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Cecropins as a marker of *Spodoptera frugiperda* immunosuppression during entomopathogenic bacterial challenge

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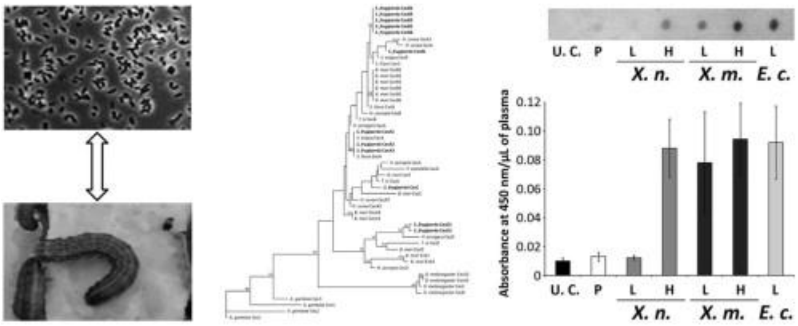
Abstract :

An antimicrobial peptide (AMP) of the cecropin family was isolated by HPLC from plasma of the insect pest, *Spodoptera frugiperda*. Its molecular mass is 3910.9 Da as determined by mass spectrometry. Thanks to the EST database Spodobase, we were able to describe 13 cDNAs encoding six different cecropins which belong to the sub-families CecA, CecB, CecC and CecD. The purified peptide identified as CecB1 was chemically synthesized (syCecB1). It was shown to be active against Gram-positive and Gram-negative bacteria as well as fungi. Two closely related entomopathogenic bacteria, *Xenorhabdus nematophila* F1 and *Xenorhabdus mauleonii* VC01[T] showed different susceptibility to syCecB1. Indeed, *X. nematophila* was sensitive to syCecB1 whereas *X. mauleonii* had a minimal inhibitory concentration (MIC) eight times higher. Interestingly, injection of live *X. nematophila* into insects did not induce the expression of AMPs in hemolymph. This effect was not observed when this bacterium was heat-killed before injection. On the opposite, both live and heat-killed *X. mauleonii* induced the expression of AMPs in the hemolymph of *S. frugiperda*. The same phenomenon was observed for another immune-related protein lacking antimicrobial activity. Altogether, our data suggest that *Xenorhabdus* strains have developed different strategies to supplant the humoral defense mechanisms of *S. frugiperda*, either by increasing their resistance to AMPs or by preventing their expression during such host-pathogen interaction.

Graphical abstract :



Highlights

► We first describe the *Spodoptera frugiperda* cecropin family (*Sf*Cec). ► *Sf*Cec family is composed of 12 members. ► Two bacteria of *Xenorhabdus* genera show different susceptibility to *Sf*Cec. ► *Xenorhabdus* sp. set up different strategies to overcome insect innate immunity.

Keywords : Humoral immunity ; Innate immunity ; Immunosuppression ; Lepidopteran ; Invertebrate

Introduction

To fight infection, insects rely on multiple innate defence reactions which include the use of physical barriers together with local and systemic immune responses. These involve phagocytosis and encapsulation by hemocytes (Costa et al., 2005; Russo et al., 1996), proteolytic cascades leading to coagulation (Loof et al., 2011) and melanisation (Kanost et al., 2004), and *de novo* secretion of antimicrobial peptides (AMPs) (Lemaitre and Hoffmann, 2007). AMPs are widely distributed among living organisms (Garcia-Olmedo et al., 1998; Zasloff, 2002).

Since the first report of an inducible defense reaction in *Drosophila* (Boman et al., 1972), AMPs have been identified and characterized from the main insect orders Diptera (Kylsten et al., 1990), Lepidoptera (Dickinson et al., 1988; Steiner et al., 1981), Coleoptera (Bulet et al., 1991), Hemiptera (Cociancich et al., 1994) and Hymenoptera (Casteels et al., 1989). On the basis of sequence and structural features, these peptides have been classified into three broad classes: (i) linear peptides with amphipathic α-helices, (ii) cysteine-stabilized α-helical/β-sheet motif containing peptides and (iii) linear peptides with an overrepresentation in proline and/or glycine residues (Bulet and Stocklin, 2005).

Cecropins are among the best known α-helical cationic antimicrobial peptides. Mature cecropins are composed of highly basic amino acid residues that can fold into two amphiphatic α-helices and integrate into the acidic cell membranes of bacteria leading to their disruption (Steiner et al., 1988). Recent works indicate that cecropins seem to be a target of insect pathogens. Indeed, the bacterial-challenged induction of cecropins from the lepidoptera *Plutella xylostella* was shown to be inhibited by a symbiotic polyDNA virus (CpBV) of the hymenoptera endoparasitoid *Cotesia plutellae* (Barandoc et al., 2010). On the other hand, *Xenorhabdus nematophila*, a Gram-negative bacterium belonging to the Enterobacteriaceae family, kills various insects. These bacteria form a species-specific mutualistic association with the entomopathogenic nematode, *Steinernema carpocapsae* (Thomas and Poinar, 1979) and are transported and released by nematode vectors into the hemocoel (body cavity) of insect hosts. *Xenorhabdus* initially colonizes the connective tissue surrounding the anterior midgut and hemolymph (bloodstream) of the Lepidoptera *Spodoptera littoralis* (Sicard et al., 2004), leading to the death of the insect, probably due to a combination of the effects of toxins and septicaemia. *X. nematophila* can independently kill insects and grow within their bodies if introduced into the hemolymph by direct injection (Sicard et al., 2004). *X. nematophila* proliferates in the hemolymph before the insect dies and must therefore be able to escape the insect immune response. Hence, it was shown that *Xenorhabdus* inhibits the expression of cecropins (Ji and Kim, 2004). This indicates that different pathogens might use a common strategy in order to establish their pathogenicity.

In this work, we purified a cecropin from the hemolymph of bacterial-challenged larvae of the crop pest, *Spodoptera frugiperda*. The analysis of Spodobase (http://bioweb.ensam.inra.fr/spodobase/), a *Spodoptera* specific cDNA database (Negre et al., 2006), allowed the identification of 13 distinct nucleotide sequences encoding members of the cecropin family. The deduced amino acid sequences led us to classify the *S. frugiperda* cecropins in 4 families (CecA, B, C and D) and to identify the purified cecropin as CecB1. CecB1 was chemically synthesized (syCecB1) and its antimicrobial activity was tested against a panel of microorganisms. Thus, we showed that two closely related entomopathogenic bacteria, *Xenorhabdus nematophila* F1 strain and *Xenorhabdus mauleonii* strain VC01^T, were not sensitive to the same extent to syCecB1. Additionally, *X. nematophila* F1 had an inhibitory effect on cecropin expression whereas *X. mauleonii* VC01^T did not have such an effect. This difference between the two *Xenorhabdus* strains could be extended to the global antimicrobial activity present into the hemolymph of infected larvae. Finally, the same results were obtained when we followed the outcome of Spod-11-tox, an immune-related protein lacking antimicrobial activity and mainly expressed by hemocytes (Destoumieux-Garzon et al., 2009). Altogether, these results showed that the two entomopathogenic *Xenorhabdus* strains use two different strategies in order to circumvent the humoral immune response of their insect host.

1. Materials and Methods

1.1. Insects and Immune Challenge

Spodoptera frugiperda were reared on artificial diet (Pagès and Ginibre, 2006) at 23°C with a photoperiod of 12 h. One day-old sixth-instar larvae were used for the expression studies. Larvae used for antimicrobial peptide purification were bacterial-challenged with *Escherichia coli* CIP7624 (Gram negative) and *Micrococcus luteus* CIP5345 (Gram positive)(10^6 bacteria/larva). Eight hours post challenge, hemolymph was collected from a cut abdominal proleg into ice-cold anticoagulant buffer (69 mM KCl, 27 mM NaCl, 2 mM $NaHCO_3$, 100 mM D-glucose, 30 mM tripotassium citrate, 26 mM citric acid, 10 mM Na_2–EDTA, pH 4.6, 420 mOsm)(van Sambeek and Wiesner, 1999). This time point was chosen because Girard et al. (2008) previously showed that this was the time at which maximum expression of antimicrobial peptides occurred.

Experimental infections were also performed by injection of 10^6 PBS-washed *Xenorhabdus nematophila* strain F1 or *Xenorhabdus mauleonii* strain VC01^T (from the laboratory collection). The use of this high dose of bacteria was to avoid difference of virulence of the *Xenorhabdus* sp. In some cases, the bacteria were heat killed by 10 min incubations at 95°C.

1.2. Plasma (cell-free hemolymph) Purification Procedure as described in (Destoumieux-Garzon et al., 2009)

1.2.1. Extraction and pre-purification by solid-phase extraction (SPE)

Hemocytes and plasma were separated by centrifugation at 600 x g during 30 sec at 4°C. Plasma from bacterial-challenged larvae (28 mL) was acidified to pH 2 with 1 M HCl. The acidic extraction was performed overnight under gentle shaking at 4°C. After centrifugation (16,000 x g for 30 min at 4 °C), the supernatant was pre-purified by SPE on Sep-Pak C_{18} cartridges (Waters) equilibrated with 0.05% trifluoroacetic acid (TFA). Elutions were performed with 10%, 40% and 80% acetonitrile in acidified water. All fractions were freeze-dried in a vacuum centrifuge (Speed-Vac, Cryo Rivoire) and subsequently reconstituted with MilliQ water at 1/20 of the initial hemolymph volume.

1.2.2. HPLC purification

The 40% Sep-Pak fractions were subjected to reverse phase chromatography on an UP5NEC25QS column (Interchim) equilibrated in 0.05% TFA. Separation of the 40% Sep-Pak fractions was performed with a linear gradient of 0-60% acetonitrile in acidified water over 80 min at a flow rate of 1 mL/min.

Antimicrobial activity-containing fractions were purified on the same reversed phase column as above at a controlled temperature of 35°C. The linear biphasic gradient was composed of 0%-28% acetonitrile in 0.05% TFA over 5 min, and of 28%-45% over 55 min at a flow rate of 1 mL/min.

When needed, the fractions of interest were subjected to a final purification step on a narrow-bore reversed phase column (Xbridge BEH130, Waters Associates) at 40°C with a flow rate of 0.25 mL/min using the biphasic gradients described above.

All HPLC purifications were performed on a Waters HPLC system (Waters 600 pump) equipped with a photodiode array (Waters 996 PDA). Column effluent was monitored by its UV absorption at 225 nm. Fractions corresponding to absorbance peaks were hand collected in polypropylene tubes (Microsorb 75 mm x 12 mm, Nunc immunotubes), concentrated under vacuum (Savant) and reconstituted in MilliQ water (Millipore™) before antimicrobial activity testing.

1.3. Antimicrobial Assays.

Antimicrobial activity was assayed against Gram positive bacteria, Gram negative bacteria and fungi (listed in Table 2) based on the liquid growth inhibition assay described previously (Hetru and Bulet, 1997). Poor Broth (PB : 1% bacto-Tryptone, 0.5% NaCl w/v, pH 7.5), and ½ Potato Dextrose Broth (Difco) were used for bacterial and fungal growth, respectively. Growth of bacteria was monitored spectrophotometrically at 600 nm on a multifunctional microplate reader (Tecan infinite 200) while fungal growth was evaluated after 24 and 48 hours at 30°C by optical microscopy and measurement of the culture absorbance at 595 nm. MIC values are expressed as the lowest concentration tested that caused 100% of growth inhibition (micromolar). The bactericidal activity of the peptide was assessed by plating cultures. The absence of CFUs on agar plates, after an overnight incubation at 30°C, was considered indicative of a bactericidal effect.

1.4. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry

Mass spectrum was acquired on Ultraflex (Bruker) in positive mode. The irradiation source was a pulsed nitrogen laser with a wavelength of 337 nm. A solution of α-cyano-4-hydroxycinnamic acid (10 mg/ml) used as matrix and a product solution were mixed in a ratio of 1:1. A 1 µL aliquot of the matrix/product mixture was deposited and air dried. External mass calibrations were performed with a standard peptide mixture. The analyses were recorded in reflector mode. Mass spectrum was analyzed with Flex Analysis software.

1.5. Dot-blot assay

The presence of cecropins in *Spodoptera frugiperda* plasma from treated or untreated larvae was evaluated by spotting 3 µL of 40% ACN Sep-Pak fraction on a PVDF membrane (0.22 µm, Immun-Blot™, Bio-Rad). Membranes were probed with rabbit anti-*Bombyx mori* CecB antibodies (Acris Antibodies, Germany) diluted at 300 ng/mL in PBS containing 0.05% Tween-20 and 1% BSA. A horseradish peroxidase coupled anti-rabbit IgG (GE Healthcare) was used at a 1/5,000 dilution for detection by chemiluminescence (ECL Western blotting detection, GE Healthcare) and finally, the PVDF membrane was exposed to Hyperfilm-ECL (GE Healthcare).

1.6. Enzyme-linked immunosorbent assay (ELISA)

Spod-11-tox content in the plasma of challenged and unchallenged larvae was measured by ELISA. Eight hours post-challenge, larvae were bled from a cut abdominal proleg into 150 µL of ice-cold anti-coagulant buffer. Tubes were weighed before and after bleeding to measure the volume of hemolymph from each larva. Hemocytes were separated from plasma by centrifugation (600 x g for 30 sec at 4°C). For ELISA, microtiter plates (Maxisorp Nunc-Immuno Plate™) were coated with plasma (50 µL/well), and incubated overnight at room temperature, washed three times with 0.05% Tween-

20 in PBS (PBS-T) and blocked by incubation with 0.25% BSA in PBS-T for 2 h at room temperature. Anti-Spod-11-tox antibodies (100 ng/mL in PBS-T containing 1% BSA) were added to each well and the plates were incubated for 1 h at room temperature. The plates were washed four times with PBS-T and incubated for 1 h with peroxidase-linked donkey anti-rabbit IgG (1/5,000 dilution; GE Healthcare). The plates were washed four times with PBS-T and 100 µL of 1-Step™ Ultra TMB-ELISA (Pierce) solution was added to each well. Color development was stopped after 30 min by the addition of 100 µL of 2 M H_2SO_4, and absorbance at 450 nm was measured with a microplate reader (Tecan Infinite 200).

1.7. Production of synthetic S. frugiperda CecB1

Synthetic CecB1 peptide was produced by NeoMPS S.A. (Strasbourg, France) using the t-Boc solid-phase peptide synthesis technology.

2. Results

2.1. *Purification of a Spodoptera frugiperda cecropin*

In previous work, we detected several antimicrobial activities in the plasma (cell-free hemolymph) of bacterial-challenged *S. frugiperda* larvae (Destoumieux-Garzon et al., 2009). To further characterize the molecules responsible for those activities, we used a classical procedure for purification of antimicrobial peptides (AMPs) (as described in Materials and Methods). Nine fractions were found to contain antimicrobial activity against the three microorganisms tested (Figure 1a). A fraction that eluted around 40% acetonitrile (indicated by an arrow on Figure 1a) was further purified (Figure 1b). The molecular weight and the N-terminal sequence of the purified molecule were determined by MALDI-TOF-MS (Figure 1c) and Edman degradation (Figure 1d), respectively. Both, the MS data (one single ion at m/z=3,910.9) and the single N-terminal sequence, Arg-Xaa-Lys-Phe-Phe-Lys-Lys-Ile-Glu-Lys-, showed the purity of the collected peptide. These data are consistent with the isolation of a cecropin.

2.2. *Spodoptera frugiperda genome contains genes encoding members of the antimicrobial peptide cecropin family*

In order to further characterize the purified peptide, we analyzed the Spodobase, a *Spodoptera* ESTs database (http://bioweb.ensam.inra.fr/spodobase/). We identified 189 sequences corresponding to cecropin transcripts. These sequences were clustered by the use of Seqman (Lasergene, DNASTAR inc.) into 13 different contigs whose characteristics are summarized in Table 1. Their lengths ranged from 433 bp up to 992 bp and the number of sequences in each contig varied from 4 up to 31. On the other hand, the analysis of their nucleotide sequences allowed the identification of five clusters within which contig sequences differed by few nucleotide substitutions likely due to polymorphism as in cluster II (Supplementary Figure 2) or by the presence of gaps mainly in the 3' UTR for clusters I, III and V which might indicate gene duplication (Supplementary

Figures 1, 3 and 5). However, the alignment of the deduced amino acid sequences (Figure 2) indicated that the nucleotide sequences encode only 6 different polypeptides composed of 62 or 63 amino acids.

In order to classify the different members of the *S. frugiperda* cecropin family, deduced amino acid sequences of the mature polypeptides were aligned with a selection of cecropins from two Diptera (*Drosophila melanogaster* and *Anopheles gambiae*) and eight Lepidotera (*Bombyx mori*, *Helicoverpa armigera*, *Hyalophora cecropia*, *Hyphantria cunea*, *Plutella xylostella*, *Spodoptera exigua*, *Spodotera litura* and *Trichoplusia ni*) (Supplementary Table 1) using Seaview software (Gouy et al., 2010). The alignment was performed by clustalW (Larkin et al., 2007) and the phylogenetic tree (Figure 3) was obtained by Maximum Likelihood method (Guindon et al., 2010). The cladogram shows that all the lepidopteran cecropins cluster apart from the dipteran sequences. The previously described lepidopteran cecropins A, B, C and E cluster together, whereas cecropins D cluster in a separate clade. The *S. frugiperda* cecropins that are described here for the first time were named (Table 1) according to their positions in the cladogram. The previously described *S. litura* CecD however appears to be misnamed, clustering with the lepidopteran CecA/B sub-group.

Comparison of the amino acid sequences of *S. frugiperda* Cec with the N-terminal sequence obtained from the purified cecropin showed that it corresponded to CecB1 which is the only one that contains, in the mature peptide obtained after removal of the 26-residue signal peptide, a Phe and an Ile at positions 4 and 8, respectively. According to the amino acid sequence deduced from its cDNA, *S. frugiperda* CecB1 should be a 36-residues peptide with a molecular mass of 3,951.7 Da, while the mass of the purified cecropin is 3,910.9 Da (Figure 1d). This strongly suggests that the mature peptide is generated by an enzymatic cleavage of the carboxy-terminal Gly residue and a C-terminal amidation as previously reported (Boman et al., 1989), and therefore, has a final molecular mass of 3,894.7 Da. The 16 Da difference found with the mass determined by MALDI-TOF is likely due to an oxidation of the peptide during the mass spectrometry analysis. The oxidation likely occurred on the Trp-residue in position 2, which gave no standard PTU-amino acid by Edman degradation (Xaa in Figure 1d).

2.3. Antimicrobial activity of S. frugiperda CecB1

To investigate the antimicrobial activity of *S. frugiperda* CecB1, we used a chemically synthesized CecB1 (syCecB1) produced by the t-Boc solid-phase peptide synthesis technology. syCecB1 is a 35 amino acid peptide corresponding to the mature peptide formed by deletion of the C-terminal Gly and amidated on the Leu at 35 position (R-35-L-NH_2). syCecB1 antimicrobial activity was determined against a panel of microorganisms, including Gram-positive and Gram-negative bacteria, and filamentous fungi. The MIC values obtained are reported in Table 2. Like native CecB1, the synthetic peptide was active against *M. luteus*, *E. coli* SBS363 and *F. oxysporum*, the three microorganisms used during the different purification steps of CecB1. syCecB1 was more active against Gram-negative bacteria than against Gram-positive bacteria as described for cecropins characterized in other insects (Bulet et al., 2003). syCecB1 had bactericidal effects against all the Gram-negative bacteria tested (Table 2).

Interestingly, entomopathogenic microorganisms such as the Gram negative bacterium *S. marcescens*, Gram positive bacteria *Bacillus* sp., and the two fungi *Beauveria bassiana* and *Nomuraea rileyi*, appeared to be less susceptible to syCecB1 than non-pathogenic ones. Finally, *X. nematophila* F1 and *X. mauleonii* VC01^T, two closely related entomopathogenic bacteria were not sensitive to the same extent. Indeed, the MIC value for *X. mauleonii* was eight times higher than the one of *X. nematophila*.

2.4. Effect of X. nematophila F1 and X. mauleonii VC01^T infection on the induction of humoral response in S. frugiperda.

To investigate if the differential sensitivity of the two *Xenorhabdus* sp. to syCecB1 was correlated to different infection strategies, we evaluated the total Cec plasma content of *S.*

frugiperda larvae challenged by *X. nematophila* or *X. mauleonii*. Larvae were challenged with PBS or *E. coli* (CIP7624) as controls, or with live or heat-killed *Xenorhabdus*. The 40% Sep-Pak fractions (SPE40) (see Materials and Methods) were freeze-dried and reconstituted with MilliQ water at 1/20 of the initial hemolymph volume. Reconstituted SPE40 (3 µl) were spotted on nylon membrane which was then probed by commercial antibodies directed towards *Bombyx mori* cecropins (Figure 4). Immuno-reactivity was observed within the plasma of larvae challenged with heat-killed bacteria of the two strains, *X. nematophila* F1 and *X. mauleonii* VC01^T as well as with live *X. mauleonii* VC01^{T}and *E. coli* CIP7624. On the contrary, when insects were challenged with live *X. nematophila* F1, the level of immune-staining was similar to that found in unchallenged larvae. This indicates that larvae challenged with live *X. nematophila* F1 do not induce cecropins.

The above plasma samples were also used in liquid growth inhibition assays using *E. coli* SBS363 as bacterial target (Table 3). Results show that plasmas of larvae challenged with either live or heat-killed *X. mauleonii* VC01^T displayed levels of total antimicrobial activity similar to that found in plasma from *E. coli*-challenged larvae. In larvae challenged with live *X. nematophila* F1, total plasma antimicrobial activity was only about 10% of that seen in insects challenged with *X. mauleonii* or *E. coli*. However, when *X. nematophila* bacteria were heat-killed, the antimicrobial activity reached a level comparable to that present in plasma of *E. coli*-challenged larvae. Again, these results suggest that larvae challenged with live *X. nematophila* F1 have a reduced expression of antimicrobial peptides. Finally, we quantified the production of Spod-11-tox, an immune-related protein lacking antimicrobial activity (Destoumieux-Garzon et al., 2009), in the plasma of larvae challenged by the two *Xenorhabdus* sp. (Figure 5). The plasma content of Spod-11-tox in *E. coli*-challenged larvae was 9 times higher than in unchallenged or PBS-injected larvae. Similar high levels of Spod-11-tox were measured in plasma of *X. mauleonii* VC01^T-challenged larvae whether the bacteria were alive or dead. Conversely, Spod-11-tox plasma content of larvae injected with live *X. nematophila* F1 was comparable to that measured in plasma of unchallenged or PBS-challenged larvae whereas in larvae challenged by heat-killed *X. nematophila*, Spod-11-tox plasma content was similar to that measured in plasma of *E. coli*-challenged larvae.

Altogether, these results show that while *X. mauleonii* VC01^T elicits a regular humoral response for insects challenged by bacteria, *X. nematophila* F1 prevents the expression of plasma-soluble immune-related peptides and proteins.

2.5. X. nematophila secretes an inhibitor of S. frugiperda humoral response.

To test whether *X. nematophila* was able to prevent humoral immune response, *E. coli* bacteria were injected alone or with a culture supernatant of *X. nematophila* (Table 4). The antimicrobial activity measured in the hemolymph of such challenged insects was comparable to the one measured in the hemolymph of insects challenged with PBS or live *X. nematophila.* In other words, the presence of *X. nematophila* culture supernatant was able to inhibit the AMPs expression observed when *E. coli* was injected alone. Altogether, these results suggest that the reduced expression of antimicrobial activity observed above is due to a bacterial factor produced and secreted by *X. nematophila*.

3. Discussion

In this study, we have identified a family of cecropins (Cec) encoded by sequences present in Spodobase, a *Spodoptera frugiperda* specific EST library (Negre et al., 2006). This family appears to be composed of 13 members, 3 CecA, 7 CecB, one CecC and 2 CecD according to their cDNA sequences. Recently, 13 cecropin genes were found in the genome of *Bombyx mori* (Cheng et al., 2006; Tanaka et al., 2008), the only Lepidopteran genome available to date (The International Silkworm Genome Consortium, 2008). However, the 2 CecA and the 6 CecB silkworm cDNA encode only two amino acid sequences (Tanaka et al., 2008). In our work, we show that a similar situation is present in *S. frugiperda*, since the 3 CecA give only one protein as do 5 of the CecB cDNA. Therefore, we may suggest that Lepidopteran insects have a high degree of cecropin gene duplication. Moreover, since the database does not represent the whole *S. frugiperda* transcriptome, we cannot rule out the possibility that *S. frugiperda* genome might contain more cecropin genes. The accessibility of *S. frugiperda* genome would help to check this hypothesis.

S. frugiperda Cec are 62 or 63 amino acids long in their prepro-forms which, after post-translational modifications, generate mature polypeptides of 36, 37 and 42 amino acids that possess one feature that is characteristic of most insect cecropins which is the presence of a tryptophan residue in the first or second position. A TMHMM analysis (http://www.cbs.dtu.dk/services/TMHMM/) indicates that, as already described for other insect cecropins (Bulet and Stocklin, 2005), all *S. frugiperda* Cec have a long N-terminal, basic and amphipathic α-helix (residues 2 to 22 in the mature peptide, positions determined from CecA1 in Figure 2) and a shorter and more hydrophobic C-terminal helix (residues 25 to 34), linked by a highly conserved Gly^{23}-Pro^{24} hinge region.

A phylogenetic analysis conducted with 51 cecropins from a selection of Dipteran and Lepidopteran allowed the classification of Cec from *S. frugiperda* as members of the cecropin peptide families CecA, CecB, CecC and CecD. Lepidopteran cecropins were found distributed into two main clades, one containing all types of cecropins except CecD. Lepidopteran CecD present several specific features such as one highly conserved acidic amino acid, Glu^6 or Asp^6, and a Ser^{20} (positions determined from the CecD1 mature polypeptide in Figure 2). In addition, the highly conserved Ala-Pro-Glu-Pro sequence which precedes the mature peptide and corresponds to a pro-peptide removed in two steps by a dipeptidyl aminopeptidase in other cecropins (Boman et al., 1989), is absent from CecD sequences. This suggests that they are produced as mature active peptide directly after digestion by a signal peptidase at the carboxy-terminus of Ala^{-1}. Finally, although this phylogenetic tree does not give a lot of information on the evolutionary history of the cecropin gene, it may indicate that some insects Cec have likely been wrongly named such as for example *S. litura* CecD that we propose to rename *S. litura* CecB according to its position in the cladogram.

The α-helical linear antimicrobial peptides of insects, such as cecropins, are mostly active against bacteria, with a higher efficacy on Gram negative than Gram positive strains (Choi et al., 2000; Kylsten et al., 1990; Samakovlis et al., 1990). They have also been shown to be active against some fungi (Ekengren and Hultmark, 1999). As expected, syCecB1 had a similar profile of antimicrobial activity. It is noteworthy that the two strains of entomopathogenic fungi tested here were not susceptible to syCecB1 at concentrations as high as 25 µM. By contrast, entomopathogenic *Xenorhabdus nematophila* strain F1 was as sensitive as the most susceptible strains, *Escherichia coli* CIP7624 and *Micrococcus luteus*. In contrast, *X. mauleonii* $VC01^T$, an insect pathogen closely related to *X. nematophila*, was much less sensitive to syCecB1. We therefore studied the effect of these two bacteria on the cecropins expression in *S. frugiperda*. Our results showed that *X. nematophila* F1 was able to inhibit the expression of cecropins in *S. frugiperda*. This is reminiscent of two recent studies showing that microorganisms such as a polyDNA bracovirus from *Cotesia plutellae* or *X. nematophila* inhibited the expression of cecropins in *Plutella xylostella* (Barandoc et al., 2010) and *Spodoptera exigua* (Ji and Kim, 2004), respectively. Moreover, *X. nematophila* had also an inhibitory activity on the expression of Spod-11-tox, an immune-related protein inducible by infection (Girard et al., 2008). Because only live *X. nematophila* controls the expression of host immune-related genes, it is likely that this occurs through the secretion by the bacteria of immunosuppressive products that prevent the host immune response. This hypothesis is reinforced by the fact that the injection of supernatant from *X. nematophila* culture prevents the induction of antimicrobial activity by *E. coli*. It is known that *X. nematophila* secretes some inhibitory metabolites against insect immune-associated phospholipase A_2 (PLA_2) to suppress both the cellular and humoral immune responses in the hemocoel of target insects (Kim et al., 2005; Song et al., 2011). However, Ji and Kim (Ji and Kim, 2004) demonstrated that the use of specific PLA_2 inhibitors did not inhibit antimicrobial activity or cecropin gene expression when *Spodoptera* were infected with heat-killed *X. nematophila* suggesting that the inhibition of *X. nematophila* on the expression of the antimicrobial peptide is not related to the inhibition of the eicosanoid pathway. Consequently, the *X. nematophila*-induced inhibition of two different immune-related genes allows us to raise the hypothesis that the bacterial virulence factor may interact with an insect molecular target upstream of the genes, *cecropins* and *Spod-11-tox*. Over the past years, several works have been performed to study the interactions between insect pathogens and different defense mechanisms of insects. Hence, it was shown in *Drosophila melanogaster* that during the early steps of infection, the highly virulent *Pseudomonas aeruginosa* PA14 strain down-regulates not only cecropins but also IMD pathway-dependent AMPs such as attacins and defensin (Apidianakis et al., 2005). We are currently involved in the characterization of the immunosuppressive factor present in supernatant from *X. nematophila* culture.

Another major result from this study is that two closely related pathogens of insects have evolved divergent strategies to overcome the host defense reactions. Indeed, while *X. nematophila* evades the host antimicrobial response by repressing the expression of AMPs, the other pathogen, *X. mauleonii* strain VC01^T, induces cecropin expression but is resistant to high concentration of *Sf*CecB1. Therefore, these two species of entomopathogenic bacteria use different strategies to evade the host antimicrobial response either by developing AMP resistance mechanisms or by preventing AMP expression.

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Nucleotide sequences reported in this paper have been submitted to the GenbankTM/EBI Data Bank with the following accession numbers DY773722, DY773924, DY776416, DY774879, DY778990, DY774910, DY775152, DY779994, DY775699, DY774316 and DY777111 for Sf1F00836, Sf1F01169, Sf1F04795, Sf1F02507, Sf1F09170, Sf1F02553, Sf1F02902, Sf1F11023, Sf1F03731, Sf1F01749 and Sf1F05978

Figure legends

Figure 1: Cecropin purification from fractionated *S. frugiperda* plasma.

(a) a 40% Sep-Pak fraction from the extraction of immunized *S. frugiperda* plasma was subjected to reversed phase HPLC on a UPNEC25QS column (Interchim) using a 0-60% linear gradient (dashed line) of acidified acetonitrile over 80 min. Abs, Absorbance monitored at 225 nm. Antimicrobial activity against *Escherichia coli* SBS363 (black rectangles), *Micrococcus luteus* CIP5345 (grey rectangles) and *Fusarium oxysporum* (white rectangles) was measured by liquid growth inhibition assays. (b) Final purification step with a Xbridge BEH130 narrowbore column with a 0-28-45% biphasic gradient of acidified acetonitrile over 5 and 40 min. (c) The molecular mass of the purified peptide was measured by MALDI-TOF MS (Ultraflex). (d) The N-terminal sequence of the purified peptide was determined by Edman degradation.

Figure 2: Alignment of deduced amino acid sequences of mature cecropins from *Spodoptera frugiperda*. Sequences were analysed by ClustalW. Arrow head indicates maturation cleavage site. Asterisks indicate differences between CecD1 (Sf1F01749) and CecD2 (Sf1F05978). Identical residues are indicated by Dots.

Figure 3: Phylogenetic analysis of *S. frugiperda* cecropins. 39 amino acid sequences of mature cecropins from two Dipteran (*Drosophila melanogaster* and *Anopheles gambiae*) and eight Lepidopteran (*Bombyx mori*, *Helicoverpa armigera*, *Hyalophora cecropia*, *Hyphantria cunea*, *Plutella xylostella*, *Spodoptera exigua*, *Spodotera litura* and *Trichoplusia ni*) were aligned together with *Spodoptera frugiperda* cecropins using ClustalW and the phylogenetic tree was obtained by the Maximum Likelyhood method. Bootstrap values of 1000 trials are indicated as numbers. Sequences used in this analysis are listed in Supplementary Table 1.

Figure 4: Effect of *Xenorhabdus nematophila* F1 and *Xenorhabdus mauleonii* VC01^{T} on the expression of *Spodoptera frugiperda* cecropins. Fractionated plasma samples (40% ACN eluted fraction after

SEP) of *S. frugiperda* after challenges as indicated (at least 20 larvae per challenge condition) were spotted on nylon membrane. Then, immuno-blot was stained with anti-*Bombyx mori* CecB antibodies (Acris Antibodies, Germany). U.C.: unchallenged, P: PBS-challenged, L: live, H: heat-killed, *X. n.*: *Xenorhabdus nematophila* F1, *X. m.*: *Xenorhabdus mauleonii* VC01^{T}, *E. c.*: *Escherichia coli* CIP7624. Samples used were: reconstituted plasmas (RP) or plasmas diluted 1:1 or 1:10 in PBS as indicated to the left.

Figure 5: Effect of *Xenorhabdus nematophila* F1 and *Xenorhabdus mauleonii* VC01^{T} on Spod-11-tox plasma content. Spod-11-tox content in *S. frugiperda* plasmas was measured by ELISA as described in material and methods section. For each experimental condition, Spod-11-tox content was individually measured into the plasma of at least 20 larvae. Values represent the mean of three independent experiments while error bars show standard deviation. *$p < 0.001$ (as determined by Student t-test) compared to PBS injected larvae. U.C.: unchallenged, P: PBS-challenged, L: live, H: heat-killed, *X. n.*: *Xenorhabdus nematophila* F1, *X. m.*: *Xenorhabdus mauleonii* VC01^{T}, *E. c.*: *Escherichia coli* CIP7624.

Supplementary Figures 1 to 5: Cecropin-encoding contigs (see Table 1) present in the Spodobase (Negre et al., 2006) were aligned using Clustal W. Identical nucleotides in Clusters I, II and V are boxed. In the case of Cluster III, boxes indicate residues which are identical in, at least, 3 sequences. ORFs are indicated by the black line.

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Table 1

Table 1: Analysis of the SpodoBase, a *Spodoptera* EST database (Negre et al., 2006). This database is composed of cDNA sequences from three different tissues (hemocytes, fat body and midgut) as well as the cell line Sf9.

Contig	Name	Nbr of EST	Length	Best BLAST	E_value	Score	Deduced peptide (AA)
Sf1F00836	CecA1	14	498	AF142341.1 Antimicrobial protein cecropin A [*S. litura*]	4e-31	117	62
Sf1F01169	CecA2	16	494	AF142341.1 Antimicrobial protein cecropin A [*S. litura*]	4e-36	117	62
Sf1F04795	CecA3	31	505	AF142341.1 Antimicrobial protein cecropin A [*S. litura*]	1e-31	117	62
Sf1F02507	CecB1	25	471	GU182910.1 Antimicrobial protein cecropin 3 [*H. armigera*]	4e-21	108	62
Sf1F09170	CecB1	4	436	GU182910.1 Antimicrobial protein cecropin 3 [*H. armigera*]	4e-21	108	62
Sf1F02553-1	CecB4	10	598	AF142342.1 Antibacterial protein cecropin B [*S. litura*]	2e-17	97	63
Sf1F02553-2	CecB2	9	992	AF142342.1 Antibacterial protein cecropin B [*S. litura*]	2e-19	94	63
Sf1F02902	CecB3	10	915	AF142342.1 Antibacterial protein cecropin B [*S. litura*]	3e-18	96	63
Sf1F11023-1	CecB5	12	804	AF142342.1 Antibacterial protein cecropin B [*S. litura*]	3e-25	123	63
Sf1F11023-2	CecB6	10	858	AF142342.1 Antibacterial protein cecropin B [*S. litura*]	1e-15	93	63
Sf1F03731	CecC	14	433	GU182909.1 Antibacterial peptide cecropin 2 *[H. armigera*]	2e-18	97	63
Sf1F01749	CecD1	15	505	EU041763.1 Antimicrobial peptide CecD [*H. armigera*]	6e-25	121	63
Sf1F05978	CecD2	19	498	EU041763.1 Antimicrobial peptide CecD [*H. armigera*]	8e-24	117	63

Clusters and deduced amino acid sequences were determined using SeqMan and EditSeq (Software suite, Lasergene, DNASTAR inc.), respectively. tblastx were performed at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 2

Table 2: Activity of synthetic Cecropin B1 on various micro-organisms.

Micro-organisms	MIC (µM)	Activity
Gram positive bacteria		
Bacillus cereus CIP14579[*]	20	Bacteriostatic
Bacillus thuringiensis[*]	20	Bactericidal
Micrococcus luteus CIP5345[†]	1.25	Bactericidal
Staphylococcus aureus CIP103428	20	Bacteriostatic
Gram negative bacteria		
Escherichia coli CIP7624	1.25	Bactericidal
Escherichia coli SBS363[†]	0.08	Bactericidal
Klebsiella pneumoniae 100633	10	Bactericidal
Salmonella enterica CIP5858	10	Bactericidal
Serratia marcescens 363[*]	> 20	n. d.
X. mauleonii VC01[T][*]	10	Bactericidal
X. nematophila F1[*]	1.25	Bactericidal
Fungi and Yeast		
Fusarium oxysporum[†]	2.5	Fungicidal
Beauveria bassiana[*]	> 25	n. d.
Nomuraea rileyi[*]	> 25	n. d.

[†] Used during the different purification steps.

[*] Entomopathogenic microorganisms.

n. d., not determined

Table 3

Table 3: Effect of *Xenorhabdus nematophila* F1 and *Xenorhabdus mauleonii* VC01^T on the expression of *Spodoptera frugiperda* antimicrobial peptides directed towards *Escherichia coli*.

	Larval treatment						
Experiments	**U.C.**	**PBS**	***E. coli***	***X. nematophila***		***X. mauleonii***	
				Live	**Heat-killed**	**Live**	**Heat-killed**
N° 1	1/4	1/16	1/256	1/8	1/128	1/64	1/64
N° 2	1/4	1/2	1/128	1/16	1/128	1/128	1/256
N° 3	1/4	1/8	1/128	1/8	1/64	1/64	1/128

The AMP activity present in the different plasma samples was measured by bacterial growth inhibition assay with *E. coli* SBS363 as bacterial target. Numbers indicate the minimal dilution of the samples that allowed normal bacterial growth. Three independent experiments were performed. U.C.: unchallenged, PBS: PBS-challenged, *E. coli*: *Escherichia coli* CIP7624, *X. nematophila*: *Xenorhabdus nematophila* F1, *X. mauleonii*: *Xenorhabdus mauleonii* VC01^T.

Table 4

Table 4: Effect of supernatant from *Xenorhabdus nematophila* F1 culture on the expression of *Spodoptera frugiperda* antimicrobial peptides.

	Larval treatment				
Experiments	**U.C.**	**PBS**	***E. coli***	**Live *X. n.***	***E. coli*/ Sup[(a)] *X. n.***
N°1	1/1	1/16	1/128	1/16	1/4
N°2	1/2	1/4	1/64	1/4	1/16
N°3	1/2	1/16	1/128	1/16	1/16

The AMP activity present in the different plasma samples was measured by bacterial growth inhibition assay with *E. coli* SBS363 as bacterial target. Numbers indicate the minimal dilution of the samples that allowed normal bacterial growth. Three independent experiments were performed. U.C.: unchallenged, PBS: PBS-challenged, *E. coli*: *Escherichia coli* CIP7624, *X. n.*: *Xenorhabdus nematophila* F1. [(a)] Sup: Culture supernatant of *X. nematophila* was obtained after centrifugation and filtration (0.22 µm) of an overnight bacterial culture. 20 µL of *X. nematophila* culture supernatant was injected at the same time than *E. coli*.

Figure 1

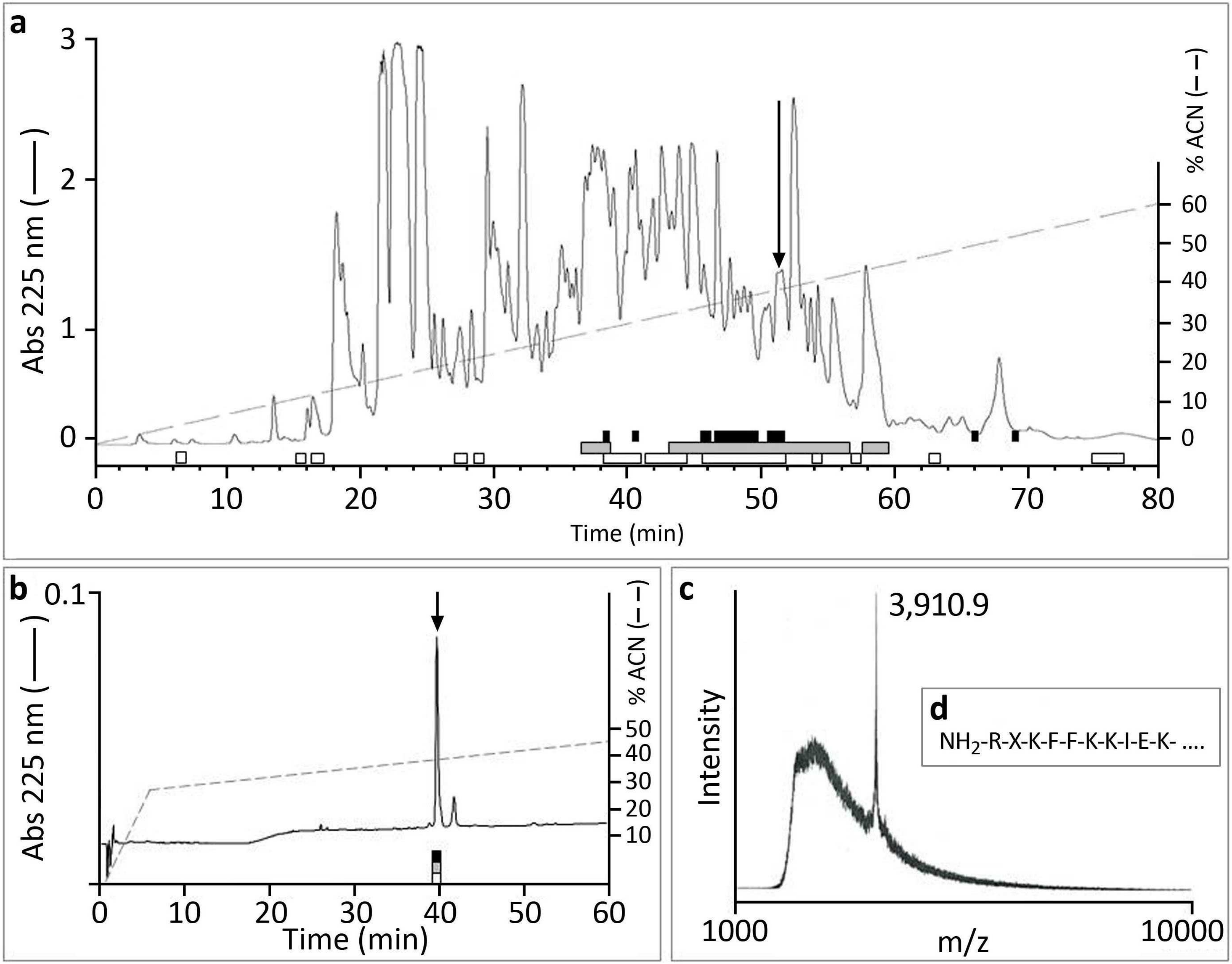

a
Abs 225 nm (——)
% ACN (– –)
Time (min)
b
Abs 225 nm (——)
% ACN (– –)
Time (min)
c
3,910.9
Intensity
m/z
1000
10000
d
NH2-R-X-K-F-F-K-K-I-E-K- ....

Figure 2

```
                                 20         ▼                 40                  60
CecA1  MKFSRVFLFVFACLVALSAVSAAPEPRWKVFKKIEKVGRNVRDGIIKAGPAIGVLGQAKAL--G----- 62
CecA2  .............................................................--.----- 62
CecA3  .............................................................--.----- 62
CecB1  .............................F..........I...........E...A....--.----- 62
CecB2  ....................................M...I....V.....VE...A....---.K---- 63
CecB3  ....................................M...I....V.....VE...A....---.K---- 63
CecB4  ....................................M...I....V.....VE...A....---.K---- 63
CecB5  ....................................M...I....V.....VE...A....---.K---- 63
CecB6  ....................................M...I....V.....VE...A....---.K---- 63
CecC   .N.TKI.......F.LMAT..G.......F...V..L.Q.I..........VA.V.S.A.I---.K---- 63
CecD1  .N-.KIII.LCI.FL.V.T..-.----.DL..E..G..QR...AV.S....VD..TK..K.AD.SSEED 63
CecD2  .N-.KIII.LCI.FL.V.T..-.----.DL..EL.G..QR...AV.S....VD..TK..K.AG.SSEED 63
                                        *                             *
```

Figure 3

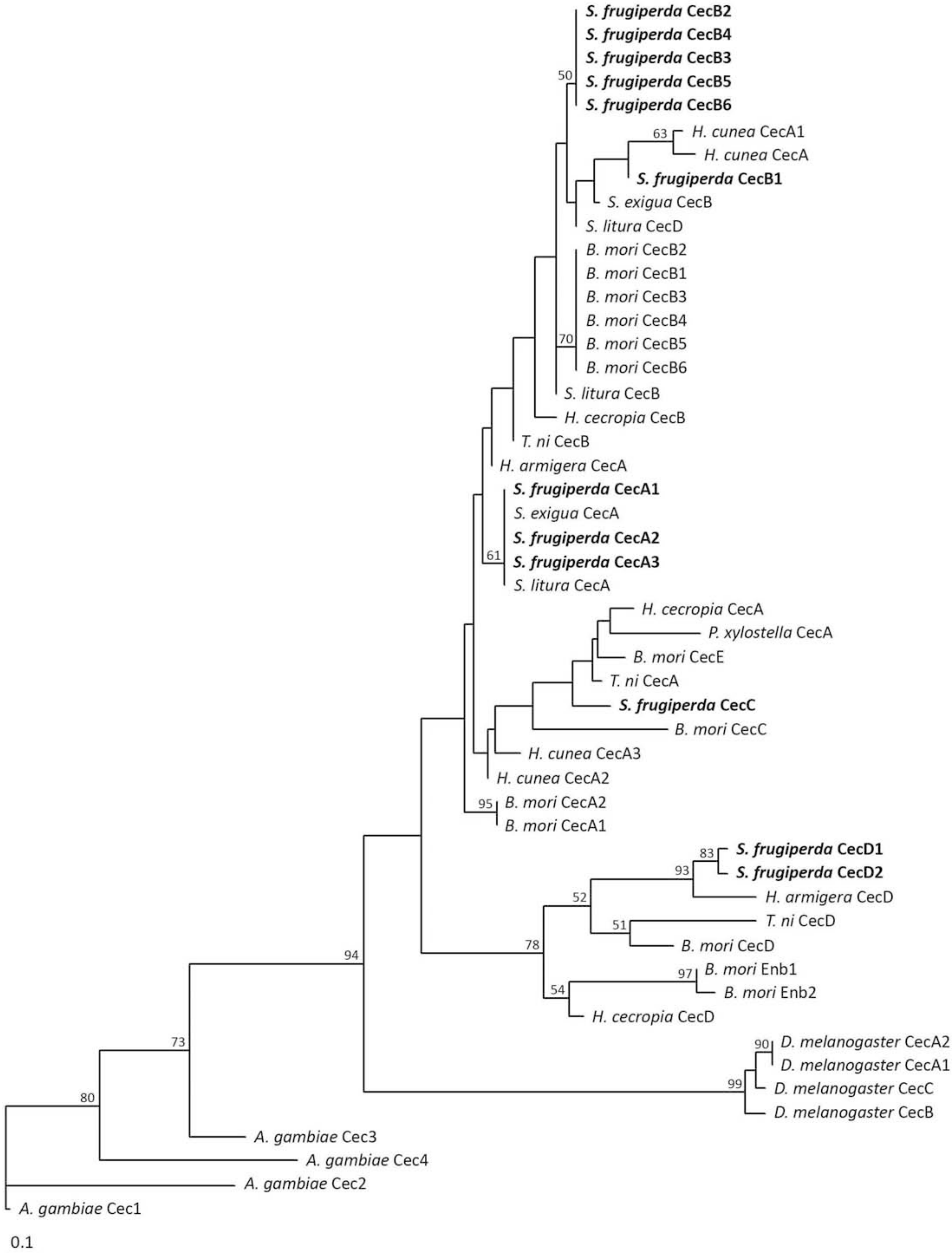

S. frugiperda CecB2
S. frugiperda CecB4
S. frugiperda CecB3
S. frugiperda CecB5
S. frugiperda CecB6
50
63
H. cunea CecA1
H. cunea CecA
S. frugiperda CecB1
S. exigua CecB
S. litura CecD
B. mori CecB2
B. mori CecB1
B. mori CecB3
B. mori CecB4
70
B. mori CecB5
B. mori CecB6
S. litura CecB
H. cecropia CecB
T. ni CecB
H. armigera CecA
S. frugiperda CecA1
S. exigua CecA
S. frugiperda CecA2
61
S. frugiperda CecA3
S. litura CecA
H. cecropia CecA
P. xylostella CecA
B. mori CecE
T. ni CecA
S. frugiperda CecC
B. mori CecC
H. cunea CecA3
H. cunea CecA2
95
B. mori CecA2
B. mori CecA1
83
S. frugiperda CecD1
93
S. frugiperda CecD2
52
H. armigera CecD
51
T. ni CecD
B. mori CecD
94
78
97
B. mori Enb1
54
B. mori Enb2
H. cecropia CecD
73
90
D. melanogaster CecA2
D. melanogaster CecA1
99
D. melanogaster CecC
80
D. melanogaster CecB
A. gambiae Cec3
A. gambiae Cec4
A. gambiae Cec2
A. gambiae Cec1
0.1

Figure 4

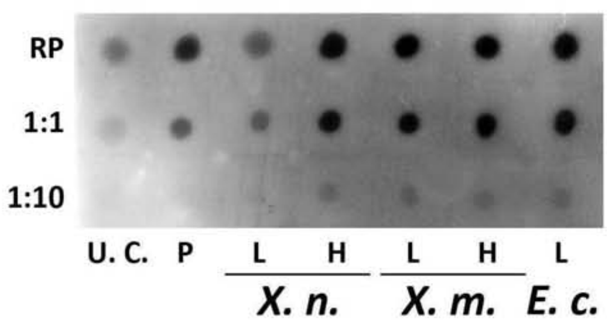
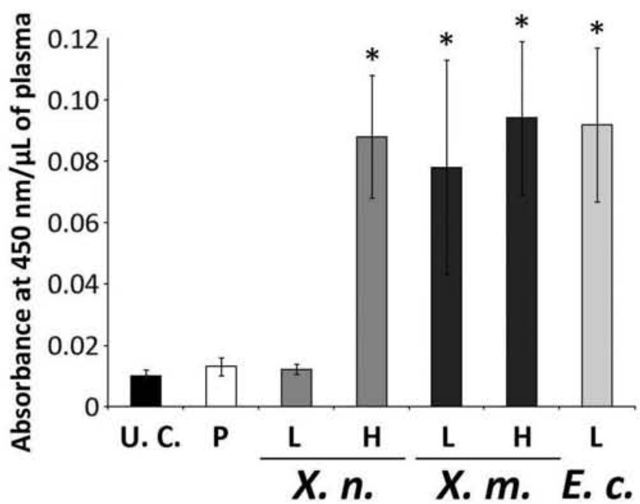


Figure 5



Absorbance at 450 nm/μL of plasma
0.12
0.10
0.08
0.06
0.04
0.02
0
*
*
*
*
U. C.
P
L
H
L
H
L
X. n.
X. m.
E. c.

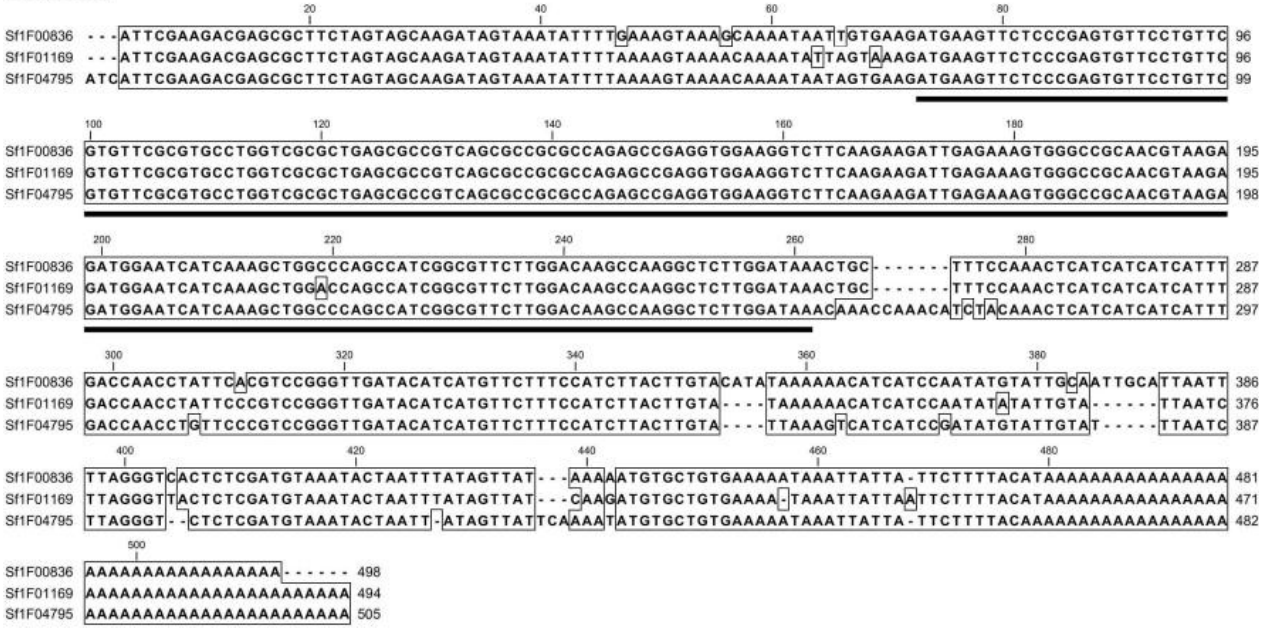
Supplementary Table 1: Sequences of insect cecropins used in this study.

Species	Sequences	Acc numbers
A. gambiae Cec1	GRLKKLGKKIEGAGKRVFKAAEKALPVVAGVKALG	XP_311223
A. gambiae Cec2	FKKFLKKVEGAGRRVANAAQKGLPLAAGVKGLVG	XP_311222
A. gambiae Cec3	RWKFGKRLEKLGRNVFRAAKKALPVIAGYKALGA	XP_311224
A. gambiae Cec4	LKKFGKKLEKVGKNVFHAVEKVVPVLQGIQDLRDKKNGQRG	XP_565481
B. mori CecA1	RWKLFKKIEKVGRNVRDGLIKAGPAIAVIGQAKSLGK	ref. [31]
B. mori CecA2	RWKLFKKIEKVGRNVRDGLIKAGPAIAVIGQAKSLGK	ref. [31]
B. mori CecB1	RWKIFKKIEKMGRNIRDGIVKAGPAIEVLGSAKAIGK	ref. [31]
B. mori CecB2	RWKIFKKIEKMGRNIRDGIVKAGPAIEVLGSAKAIGK	ref. [31]
B. mori CecB3	RWKIFKKIEKMGRNIRDGIVKAGPAIEVLGSAKAIGK	ref. [31]
B. mori CecB4	RWKIFKKIEKMGRNIRDGIVKAGPAIEVLGSAKAIGK	ref. [31]
B. mori CecB5	RWKIFKKIEKMGRNIRDGIVKAGPAIEVLGSAKAIGK	ref. [31]
B. mori CecB6	RWKIFKKIEKMGRNIRDGIVKAGPAIEVLGSAKAIGK	ref. [31]
B. mori CecC	KRKVFKIIEKIGRNVRGGVITAGPAVVVVGQAASVGM	ref. [31]
B. mori CecD	GNFFKDLEKMGQRVRDAVISAAPAVDTLAKAKALGQG	ref. [31]
B. mori CecE	RWKIFKKIEKVGQNIRDGIIKAGPAVAVVGQAATIAHGK	ABB19289
B. mori Enbocin1	WNFFKEIERAVARTRDAVISAGPAVATVAAASAVASG	AAC02238
B. mori Enbocin2	WNFFKEIERAVARTRDAVISAGPAVATVGAAAAVASG	BAF51563
D. melanogaster CecA1	GWLKKIGKKIERVGQHTRDATIQGLGIAQQAANVAATARG	NP_524588
D. melanogaster CecA2	GWLKKIGKKIERVGQHTRDATIQGLGIAQQAANVAATARG	NP_524589
D. melanogaster CecB	GWLRKLGKKIERIGQHTRDASIQVLGIAQQAANVAATARG	NP_524590
D. melanogaster CecC	GWLKKLGKRIERIGQHTRDATIQGLGIAQQAANVAATARG	NP_524591
H. armigera CecA	RWKVFKKIEKVGRNVRDGVIKAGPAIAVLGEAKALG	AAX51304
H. armigera CecD	WDFFKELEGAGQRVRDAIISAGPAVDVLTKAKGLYDSSEEKD	AAX51193
H. cecropia CecA	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAKG	CAA29871
H. cecropia CecB	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKALG	AAA29187
H. cecropia CecD	WNPFKELEKVGQRVRDAVISAGPAVATVAQATALAKGK	AAX51193
H. cunea CecA	RWKIFKKIERVGQNVRDGIIKAGPAIQVLGTAKALGK	P50720
H. cunea CecA1	RWKFFKKIERVGQNVRDGLIKAGPAIQVLGAAKALGK	P50721
H. cunea CecA2	RWKVFKKIEKVGRNIRDGVIKAGPAIAVVGQAKALGK	P50722
H. cunea CecA3	RWKVFKKIEKVGRHIRDGVIKAGPAITVVGQATALGK	P50723
P. xylostella CecA	RWKPFKKLEKVGRNIRNGIIRYNGPAVAVIGQATSIARPTGK	ACX31606
S. exigua CecA	RWKVFKKIEKVGRNVRDGIIKAGPAIGVLGQAKAL	ref. [20]
S. exigua CecB	RWKVFKKIEKVGRNIDGIIKAGPAVEVLGTAKAL	ref. [20]
S. frugiperda CecA1	RWKVFKKIEKVGRNVRDGIIKAGPAIGVLGQAKALG	This study
S. frugiperda CecA2	RWKVFKKIEKVGRNVRDGIIKAGPAIGVLGQAKALG	This study
S. frugiperda CecA3	RWKVFKKIEKVGRNVRDGIIKAGPAIGVLGQAKALG	This study
S. frugiperda CecB1	RWKFFKKIEKVGRNIRDGIIKAGPAIEVLGAAKALG	This study
S. frugiperda CecB2	RWKVFKKIEKMGRNIRDGIVKAGPAVEVLGAAKALGK	This study
S. frugiperda CecB3	RWKVFKKIEKMGRNIRDGIVKAGPAVEVLGAAKALGK	This study
S. frugiperda CecB4	RWKVFKKIEKMGRNIRDGIVKAGPAVEVLGAAKALGK	This study
S. frugiperda CecB5	RWKVFKKIEKMGRNIRDGIVKAGPAVEVLGAAKALGK	This study
S. frugiperda CecB6	RWKVFKKIEKMGRNIRDGIVKAGPAVEVLGAAKALGK	This study
S. frugiperda CecC	RWKFFKKVEKLGQNIRDGIIKAGPAVAVVGSAAAIGK	This study
S. frugiperda CecD1	WDLFKEIEGVGQRVRDAVISAGPAVDVLTKAKKLADGSSEED	This study
S. frugiperda CecD2	WDLFKELEGVGQRVRDAVISAGPAVDVLTKAKKLAGGSSEED	This study
S. litura CecA	RWKVFKKIEKVGRNVRDGIIKAGPAIGVLGQAKALG	Q9XZG9
S. litura CecB	RWKVFKKIEKMGRNIRDGIVKAGPAIEVLGSAKALGK	Q9XZH0
S. litura CecD	RWKVFKKIEKMGRNIRDGIIKAGPAVEVLGSAKALGK	ABQ51092
T. ni CecA	RWKFFKKIEKVGQNIRDGIIKAGPAVAVVGQAASITGK	P50724
T. ni CecB	RWKVFKKIEKMGRNVRDGIIKAGPAIAVLGEAKALGK	ABV68872
T. ni CecD	GNFFKDLEGIGQRVRDAIESAGPAVDVLGRAAALSRGEQQQRE	ABV68873

Legend of Supplementary Figures 2 to 6: Cecropin-encoding contigs (see Table 1) present in the Spodobase (Negre et al., 2006) were aligned using Clustal W. Identical nucleotides in Clusters I, II and V are boxed. In the case of Cluster III, boxes indicate residues which are identical in, at least, 3 sequences. ORFs are indicated by the black line.

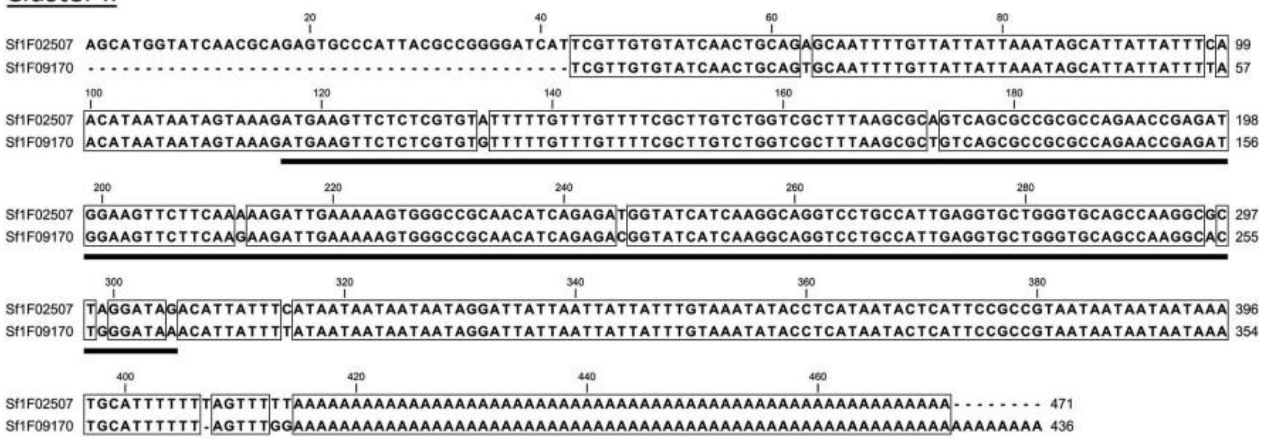
Supplementary Fig. 2. Supplementary material.

Cluster I



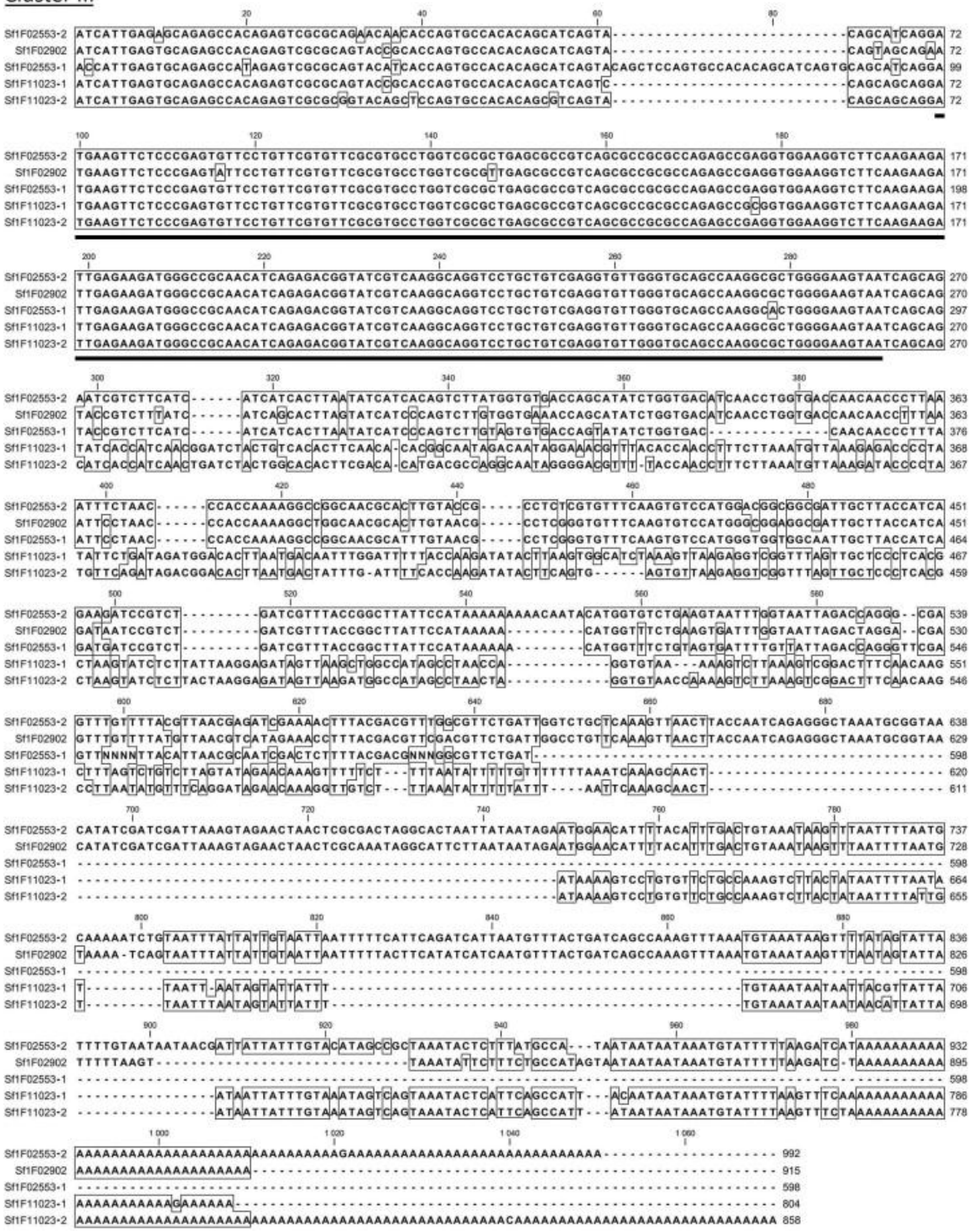
Supplementary Fig. 3. Supplementary material.

Cluster II



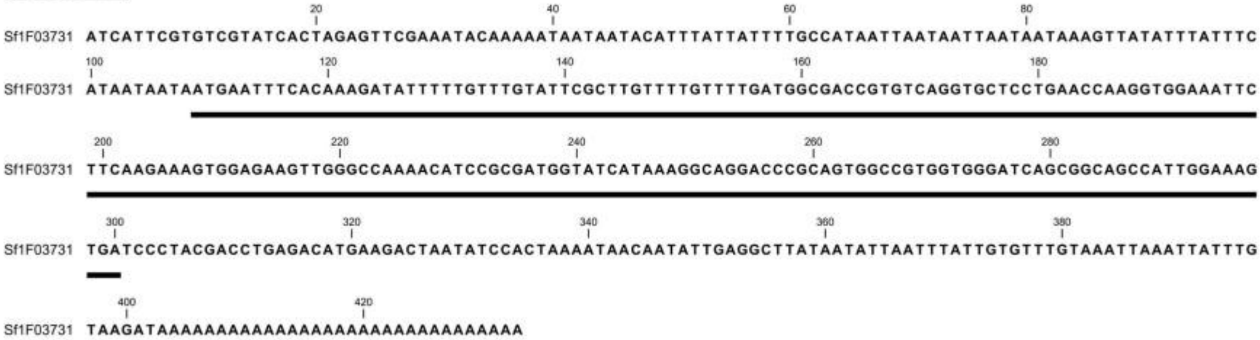
Supplementary Fig. 4. Supplementary material.

Cluster III



Supplementary Fig. 5. Supplementary material.

Cluster IV



Supplementary Fig. 6. Supplementary material.

Cluster V