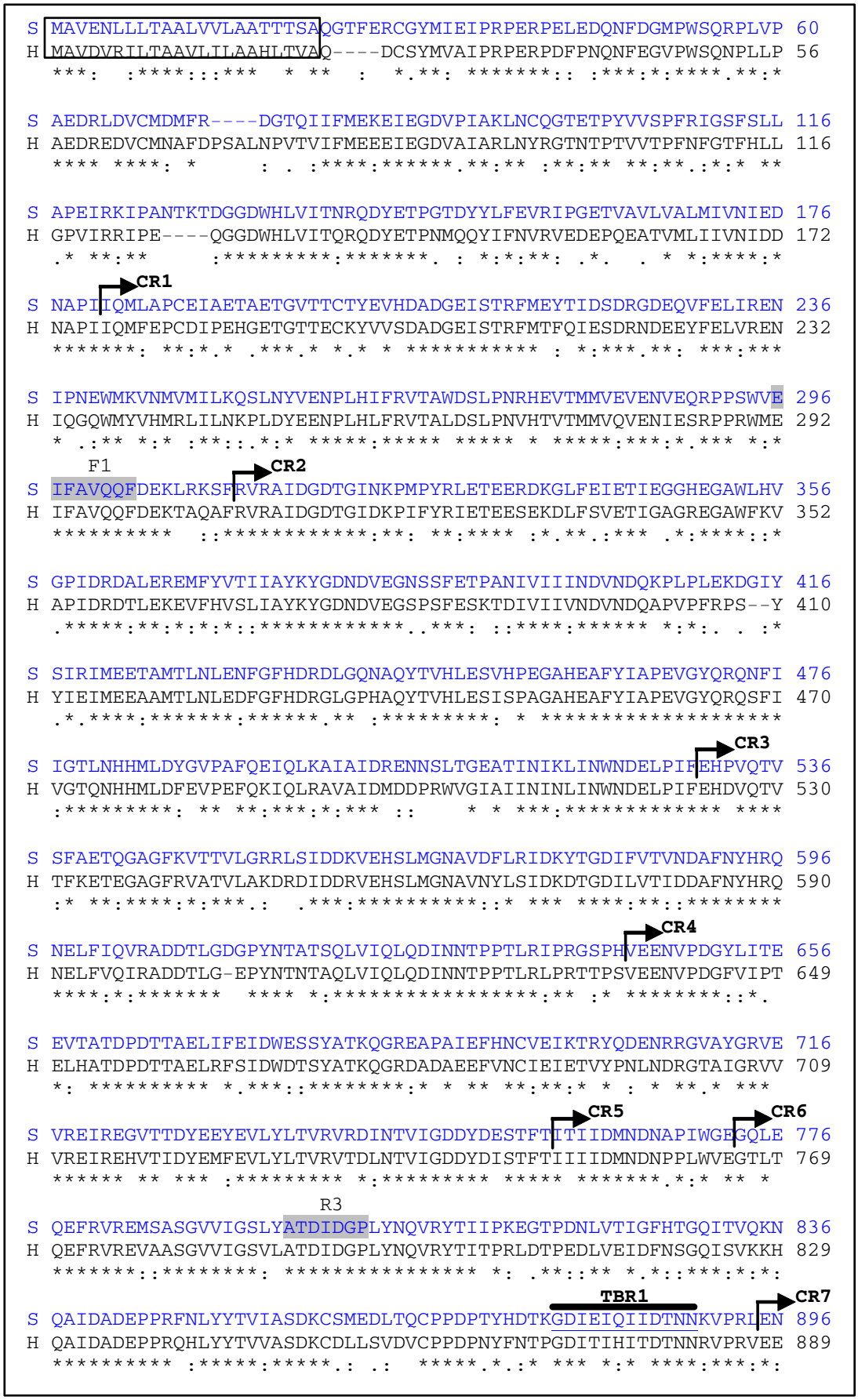
2.7 HRM Assay

Genomic DNA was isolated from 4th instar larvae via the DNeasy tissue kit (Qiagen) using 0.1 gr of powderized larvae according to the manufacturers' instructions. The DNA concentration was estimated by the nanodrop (Nanodrop 2000-Thermo Scientific) and DNAs integrity by gel electrophoresis in a 0.8% agarose gel. Samples were then diluted to 20 ng/µl working concentration. PCR amplification, DNA melting, and end point fluorescence acquiring were performed with a Rotor-Gene 6000 real-time 5P HRM PCR Thermocycler (Corbett Research, Sydney, Australia). Reactions were conducted in a final volume of 20µl containing 1x buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 mM forward and reverse primers, 1.5 mM Syto®9, and 0.5U Kapa Taq DNA polymerase (Kapa Biosystems, USA). Each reaction was performed in duplicates. Thermocycler conditions were as follows: initial denaturation step at 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s, then a final extension step of 72 °C for 10 min. HRM was performed as follows: pre-melt at the first appropriate temperature for 90 s, and ramping at 0.1 °C increments every 2 s from 70 to 90 ºC. The fluorescent data were acquired at the end of each increment step. The negative derivative of fluorescence (F) over temperature (T) (dF/dT) curve primarily displaying the Tm, the normalised raw curve depicting the decreasing fluorescence vs increasing temperature, and the difference curves (Wittwer et al., 2003) were generated via the Rotor-Gene 6000 proprietary software (version 1.7.87). The Rotor-Gene 6000 proprietary software (version 1.7.87) was used to genotype the different populations.

3. Results

3.1 Identification of a Cadherin-Like cDNA From S. nonagrioides and Protein Analysis

Protein alignments of known cadherin proteins from several Lepidopteran species (*H. viresceses*, *H. armigera*, *B. mori*, *P. gossypiella*, *Lymantria* dispar, *M. sexta* and *Chillo suppressalis*) revealed several regions of high amino acid conservation. The conserved regions EIFAVQQF and ATDIDGP (Figure 1) were used to design forward and reverse degenerate primers (CADF1-forward and CADR3-reverse) in order to amplify a 1.5 kb fragment towards the 5' end of the *S. nonagrioides* cadherin coding region. Likewise the conserved regions MTCNIDQ and GTNKHAVEG were used to design forward and reverse degenerate primers (CADF4 and CADR, respectively) in order to amplify a 0.45 kb fragment in the 3'end of the coding region (Figure 1). These sequences were subsequently used in order to generate specific primers to amplify the region between the 1.5 and 0.45kb fragments mentioned above. Finally specific primers were also designed and utilized in 5'RACE-PCR and 3'-RACE PCR in order to complete the *S. nonagrioides* cadherin (*SnCAD)* cDNA sequence to the 5'and 3' ends, respectively. A full length cadherin cDNA was isolated, in this manner, from *S. nonagrioides* larval midguts. The full length cDNA is 5405 bp long, translates to a putative protein of 1739 aa and contains 206 bp 5' and 188 bp 3' untranslated regions, respectively. The amino acid sequence of the protein is shown in Figure 1. The *Sn*CAD protein contains a putative 22-residue signal peptide, and a 1560-residue extracellular domain. In addition it harbors a predicted 24 aa long transmembrane domain (TMR), and a 133 aa cytoplasmic domain (CYT). Moreover, two putative toxin binding sites, TBR1 and TBR2, were identified as shown in Figure 1. ProSite analysis of the deduced amino acid sequence identified eleven consensus cadherin repeat motifs


CR1
CR2
F1
CR3
CR4
CR5
CR6
R3
TBR1
CR7

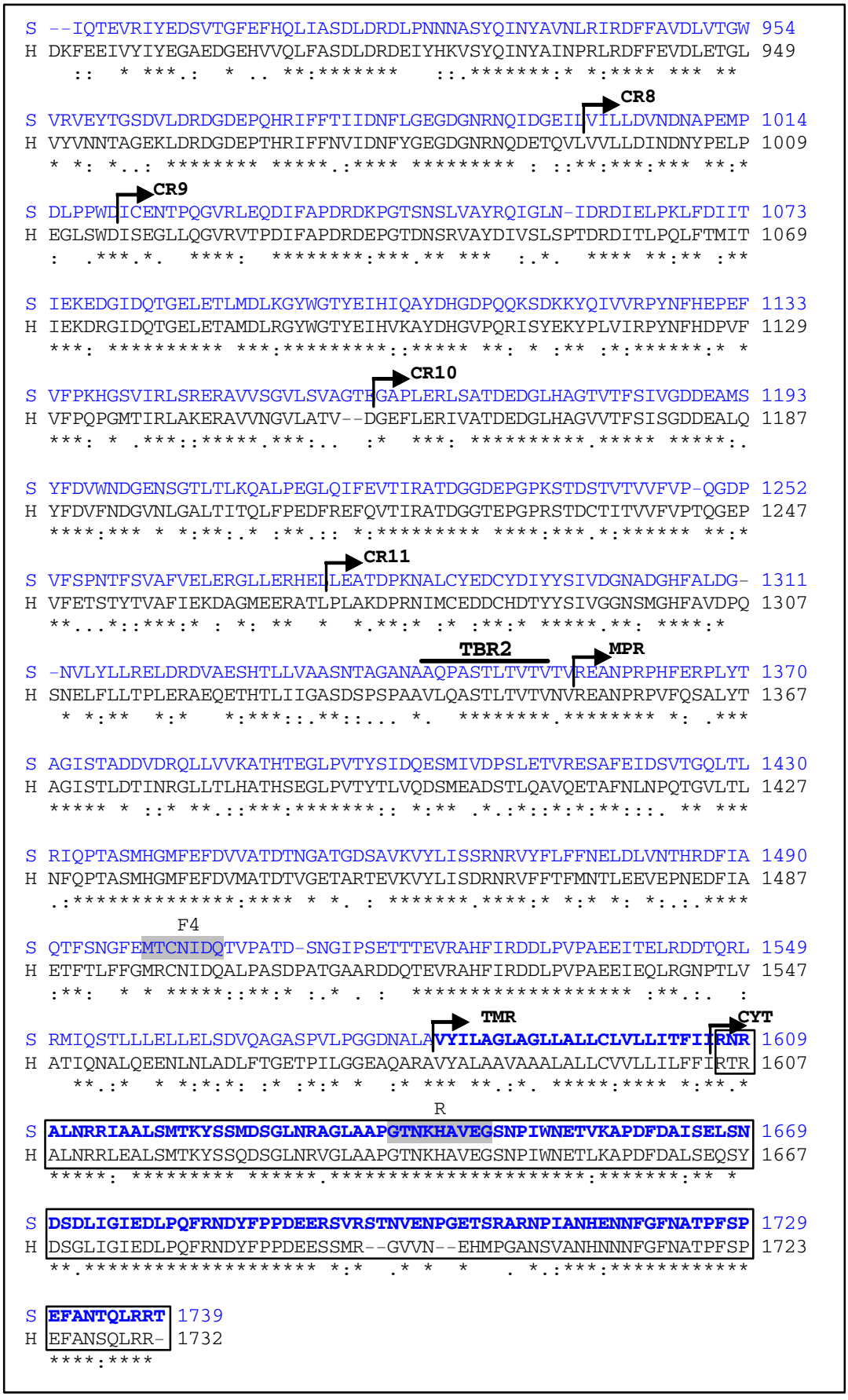
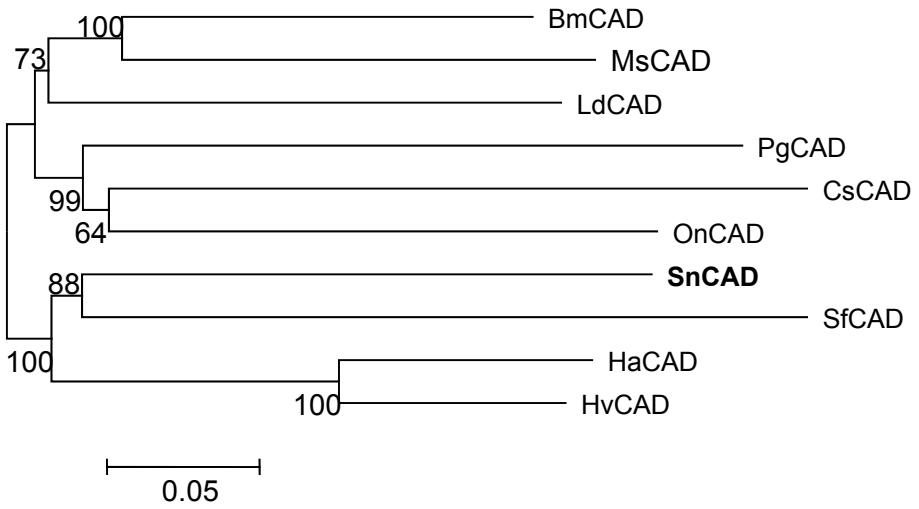

CR8
CR9
CR10
CR11
TBR2
MPR
TMR
CYT
F4
R

Figure 1. *S. nonagrioides* cadherin (*SnCAD*) amino acid sequence analysis. Alignment between the deduced cadherin amino acid sequences from *S. nonagrioides* (S) and *H. virescens* (H), in blue and black, respectively. Identical amino acids are indicated by asterisk (*). A putative signal peptide is boxed and CR1-CR11 indicate cadherin repeats. MPR and TMR denote the putative membrane proximal region and transmembrane region, respectively. The putative cytoplasmic domain (CYT) is in bold and boxed. Putative toxin binding regions TBR1 and TBR2 are overlined. Conserved regions used to design degenerate primers are highlighted in grey

3.2 Phylogenetic Analysis

A)



B)

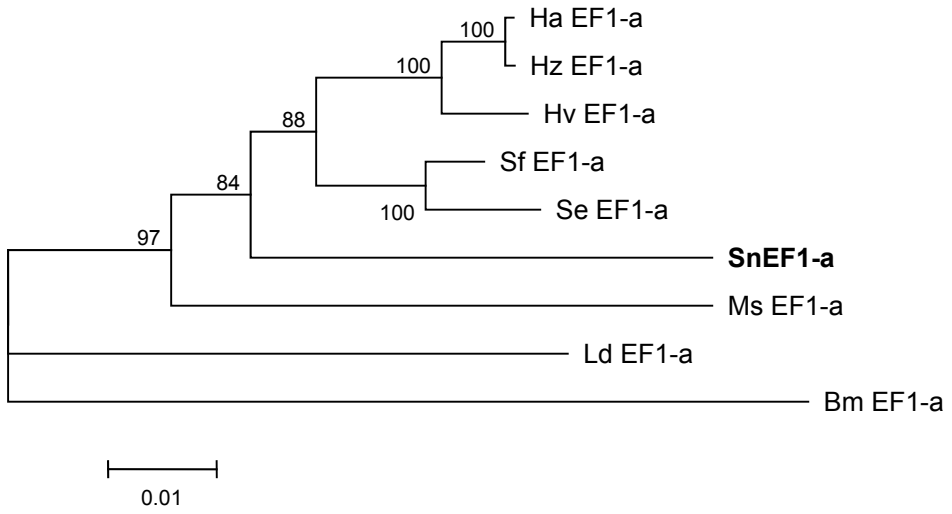


Figure 2. Phylogenetic analysis of cadherin sequences. A) A phylogenetic tree of cadherin proteins from different lepidopteran species. B) A phylogenetic tree constructed based on the nucleotide sequences of *EF1-a*, from different lepidopteran species. Sn, *S. nonagrioides*; Sf, *S. frugiperda*; Hv, *H. virecsens*; Ha, *H. armigera*; Ld, *L. dispar*; Pg, *P. gossypiella*; Cs, *C. suppressalis*; On, *O. nubilalis*; Bm, *B. mori*; Ms, *M. sexta*; Se: *S. exigua*; Hz: *H. zea*

The deduced cadherin amino acid sequence from *S. nonagrioides* has the highest degree of homology with the cadherin proteins from other Heliothinae species like *H. virescens* (64 % identity) and *H. armigera* (63 % identity) as well as with other lepidopteran species like *L. dispar* (62 % identity), *B. mori* (60 % identity), and less homology with *M. sexta* (58 % identity*), P. gossypiella* (57 % identity), *O. nubilalis* (57 % identity), *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) (55 % identity), and *C. suppressalis* (52 % identity). Based on the alignments of the cadherins from those different Lepidoptera a phylogenetic tree was constructed (Figure 2A) which showed that the amino acid sequence of *SnCAD* is more closely related to the cadherin sequences from species of the superfamily of Noctuoidea (Hadeninae, Heliothinae, Lymantriidae) and more distantly related to the species from the Bombicoidea, Sphingioidea, Gelechioidea and Pyraloidea superfamilies, respectively. The decreased similarity with the cadherin from *S. frugiperda* is intriguing because *S. frugiperda* is evolutionary closer to *S. nonagrioides* as they belong to the same subfamily of Hadeninae (Table 1). Therefore we attempted to examine the phylogenetic analysis of Elongation Factor 1- alpha (*EF1-a*), a well conserved translation factor. We isolated and sequenced a partial cDNA coding for the Elongation Factor 1- alpha (*EF1-a*) (Kapazoglou and Tsaftaris, unpublished data) and constructed a phylogenetic tree based on the *EF1-a* nucleotide sequence from *S. nonagrioides* and those of other Lepidoptera that were retrieved from GenBank (Figure 2B). We observed that the *EF1-a* sequence from *S. nonagrioides* is more distantly related to the other sequences of the Hadeninae (*S. frugiperda* and *Spodoptera exigua*) subfamily as is the case for the cadherin sequence. Together these observations may reflect a discrepancy between classical taxonomy and sequence–based taxonomy. It is possible that although *S. nonagrioides* had been classified as a close relative of the Hadeninae at the level of morphology, there may be divergence at the level of DNA. Certainly more extensive pylogenetic analysis with a large number of sequences would have to be performed towards this contention.

3.3 Southern Analysis

A 275 bp probe prepared from the 888-1061 nt region of the *SnCAD* cDNA, was utilized to determine the number of gene copies in the genome of *S. nonagrioides* by Southern blot analysis (Figure 3). Non of the restriction enzymes used has a restriction site within the sequence of the cDNA region containing the probe. A single band was detected for the *Eco*RV and *Eco*RI digests which suggests a single cadherin gene. The double bands in the *Dra*I and *Hind*III digests suggest that possibly an intron with a *Dra*I and a *Hind*III site interrupts the probe in the genomic sequence, or that there are two different alleles with a change in the *Dra*I and *Hind*III restriction sites.

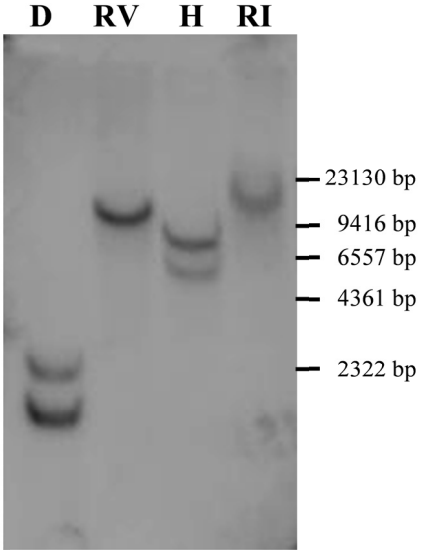


Figure 3. Southern analysis of *SnCAD*. Genomic DNA from *S. nonagrioides* digested with *Dra*I(D), *Eco*RI(RI), *Eco*RV(RV), and *Hind*III(H), was hybridized with a *SnCAD* probe described in section *Southern hybridization*. A single band is detected for *Eco*RI (RI) and *Eco*RV (RV) and double bands for *Dra*I (D) and *Hind*III (H)

3.4 Expression Analysis

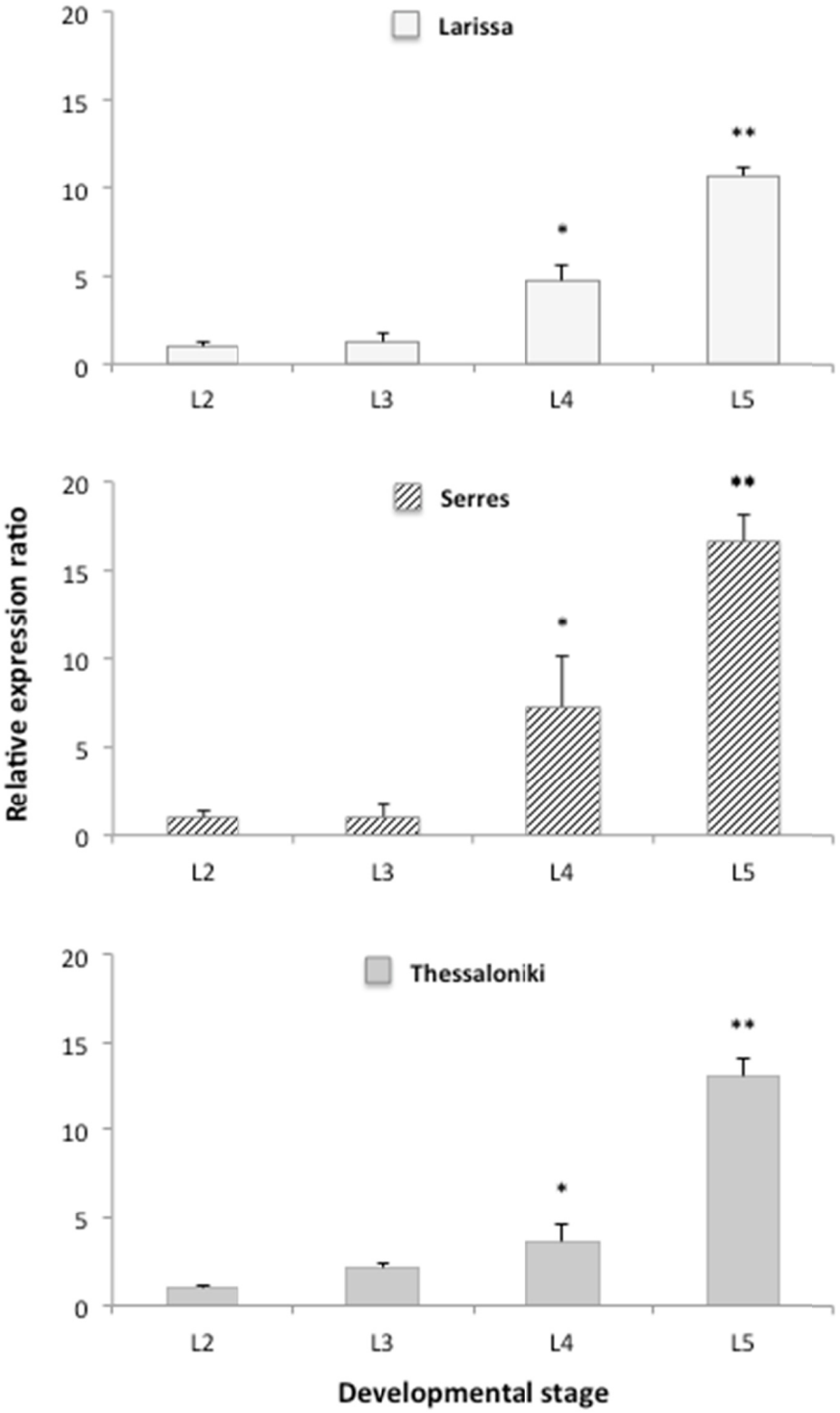


Figure 4. Quantitative Real Time RT-PCR expression analysis of *SnCAD* in different larval instars of *S. nonagrioides*. Expression values were normalized to those of *SnEF1-a*. The relative expression ratio of each sample is compared to the control group which was L_2. Data represent mean values from two independent experiments with standard errors. Significant differences in the expression ratio are indicated by **P<0.01 and *P<0.05 (t-test, a = 0.05). (L_2, L_3, L_4, and L_5 represent 2nd, 3rd, 4th and 5th instar, respectively; *SnCAD*, *S. nonagrioides* cadherin gene; *SnEF1-a*, elongation factor 1-alpha gene from *S. nonagrioides*)

Quantitative real-time PCR analysis was employed to examine the expression of *SnCAD* at different larval developmental stages in three different greek geographical populations of *S. nonagrioides* (Figure 4). In all three populations, Larissa, Serres, and Thessaloniki, the *SnCAD* transcript levels were low in the 2nd and 3rd instar, medium in 4th and reached a maximum in the 5th instar. Transcripts were increased by approximately 5 fold and 10-15 fold in 4th and 5th instar, respectively, as compared to the 2nd instar in all three samples (Figure 4).

3.5 Cadherin Sequence Polymorphisms in Different Geographical Populations

In a preliminary attempt to identify polymorphisms in cadherin sequences of *S. nonagrioides* from different geographical areas, late instar larvae were collected from three different regions in Greece (Serres, Larissa and Thessaloniki). Two cadherin fragments (3099-3871 nt and 4116-4845 nt from the ATG site, respectively) were isolated and sequenced from each geographical population. These fragments contain cadherin regions that have been linked to Bt resistance and Cry1A binding (Morin et al., 2003; Xie et al., 2005). Comparative analysis of these sequences detected sixteen SNPs, seven of which also lead to amino acid changes as shown in Figure 5A. Five of those are located between cadherin repeats CR9 and CR10 and the other two reside in the MPR close to TBR2 (Figure 5B).

A)

```
a)
SERRES              ----------------------------------------..... 21
LARISSA             ----------------------------------------..... 21
THES/NIKI           ATCTGTAAAACACACCACAGGGTGTACGTTTAGAACAAGATATCTTCGCACCGGATCGG 3120
                                                    ********************
SERRES              ..........C...C.....................A..CC...C..C.C..... 81
LARISSA             ..........C...C.....................A..C....C..C.C..... 81
THES/NIKI           GACAAGCCAGGAACATCTAACTCTCTGGTCGCCTACAGACAGATCGGTCTAAACATAGAT 3180
                    ********** *** ********************** ** **** ** * *******
                               P                       K  P        T
                               S                       Q  L        N
                                                          I
SERRES              ..................................................... 141
LARISSA             ..................................................... 141
THES/NIKI           CGAGACATCGAACTACCGAAACTGTTTGACATCATTACTATAGAGAAAGAAGACGGCATA 3240
                    ************************************************************
SERRES              ..................................................... 201
LARISSA             .....................G................................ 201
THES/NIKI           GACCAGACTGGAGAACTTGAAACCCTCATGGACTTGAAAGGATACTGGGGCACCTACGAA 3300
                    ******************* ************************************
SERRES              ..................................................... 261
LARISSA             ..................................................... 261
THES/NIKI           ATTCACATACAGGCATACGACCATGGCGATCCACAGCAAAAGTCAGATAAAAAATACCAG 3360
                    ************************************************************
SERRES              ..................................................... 321
LARISSA             ..................................................... 321
THES/NIKI           ATAGTGGTCAGACCGTACAACTTCCACGAGCCTGAGTTCGTGTTCCCAAAACATGGATCT 3420
                    ************************************************************
SERRES              ........................................A.......... 381
LARISSA             ........................................A.......... 381
THES/NIKI           GTCATCAGACTATCTAGGGAGCGGGCTGTAGTCAGCGGTGTACTGTCAGTAGCAGGCACG 3480
                    ************************************* ********************
                                                       I
                                                       V
SERRES              ..................................................... 441
LARISSA             ..................................................... 441
THES/NIKI           GAGGGCGCGCCTCTGGAGCGTCTCTCTGCCACAGACGAGGACGGATTGCACGCTGGAACT 3540
                    ************************************************************
SERRES              ..................................................... 501
LARISSA             ..................................................... 501
THES/NIKI           GTCACTTTCTCTATAGTGGGAGACGATGAGGCGATGAGTTACTTCGACGTGTGGAATGAC 3600
                    ************************************************************
SERRES              ..................................................... 561
LARISSA             ..................................................... 561
THES/NIKI           GGGGAGAACTCTGGCACACTCACCTTGAAACAAGCTTTGCCTGAAGGCTTGCAAATATTT 3660
                    ************************************************************
SERRES              ..................................................... 621
LARISSA             ..................................................... 621
THES/NIKI           GAGGTGACGATCCGCGCGACAGACGGTGGTGACGAGCCCGGCCCTAAGAGCACTGACAGC 3720
                    ************************************************************
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SERRES              ..............................................................  681
LARISSA             ..............................................................  681
THES/NIKI           ACAGTCACGGTGGTGTTCGTGCCACAGGGAGACCCCGTGTTTTCGCCCAACACCTTTTCA 3780
                    ************************************************************
SERRES              ...............................................G..............  741
LARISSA             ..............................................................  741
THES/NIKI           GTTGCCTTTGTTGAGTTAGAACGAGGTTTATTGGAGCGACATGAGCTGCTAGAGGCAACA 3840
                    ********************************************** *********
SERRES              ..............................-----------------------------  771
LARISSA             ..............................-----------------------------  772
THES/NIKI           GATCCGAAGAACGCCTTGTGTTATGAAGATTGCTATGATATCTACTATAGCATCGTTGAC 3900
                    ******************************
b)

SERRES              ----------------------------------..........................  25
LARISSA             ----------------------------------..........................  26
THES/NIKI           AGGCCGCACTTCGAGAGGCCGCTGTACACAGCCGGCATTTCCACCGCAGATGACGTTGAC 4140
                                                     ************************
SERRES              .........T....................................................  85
LARISSA             ..............................................................  86
THES/NIKI           AGACAGCTGCTCGTTGTTAAGGCAACGCACACGGAAGGCCTGCCTGTCACCTACTCGATA 4200
                    ********* ************************************************
                              L
                              F
SERRES              ..............................................................  145
LARISSA             ..............................................................  146
THES/NIKI           GACCAGGAGTCTATGATAGTGGACCCATCGCTAGAGACCGTCCGGGAGAGTGCCTTCGAG 4260
                    ************************************************************
SERRES              ..............................C...A.........................  205
LARISSA             ..............................................................  206
THES/NIKI           ATAGACTCTGTCACCGGACAACTGACGCTGAGAATCCAGCCTACTGCCTCTATGCATGGA 4320
                    ********************************** *** ********************
                                                    I
                                                    T
SERRES              ..............................................................  265
LARISSA             ..............................................................  266
THES/NIKI           ATGTTCGAGTTTGATGTCGTGGCTACTGATACAAATGGAGCAACGGGCGACTCTGCAGTG 4380
                    ************************************************************

SERRES              ..............................................................  325
LARISSA             ..............................................................  326
THES/NIKI           AAGGTGTACCTGATCTCGTCACGCAACAGAGTGTACTTCCTGTTCTTCAACGAGCTAGAT 4440
                    ************************************************************

SERRES              ..............................................................  385
LARISSA             ..............................................................  386
THES/NIKI           TTAGTCAACACCCACAGAGACTTTATAGCCCAAACATTCTCTAATGGGTTCGAAATGACG 4500
                    ************************************************************
SERRES              ..T........T..................................................  445
LARISSA             ..T........T..................................................  446
THES/NIKI           TGCAACATCGACCAGACTGTACCCGCCACCGACTCCAACGGCATCCCTAGCGAAACTACA 4560
                    ** ******** ************************************************



SERRES              ..............................................................  505
LARISSA             ..............................................................  506
THES/NIKI           ACCGAAGTCAGGGCGCACTTCATACGAGACGACTTGCCCGTCCCTGCTGAAGAAATTACC 4620
                    ************************************************************

SERRES              ..............................................................  565
LARISSA             ..............................................................  566
THES/NIKI           GAACTGCGGGACGACACTCAGCGGCTGCGGATGATCCAGTCGACTCTCCTGCTGGAGCTA 4680
                    ************************************************************


SERRES              ..............................................................  625
LARISSA             ..............................................................  626
THES/NIKI           CTGGAACTGAGCGACGTGCAGGCTGGAGCCTCGCCCGTGCTGCCGGGCGGCGATAACGCG 4740
                    ************************************************************
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SERRES              ..............................................................  685
LARISSA             ..............................................................  686
THES/NIKI           CTGGCGGTGTACATCCTGGCGGGGCTGGCCGGCCTGCTCGCGTTGTTGTGCCTCGTGCTC 4800
                    ************************************************************


SERRES              ..............................................-------------- 732
LARISSA             ..............................................-------------- 733
THES/NIKI           CTCATTACCTTCATCATCAGGAATCGGGCGCTCAACCGTCGTATCGCAGCCCTATCGATG 4860
                    ********************************************
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B)

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a)
Serres              -------------DIFAPDRDKPGTPNSLVAYRKPGLTIDRDIELPKLFDIITIEKEDGI 47
Larissa             -------------DIFAPDRDKPGTPNSLVAYRKLGLTIDRDIELPKLFDIITIEKEDGI 47
Thessaloniki        ICENTPQGVRLEQDIFAPDRDKPGTSNSLVAYRQIGLNIDRDIELPKLFDIITIEKEDGI 1080
                              ************.*******: **.**********************

Serres              DQTGELETLMDLKGYWGTYEIHIQAYDHGDPQQKSDKKYQIVVRPYNFHEPEFVFPKHGS 107
Larissa             DQTGELETLMDLKGYWGTYEIHIQAYDHGDPQQKSDKKYQIVVRPYNFHEPEFVFPKHGS 107
Thessaloniki        DQTGELETLMDLKGYWGTYEIHIQAYDHGDPQQKSDKKYQIVVRPYNFHEPEFVFPKHGS 1140
                    ************************************************************
                                                CR10 ▶
Serres              VIRLSRERAVVSGILSVAGTEGAPLERLSATDEDGLHAGTVTFSIVGDDEAMSYFDVWND 167
Larissa             VIRLSRERAVVSGILSVAGTEGAPLERLSATDEDGLHAGTVTFSIVGDDEAMSYFDVWND 167
Thessaloniki        VIRLSRERAVVSGVLSVAGTEGAPLERLSATDEDGLHAGTVTFSIVGDDEAMSYFDVWND 1200
                    *************:**********************************************

Larissa             GENSGTLTLKQALPEGLQIFEVTIRATDGGDEPGPKSTDSTVTVVFVPQGDPVFSPNTFS 227
Serres              GENSGTLTLKQALPEGLQIFEVTIRATDGGDEPGPKSTDSTVTVVFVPQGDPVFSPNTFS 227
Thessaloniki        GENSGTLTLKQALPEGLQIFEVTIRATDGGDEPGPKSTDSTVTVVFVPQGDPVFSPNTFS 1260
                    ************************************************************
                                  CR11 ▶
Larissa             VAFVELERGLLERHELLEATDPKNALCYED------------------------------ 257
Serres              VAFVELERGLLERHELLEATDPKNALCYED------------------------------ 257
Thessaloniki        VAFVELERGLLERHELLEATDPKNALCYEDCYDIYYSIVDGNADGHFALDGNVLYLLREL 1320
                    ****************************
b)
                                                          MPR ▶
Serres              ------------------------------------------------------ISTADDVD 8
Larissa             ------------------------------------------------------ISTADDVD 8
Thessaloniki        DRDVAESHTLLVAASNTAGANAAQPASTLTVTVTVREANPRPHFERPLYTAGISTADDVD 1380
                                                TBR2                  ********

Serres              RQLFVVKATHTEGLPVTYSIDQESMIVDPSLETVRESAFEIDSVTGQLTLRTQPTASMHG 68
Larissa             RQLLVVKATHTEGLPVTYSIDQESMIVDPSLETVRESAFEIDSVTGQLTLRIQPTASMHG 68
Thessaloniki        RQLLVVKATHTEGLPVTYSIDQESMIVDPSLETVRESAFEIDSVTGQLTLRIQPTASMHG 1440
                    ***:******************************************** ********

Serres              MFEFDVVATDTNGATGDSAVKVYLISSRNRVYFLFFNELDLVNTHRDFIAQTFSNGFEMT 128
Larissa             MFEFDVVATDTNGATGDSAVKVYLISSRNRVYFLFFNELDLVNTHRDFIAQTFSNGFEMT 128
Thessaloniki        MFEFDVVATDTNGATGDSAVKVYLISSRNRVYFLFFNELDLVNTHRDFIAQTFSNGFEMT 1500
                    ************************************************************

Serres              CNIDQTVPATDSNGIPSETTTEVRAHFIRDDLPVPAEEITELRDDTQRLRMIQSTLLLEL 188
Larissa             CNIDQTVPATDSNGIPSETTTEVRAHFIRDDLPVPAEEITELRDDTQRLRMIQSTLLLEL 188
Thessaloniki        CNIDQTVPATDSNGIPSETTTEVRAHFIRDDLPVPAEEITELRDDTQRLRMIQSTLLLEL 1560
                    ************************************************************
                                  TMR ▶                          CYT ▶
Serres              LELSDVQAGASPVLPGGDNALAVYILAGLAGLLALLCLVLLITFIIRNRALNRRI----- 243
Larissa             LELSDVQAGASPVLPGGDNALAVYILAGLAGLLALLCLVLLITFIIRNRALNRRI----- 243
Thessaloniki        LELSDVQAGASPVLPGGDNALAVYILAGLAGLLALLCLVLLITFIIRNRALNRRIAALSM 1620
                    ************************************************
```
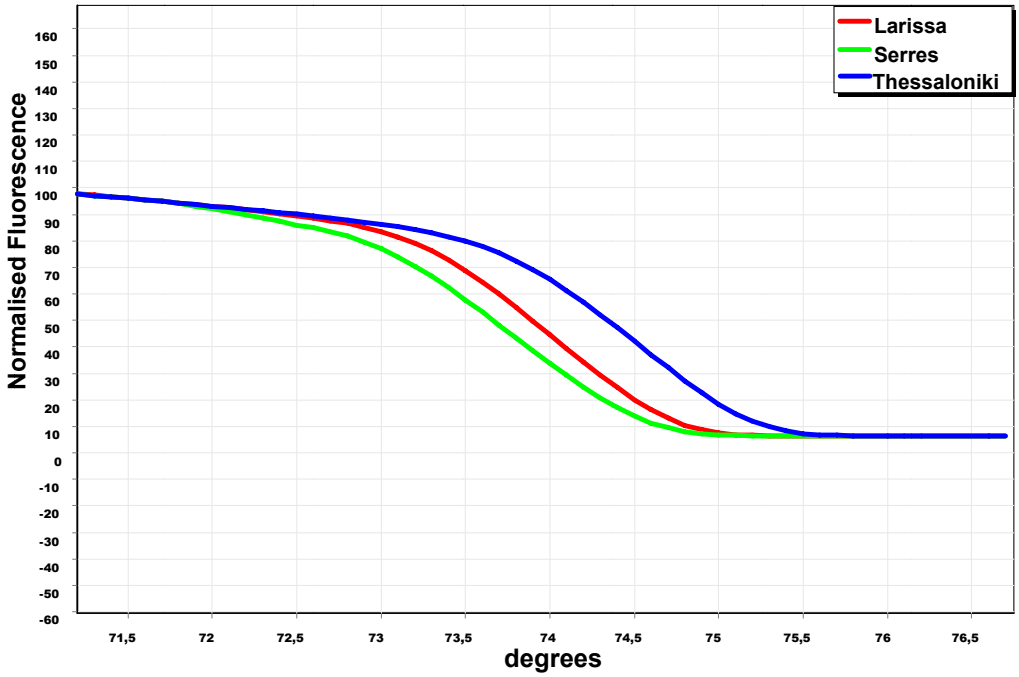

Figure 5. A) Alignment of the cadherin nucleotide sequences covering the regions 3309- 3871 nt (a) and 4116-4845 nt (b) (from the ATG translation start site) between three different populations of *S. nonagrioides* from Serres, Larissa, and Thessaloniki. Identical nucleotides are indicated with dots (.) with reference to the bottom sequence (Thessaloniki). Gaps are shown with a dash (-). Whenever nucleotide changes lead to amino acid changes the amino acids are indicated under the nucleotide sequences. B) Alignment of the respective cadherin protein sequences (corresponding to nucleotides 3309-3871 and 4116-4845) from Serres, Larissa and Thessaloniki. Identities are indicated by asterisk (*) and sites where amino acids have changed in either sequence are boxed. Cadherin repeats (CR), TBR2, MPR, TMR, and CYT, are indicated

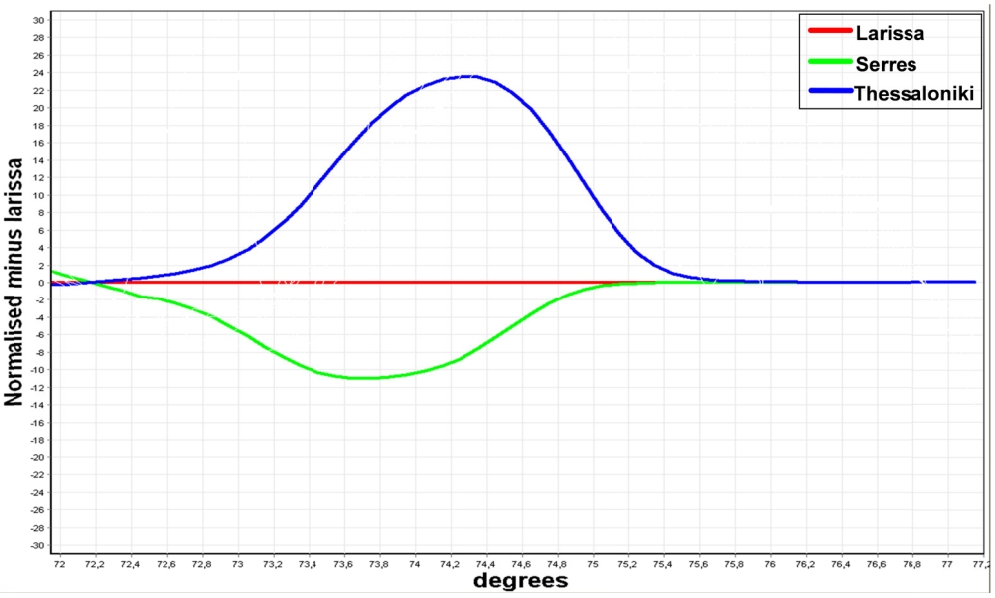
3.6 Barcoding-HRM Using COI Sequences

Universal COI primers (Hebert et al., 2003) were used to amplify COI sequences from the three different *S. nonagrioides* populations. The PCR products were sequenced and a fragment of 148 bp with nucleotide polymorphisms in all three populations was selected for HRM analysis (Figure 6). The normalized fluorescence curve and fluorescence difference plot are depicted in Figure 6A and 6B, respectively. The nucleotide differences among the three COI sequences are shown in Figure 6C. For the difference plot the Larissa population was set as a 'genotype' (reference species) and the average HRM genotype confidence percentages (GCPs) (value attributed to each species being compared to the reference genotype which is assigned the value of 100) (Hewson et al., 2009) were 8.49 for Serres and 0.79 for Thessaloniki. Distinctive curves for each population (Larissa, Serres and Thessaloniki) are observed in both normalized fluorescence plot and fluorescence difference plot.

A)



B)



C) COI sequences

```
Larissa   GAATTAGGAACTCCTGGATCTTTAATTGGAGATGATCAAATTTATAATACTATTGTTACAGCTCATGCTTTTATTATAATT
Serres    GAATTAGGAACTCCTGGATCTTTAATTGGAGATGATCAAATTTATAATACTATTGTTACAGCTCATGCTTTTATTATAATT
Thes/nik  GAATTAGGAACTCCTGGATCTTTAATTGGAGAGGATCAAATTTATAAAACTATTGTTACACGCTCATGCTTTTATTATAATT
          ******************************** ************** ************ ********************

Larissa   TTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATTGACTTGTACCTTTAATATTAGGAG
Serres    TTTTTTATAGTTGGTACCTATTATAATTGGAGGATTTGGAAATTGGCTTGAACCCTTAATATTAGGAG
Thes/nik  TTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGAAATTGACTTGTACCTTTAATATTAGGAG
          ************ ****************************** **** *** *************
```

Figure 6. A)Normalized fluorescence curve of the melting profiles of the three *S. nonagrioides* populations. [Larissa (red), Serres (green) and Thessaloniki (blue)]. B) Difference graph of the three populations using Larissa as the reference genotype. C) Sequence alignment of the 148 bp COI amplicon used in HRM from the three populations (Larissa, Serres and Thessaloniki). Nucleotide differences are highlighted in grey. Sequences used to design Forward and Reverse primers are underlined

4. Discussion

In this study we present the characterization of a cadherin-like gene from *S. nonagrioides* and a COI - based HRM analysis in an attempt to distinguish different geographical populations and potential mutants based on sequence polymorphisms.

A cadherin cDNA of 5405 bp was isolated encoding a putative protein of 1739 aa. The deduced amino acid sequence has the characteristics of cadherin proteins of other Lepidoptera in that it contains a long extracellular domain (1560 residues) with eleven cadherin repeat motifs, a membrane proximal region, a transmembrane region and a short cytosolic domain. Six N-glycosylation sites are distributed along the extracellular domain. The *SnCAD* sequence shows relatively high homology with cadherin sequences from other Lepidoptera (~ 52-64% identity). They all seem to share the same structure which classifies them in the protocadherin group of atypical cadherins (Bel & Escriche, 2006). Protocadherins have multiple functions in various developmental processes in mammals (Angst et al., 2001), however, their physiological role in Lepidoptera remains unclear. Mostly, lepidopteran cadherins have been associated with Bt toxin binding and have been implicated as one of the receptor molecules of Cry1A toxins, in different species like *M. sexta, B. mori*, *H. virescens*, *P. gossypiella, O. nubilalis* and *H. armigera* (Vadlamudi et al., 1993; Nagamatsu et al., 1999; Gahan et al., 2001; Morin et al., 2003; Flannagan et al., 2005; Xu et al., 2005). The high homology of *SnCAD* with the other lepidopteran cadherin proteins suggests that it might share a common structure and probably also bind Cry1A toxins.

It is unclear why *SnCAD* has a higher degree of identity with *H. virescens* and *H. armigera* (Heliothinae subfamily) CAD proteins than with the *S. frugiperda* CAD which is in the same subfamily as *S. nonagrioides* (Hadeninae). One possibility is that there may be more than one cadherin gene in the *S. nonagrioides* genome. However the data from Southern analysis suggesting a single copy gene makes this possibility rather unlikely.

Expression analysis using quantitative PCR showed that *SnCAD* mRNA accumulated at higher levels in 4th and 5th instar larvae and at lower levels in the 2nd and 3rd instar larvae. This pattern of expression was common for three different greek geographical populations of *S. nonagrioides*. The changes in cadherin transcript abundance in different larva stages maybe associated with the gene expression programme operating during the process of larva development. If cadherins play a role in cell-cell adhesion, the larvae with larger mass may require more cadherin molecules for this function.

Genetic studies demonstrated association of cadherin-like proteins with resistance to Cry1A toxins in *H. virescens, P. gossypiella* and *H. armigera*. The insertion of a retrotransposon in the cadherin gene of a *H. virescens* (tobacco budworm) resistant strain resulted in truncated cadherin translation product that presumably led to high levels of resistance to Cry1Ac toxin (Gahan et al., 2001). In a field resistant strain of *P. gossypiella* three alleles with deletions in the cadherin coding region were linked to resistance to the Cry1Ac toxin (Morin et al., 2003). Furthermore, it was shown that disruption of a cadherin gene by a premature codon in an *H. armigera* strain was associated with high levels of resistance to Cry1Ac (Xu et al., 2005). Finally, a deletion in the intracellular domain of cadherin was shown recently to be linked to Cry1Ac resistance in field-selected populations of *H. armigera* (Zhang et al., 2012). These data taken together point to the fact that the cadherin gene is often found linked to resistance and this makes it a good candidate for developing DNA-based screening strategies to monitor Bt resistance in the field. We inspected the cadherin sequence in three different geographical populations of *S. nonagrioides,* towards identifying genetic variation among populations. Interestingly, we found polymorphisms in two fragments of the cadherin gene that have been previously linked to Bt resistance and Cry1A binding (Morin et al., 2003; Xie et al., 2005). The identification of polymorphisms in different geographical populations, is a first

step in the development of DNA-based tools for the monitoring of potential Bt resistance in field populations of this lepidopteran species.

Barcoding in combination with High Resolution Melting (HRM) analysis has been increasingly used lately in order to conduct rapid genotyping of animal and plant species (Brechon et al., 2013; Madesis et al., 2012a). HRM curve analysis is a quick, low cost, post PCR method which permits the rapid detection of genetic variation between species. HRM is superior to other PCR methods in that it is single-tube, high-throughput and requires no post-PCR manipulations, thereby reducing analysis time and minimizing cross-contamination and technical error (Berry & Sarre, 2007). Recently our group has successfully implemented HRM in plant genotyping as well as in traceability of adulterants in food products of both plant and animal origin (Ganopoulos et al., 2011; Bosmali et al., 2012; Ganopoulos et al., 2012a; Ganopoulos et al., 2012b; Madesis et al., 2012b; Sakaridis et al., 2013a; Sakaridis et al., 2013b). In this study we extended our investigations and were able to use barcoding with COI-HRM in order to detect genetic variation among different populations of the lepidopteran species *S. nonagrioides.*

Cloning and characterization of a *S. nonagrioides* full length cDNA encoding a cadherin-like protein that may be associated with resistance to Bt and the identification of polymorphisms within the gene in different geographical populations could lead to the development of rapid DNA-based screens for detection of Bt resistant individuals. Furthermore, the easy detection of genetic variation in different geographical populations using the barcoding analysis based on COI-HRM will serve towards rapid genotyping of the Mediterranaean Corn Borer. Together these tools could contribute to the better monitoring and management of Bt resistance in the field.

Acknowledgments

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Supplementary material

Table 1. Primer sequences used in the experiments

Primer name	Sequence
Cloning	
CAD-F1	5'-GARATHTTYGCIGTICARCARTT -3'
CAD-R3	5'-GGICCRTCDATRACIGTIGC-3'
CAD-F4	5'-ATGACITGYAAYATHGAYCA3′
CAD-R	5'-CCYTCIAYIGYRTGYTTRTTIGTICC-3'
CADF3SP	5'-GTGCCAGTGGAGTGGTCATCGGCTCTC-3'
CAD450R1SP	5'-GATCATCCGCAGCCGCTGAGTGTCGTCC-3'
CAD450R2SP	5'-GCGATACGACGGTTGAGCGCCCGATTCC-3'
CADINT5	5'-GATATCTTCGCACCGGATCGGGACAAG-3'
CAD3'F1	5'-CATTACCTTCATCATCAGGAATC-3'
CAD3'F2	5'-GCACCAATAAACACACCGTCGAAG-3'
CAD5'N1	5'-TGAGTTGCCTTCTACATCATTGTC-3'
CAD5'N2	5'-AGTGGTTTCTGGTCATTGACATC-3'
CAD5'N3	5'-TCTCCTCCATAATACGAATAG-3'
AUAP	5'-GGCCACGCGTCGACTAGTAC-3'
3' RACE Adapter primer	5'-GGCCACGCGTCGACTAGTAC(T)17-3'
CADF1SP	5'- GAGATATTCGCGGTGCAACAGTTC-3'
CADR1SP	5'- CTTTGAAGCCCGCGCCTTGTGTC-3'
CADR2SP	5'- TAGAGAGCCGATGACCACTCCAC-3'
Expression	
SnCADF	5'-GTTAGAACGAGGTTTATTGGAG-3'
SnCADR	5'-GTGACAGAGTCTATCTCGAAG-3'
SnEF1-a F	5'- ACAGTCGACTCCGGCAAGTC-3'
SnEF1-a R	5'-AGGGAATTCCTGGAAGGACTC-3'
HRM	
SnCOI F	5'-GAATTAGGAACTCCTGGATC
SnCOI R	5'-CTCCTAATATTAARGGTWCAAG (W=A or T; R=C or T)