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Molecular analysis of insecticide resistance in pollen beetle (*Meligethes aeneus*)

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Molekylär analys av insekticidresistens i rapsbagge (*Meligethes aeneus*)

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Keywords: Pyrethroid, resistance, voltage-sensitive sodium channel (VSSC), cytochrome P450, pollen beetle, *Meligethes aeneus*

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SAMMANFATTNING

Ökning av användandet av pyretroider som insekticidpreparat har gett en ökad medvetenhet om resistens för pyretroider hos skadeinsekter. Pyretroider hämmar spännings-känsliga natriumkanaler i nervcellsmembraner och är en syntetiskt tillverkad molekyl baserad på pyretriner som finns i extrakt från *Chrysanthemum* arter. Spännings-känsliga natriumkanaler är transmembranproteiner som är viktiga för elektrisk signalering över membranet hos insekten. Specifika mutationer i genen kodande för natriumkanalen hos insekter har visat sig vara en vanlig orsak till resistens mot pyretroider. Hos rapsbaggen finns det idag resistens mot pyretroider i flera delar av Sverige och även utomlands. Kartläggning av genen kodande för natriumkanalen och sökandet efter mutationer kända för att ge upphov till resistens hos insekter kan ge svar på om detta kan vara anledningen till resistens hos rapsbaggen. Natriumkanalproteinet består av fyra domäner, av vilka de tre första redan är kartlagda medan den fjärde domänen är kvar att sekvensera och undersökas för eventuella mutationer. För att få fram genfragmentet kodande för den fjärde domänen används polymeraskedjereaktion för att amplifiera rätt DNA-fragment ur genomet hos rapsbagge. Därefter används ligering och transformering för att verifiera DNA-fragmentet och plasmider bärandes på fragmentet sekvenserades. Det laborativa arbetet med domän IV var lyckat och sekvensen kan ses i appendix 2. Arbetet med att amplifiera hela natriumkanalen var svårt. Endast en amplifiering gav DNA-fragment av ca 6000 bp längd så inget fortsatt arbete kunde göras. Pyretroidresistensen kan också bero på ökad metabolism via cytokrom P450 (CYP). Arbetet med CYP syftade till att isolera och sekvensera DNA-fragment kodande för olika CYP. Under den experimentella delen gjordes upprepade försök med ligeringar och transformeringar för två olika CYP utan framgång. Istället skickades ett prov med amplifierat DNA-fragment som renats fram ur en agarosgel till Macrogen, Korea för sekvensbestämning, sekvensen för CYP6B$_2$15 kan ses i appendix 2.

ABSTRACT

The escalating usage of pyrethroids has resulted in an increased awareness about resistance towards pyrethroids in insects. Pyrethroids inhibit voltage-sensitive sodium channels (VSSC) in nerve cell membranes and are composed of synthetic molecules based on pyrethrins present in pyrethrum extracts from *Chrysanthemum* species. VSSC are transmembrane proteins that are important for electric signalling over the membrane in insects. Mutations in the gene encoding the sodium channel have proved to be a common reason for resistance against pyrethroids. Pyrethroid resistance among pollen beetles is spread all over Sweden and also abroad and is increasing. Mapping of the gene encoding the VSSC and search for mutations known to provide resistance in insects is necessary to know if that is the reason for resistance in pollen beetles. The VSSC protein consists of four domains, where the first three domains already have been sequenced so what is left to do is to obtain the sequence encoding the fourth domain. Polymerase Chain Reaction (PCR) was used to amplify the DNA fragment out of the pollen beetle genome. The following step was to use ligation and transformation to verify the DNA fragment by sequencing of obtained clones. The experimental part with domain IV was successful and the sequence can be seen in appendix 2. However, the work to amplify the whole gene encoding the VSSC was difficult. Only one of all amplifications gave a DNA fragment of 6,000 bp, so no further experimental work was possible. Pyrethroid resistance can also be due to increased metabolism by cytochrome P450 (CYP). The experimental part with CYP aimed to sequence gene fragments encoding different CYP. Ligation and transformation of PCR products was repeated many times without any positive results. Amplified samples purified from agarose gels were sent for sequencing instead and the sequences can be seen in appendix 2.

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1. INTRODUCTION

1.1. VSSC, voltage-sensitive sodium channel

The usage of pyrethroids has resulted in an increased awareness about resistance to pyrethroids in insects. The widespread use of dichloro diphenyl trichloroethane (DDT) has increased the concern about the spread of cross-resistance also against pyrethroids through the mechanism for resistance against DDT. This has decreased the sensitivity in insects for pyrethroids, so called knockdown resistance (Soderlund, 2008).

The pyrethroids have their effect on VSSC in nerve cells membranes (Soderlund & Knippe, 2003). VSSC consists of four repetitive domains (I-IV), each with six transmembrane segments (S1-6). Pyrethroids are synthetic molecules based on pyrethrins present in pyrethrum extracts from *Chrysanthemum* species. VSSC are transmembrane proteins important for electric signalling and to quickly build up an action potential over the membrane. The flow of sodium ions over the membrane into the cell occurs when the channel is open, active. The channel opens as a response to the depolarization over the membrane. This mechanism is essential for the cell, which makes the sodium channel an effective target for many neurotoxins such as pyrethroids. Neurotoxins are grouped after their binding pattern and effects on the sodium channels. The pyrethroids themselves are grouped into two types, type I and II. Which type the pyrethroid belongs to depends on the given symptoms and its chemical structure. If the pyrethroid causes repeated discharges of the membrane when it responds to a stimulus it is categorized as type I. If the pyrethroid instead causes a membrane polarization followed by repression of the action potential it is categorized as type II. Studies have shown that pyrethroids destroy the stabilisation of the open configuration of the channel and inhibit deactivation, which keeps the channel open during longer time periods. Mutations in sites that are found to prevent an open configuration include L1014F in IIS6, M918T in IIS4-S5 and T929I in IIS5 (Dong, 2007).

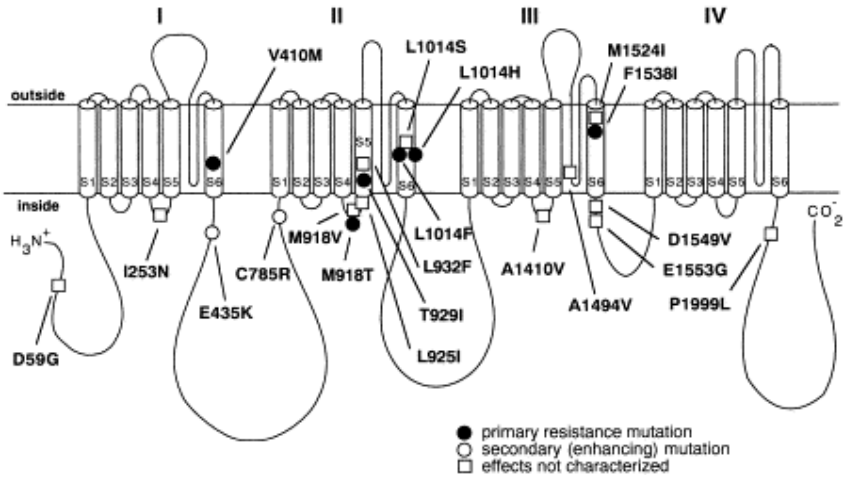

Figure 1. Structure of voltage-sensitive sodium channel. Four repetitive domains (I – IV) with six transmembrane segments (S1 – 6) (Soderlund & Knippe, 2003).

The gene encoding the transmembrane protein has a high similarity with α-subunits in the sodium channels present in mammals, both when it comes to structure and amino acid sequences. Since these proteins are very similar it makes it possible to draw a

conclusion that the gene probably encodes a sodium ion channel. The usage of *Xenopus* oocytes has been important to confirm that the gene encodes a sodium channel. Studies with *Drosophila* showed that the gene does not look like any other gene in its genome, which resulted in the conclusion that the gene probably is the only gene that encodes a sodium channel in other insects as well (Dong, 2007).

So far 20 mutations associated with pyrethroid resistance have been found. Four out of these are single mutations, Val410, Met918, Leu1014, Phe1538 (Soderlund & Knippe, 2003). Studies of the single mutations and comparison of DNA sequences encoding sodium channels in different insect species have demonstrated that the replacement of Leu1014 in the sixth transmembrane segment in domain IV to phenylalanine (L1014F) is common and the mutation most associated with knockdown resistance. Also an exchange of leucin in this position to serine or histidine is associated with pyrethroid resistance (Soderlund, 2008).

Experiments with expression of VSSC alleles in *Xenopus* oocytes have shown that this mutation in L1014 reduces the sensitivity to pyrethroids by five to ten times (Dong, 2007).
The 20 mutations found in VSSC genes from different insects are not randomly spread in the protein. Most of the mutations are found and associated with the pore of the channel. Eight of the mutations are found in one of the three distinguished parts of the protein. These are localized to the S5 and S6 transmembrane segments in domain I, II and III, where three single mutations are found. Six mutations are localized to the S6 transmembrane segment. Another mutation, A1494V is localized to the environment near the pore in the protein between S5 and S6 segments in domain III.

Four of the 20 mutations have been found in the intracellular loop that couples S4 and S5 in domain I, II and II. One of these four mutations is the single mutation M918. At the intracellular loops of the protein sequence (fig. 1) that separates the homologous domains, four mutations have been found, these are E435K, C785R, D1549V and E1553G. Two mutations that do not fit into these patterns are D59G and P1999L, these are second-site mutations (Soderlund & Knippe, 2003). It has been shown that not all mutations physically interact with the pyrethroid molecule. Such mutations have instead an indirect effect through changes of the conformational flexibility of the sodium channel where the pyrethroid has its action (Soderlund, 2008). So far no mutations associated with resistance against pyrethroids have been found in domain IV (Soderlund & Knippe, 2003).

1.2. Cytochrome P540 (CYP)

Cytochrome P450 is the largest gene family present in all living organisms. In insects hundreds of genes encoding CYPs can be found. It has been shown that CYPs can be responsible for pest resistance to insecticides but so far no mutations that cause resistance by changes in protein structure or activity have been found. Instead it has been shown that transcript levels of CYP genes metabolising pyrethroids are increased in many resistant insects, which can be the reason for resistance (Gilbert, Iatrou, Gill, 2005).

1.3. Techniques

The Polymerase Chain Reaction (PCR) method works so that the DNA fragment to be amplified serves as template so more DNA fragments can be synthesized. To the

reaction specific oligonucleotide primers are needed that bind to the template, heat-stable DNA-polymerase that builds up the strand, dNTPs (deoxynucleoside triphosphates) that are the building blocks, buffer, Mg^{2+} and K^{+}. The reaction mixture is heated to 94-98 ºC, where the DNA strands separate from each other because the hydrogen bonds are broken, the denaturation step. Next step is annealing, where the temperature decreases to 50-65 ºC to allow the primers to anneal to the single-stranded fragments. The third step is elongation where the temperature increases to 72-80 ºC, so the DNA polymerase can use the oligonucleotide as primer to elongate the complementary single-stranded fragments (5'-3' direction) to form double-stranded fragments. These three steps constitute one cycle and are repeated many times. When the reaction is finished, the sample is analyzed by gel electrophoresis to see if the amplification was successful.

Some genes are long, which makes them difficult to amplify using regular PCR methods. In these cases, long-range PCR is used instead. The method uses the same principle as regular PCR, but with the exception that the polymerase used has proof-reading ability. Sometimes two polymerases are used, one with proof-reading and one without, which makes it possible to make longer DNA fragments.

2. MATERIALS AND METHODS

2.1. VSSC, domain IV

2.1.1. Buffer and media

LB agar for the plates used in the experiments was made by mixing 5 g yeast extract, 10 g NaCl and 10 g Bacto-tryptone in 1 liter of milliQ H_2O. pH was measured and corrected to 7.2, whereafter 15 g agar was added and the solution was autoclaved. After the temperature had decreased to around 50 ºC, ampicillin was added to a final concentration of 100 µg/ml and the LB agar was poured into the plates. The plates were stored at 4 ºC.

LB-medium used in the experiments was made by mixing 5 g yeast extract, 10 g NaCl and 10 g Bacto-tryptone in 1 liter of destilled (milliQ) H_2O. After pH was adjusted to 7.2, the solution was autoclaved and stored at 4 ºC.

Buffer used for gel electrophoresis was 1x TAE buffer. The stock solution (10x TAE) consisted of 48.46 g (0.4 M) Tris Base, 3.72 g (0.01 M) EDTA-Na_2-salt and 12.01 g (0.2 M) acetic acid and made up to 1 liter with destilled (milliQ) H_2O. The pH was adjusted to 8.5.

2.1.2. Pollen beetle material

Samples of pollen beetles collected from different Swedish provinces were used in the present study. The insects were stored frozen in -70 ºC to maintain quality before making extracts.

2.1.3. DNA extraction and purification
DNA was extracted from 10 pollen beetles known to be resistant to pyrethroids using a Qiagen DNA extraction kit. The amount of DNA was measured with a NanoDrop instrument.

2.1.4. Primers
Degenerated primers were used to amplify domain IV of the VSSC gene from genomic DNA. Degenerated primers were used because the VSSC gene of pollen beetle not yet had been sequenced. The primers were designed based on a multiple sequence alignment (ClustalW) of VSSC genes from other insects. Conserved features from these sequences were assumed to correspond to similar regions in the pollen beetle gene. The VSSC protein consists of four domains and the three first domains have already been cloned and sequenced so what was left to do was to sequence and search for mutations in the fourth domain.

The degenerated primers used for amplification of pollen beetle VSSC were VSSC 1-4_F and VSSC 1-4_R that should during amplification give the sequence encoding the hole VSSC or VSSC 3417_F and VSSC 5405_R that should give the sequence encoding for domain IV. The primer sequences can be found in appendix 1.

2.1.5. PCR
PCR condition and reagents used for PCR can be seen in tables 1 and 2.
All PCR products were run on 1 % agarose gel in 1x TAE buffer and with GeneRuler 1kb DNA ladder from Fermentas as size marker.

Table 1. Reagents used for amplification.

Reagents	Volume, µl
H_2O	13.7
5x HF buffer	4
10 mM dNTPs	0.4
10 µM Forward primer	0.5
10 µM Reverse primer	0.5
50 ng template	1
Phusion DNA polymerase (2U/µl)	0.2

Table 2. PCR programme.

PCR steps	Temperature, ºC	Time
Initial denaturation	98	30 sec.
Denaturation	98	30 sec.
Annealing	50	30 sec.
Elongation	72	2 min.
Final elongation	72	10 min.

(Denaturation – Annealing – Elongation) * 35 cycles

The DNA fragments that migrated to the expected size on the gel were used as templates for reamplification. Also a new amplification was made, the conditions were the same as in tables 1 and 2. The reamplification products and the new amplification product were run on a 1.1 % agarose gel in 1x TAE buffer with 5 µl λ DNA/*Eco*RI+*Hin*dIII ladder as a size marker.

2.1.6. Band elution and purification

The DNA fragments of the expected size on the gel were cut out and purified with PureLink™ Quick Gel Extraction Kit from Invitrogen according to the "Preparing and Dissolving the Gel Slice" and "Purification Procedure using Centrifugation" manual with one exception, water was added instead of Elution Buffer (E5) in the elution step. The purification of VSSC gene fragments from the gel did not provide enough DNA so a reamplification was performed using one of the samples that gave fragments on the gel at the expected size as template. The DNA fragments of the expected size was cut out of the gel and purified.

2.1.7. Ligation

The purified DNA fragment was used for ligation. Before the ligation, 5 µl was mixed with 1 µl 10x DreamTaq PCR buffer, 0.4 µl 10 mM dATPs and 0.2 µl DreamTaq DNA polymerase to obtain an A-overhang at the ends. The mixture was incubated at 72 ºC for 15 minutes and used for ligation. The product was ligated with pBluescript SK+ vector with T-overhang (AT-cloning).
The ligation conditions are shown in table 3.

Table 3. Reagent mixture for subcloning of DNA into pBluescript SK+ T vector.

Reagents	Volume, µl
Ligation buffer	5
25 ng pBluescript SK+ T vector (3.0 kb)	1
5 ng insert	20
T4 DNA ligase (5U/µl)	1

The ligation mixture was incubated at 16 ºC over night and used for transformation.

2.1.8. Transformation

For the transformation, LB agar + amp plates were pre-heated at 37 ºC for 30 minutes before 40 µl 40 mg/ml X-gal and 40 µl 100 mM IPTG were added to each plate. The plates were incubated at 37 ºC for at least 30 minutes. Competent cells (50 µl DH5α from Invitrogen) were gently thawed on ice before the ligation mixtures were added to the cells and incubated on ice for 30 minutes. The cells were heat-shocked for 30 seconds at 42 ºC without shaking and directly placed on ice for 2 minutes. SOC medium (250 µl) at room temperature was added to the mixtures, which were incubated at 37 ºC for 1 hour with shaking (300 rpm). Aliquotes of 50 µl and 150 µl, respectively, of the transformation mixture was spread on LB agar amp +, X-gal, IPTG plates and incubated at 37 ºC over night. pUC19 (10 pg) vector was used as a positive control in transformation.
The transformations failed three times and a new ligation was made. The new ligation reaction was made using the same condition as described above, but 10 µl template was added instead of 20 µl. The new transformation was performed as described above, but with 200 µl of "home" made DH5α competent cells. After transformation confirmation of inserts were tested.

2.1.9. Confirmation

Ten confirmations were performed and 1 colony was added to each tube containing 500 µl LB + ampicillin (100 µg/µl) and the cells were grown at 37 ºC for 5 hours. A 10 µl aliquot from each culture was added to a sterile 1.5 ml eppendorf tube and 40 µl sterile H_2O was added and kept at 95 ºC for 10 minutes. A 1 µl aliquot of the heated sample

was used as template for the confirmation PCR. Reagents needed for the PCR can be seen in table 4 and the PCR conditions are shown in table 5. The primers used were VSSC 3417_F and VSSC 5405_R.

Table 4. Reagents used for PCR confirmation of VSSC inserts.

Reagents	Volume, µl
H_2O	16.2
10x PCR buffer	2
10 mM dNTPs	0.4
10 µM Forward primer	0.5
10 µM Reverse primer	0.5
Template	1
DreamTaq DNA polymerase (5U/µl)	0.2

Table 5. The PCR programme used for insert amplification.

PCR steps	Temperature, ºC	Time
Initial denaturation	94	60 sec.
Denaturation	94	45 sec.
Annealing	50	60 sec.
Elongation	72	2 min
Final elongation	72	10 min

(Denaturation – Annealing – Elongation) * 30 cycles

Positive and negative controls were also included. The same reagents were added to the two tubes, but pollen beetle DNA (198 ng/µl) served as template for the positive control and water as template for the negative control.

2.1.10. Plasmid preparation

Next step was plasmid preparation. Cells (10 µl) used in confirmation were added to 5 ml LB + amp (100 µg/ µl) and grown over night at 37 ºC and centrifuged for 10 minutes at 5000 rpm at 4 ºC. The pellet was used in Qiagen Plasmid extraction kit to break the cells and purify the plasmids.

2.1.11. Sequencing and BLAST analysis

Three plasmid samples with the inserted DNA-fragment from different colonies were sent for sequencing to Macrogen, Inc Korea. Sequencing was carried out using BigDyeTM terminator cycled sequencing technique and automatic sequencer 3730xl. The first result was not good due to some technical problem at the company, so they offered to do it again. tblastx at NCBI was used to perform database search to see if the sequenced gene is relevant and encoding a part of VSSC (Altschul et al., 1997).

2.2. VSSC

2.2.1. Primers

The second step in the laboratory work was to amplify the full-length gene encoding VSSC. The gene is large, around 6 kb, so long-range PCR together with proof-reading DNA polymerase was used. The primers used were the same as for the amplification of the gene fragment encoding domain 4 of VSSC. The primer pairs were: VSSC 1-4_F and VSSC 1-4_R or VSSC 3417_F and VSSC 5405_R. The reagents used for PCR and the PCR programme can be seen in tables 1 and 2. Products from PCR in this step were analyzed by gel electrophoresis. The gels contained 1 % agarose in 1x TAE buffer with 5 µl GeneRuler 1kb DNA ladder from Fermentas as size marker.

2.2.2. PCR

Two different PCR were made with different primer pairs. The first reaction contained primers VSSC 1-4_F and VSSC 1-4_R, while the second reaction contained VSSC 1-4_F and VSSC 5405_R.

A reamplification was made but gave no good result so a new amplification was made with fresh DNA that was isolated from two samples of pyrethroid-resistant pollen beetles. The PCR conditions and PCR programme is shown in tables 1 and 2. The primer pairs were VSSC 1-4_F and VSSC 5405_R or VSSC 3417_F and VSSC 5405_R. The reactions were made in duplicates, using the two different DNA isolates as templates.

A reamplification was performed with the products from earlier PCR as template. A new PCR was also run with primer pair VSSC 1-4_F and VSSC 5405_R to see if it was possible to obtain larger DNA fragments. It did not obtain larger DNA fragments so a new PCR was run using primer pair VSSC 3417_F and VSSC 5405. The reagent mix and the PCR programme are shown in tables 1 and 2, expect for the annealing temperature that was changed to 48 ºC. The reactions were made in duplicates, with the two different DNA samples as templates. Together with the two samples, a reamplification was also performed on one of the products from earlier PCR.

New stock solutions for the primers were made. The stock solution was 100 µM and the working solution was 10 µM so 1 µl of the stock solution was mixed together with 9 µl DNAse and RNAse free MilliQ water.

The PCR with primer pair VSSC 3417_F and VSSC 5405_R used for amplification of VSSC was repeated with newly diluted primers. A new PCR was made with another DNA polymerase lacking proof-reading. The enzyme was AmpliTaq Gold DNA Polymerase from Applied Biosystems. The reagents in the PCR can be seen in table 6 and the PCR programme in table 7.

Table 6. Reagents and volumes used for PCR.

Reagents	Volume, µl
H_2O	26.75
GeneAmp 10x PCR Gold Buffer	5
25 mM $MgCl_2$	4
10 mM dNTPs	4
10 µM Forward primer	4
10 µM Reverse primer	4
100 ng DNA template	1
AmpliTaq Gold Polymerase (5U/µl)	0.25

(Denaturation – Annealing – Elongation) * 35 cycles

Table 7. Conditions used for PCR analysis.

PCR steps	Temperature, ºC	Time
Initial denaturation	95	5 min.
Denaturation	95	30 sec.
Annealing	50	30 sec.
Elongation	72	6 min.
Final elongation	72	10 min.

The primer pairs used were VSSC 1-4_F and VSSC 1-4_R, VSSC 1-4_F and VSSC 5405_R or VSSC 3417_F and VSSC 5405_R. Together with these samples a reamplification was also made with a PCR product from earlier PCR as template and the primers used were VSSC 3417_F and VSSC 5405_R.

New PCR were set up using the same primer pairs but in these reactions Taq DNA polymerase was used instead. The reagents and volumes for PCR can be seen in table 8. PCR programme used can be seen in table 2.

Table 8. Reagents used for PCR.

Reagents	Volumes, µl
H_2O	36.5
10x PCR Buffer	5
25 mM $MgCl_2$	3
10 mM dNTPs	1
10 µM Forward primer	1
10 µM Reverse primer	1
100 ng DNA template	1
Taq DNA polymerase (5U/µl)	0.4

PCR was repeated with Phusion as DNA polymerase and the primer pairs were the same as for Taq DNA polymerase. A reamplification was made with a PCR product from earlier PCR. The reagents for the PCR can be seen in table 1 and the PCR programme in table 2.

2.3. CYP

2.3.1. Primers

The third step of the experimental work was to amplify different cytochrome P450s (CYPs) that in other insects are known to be involved in metabolic resistance to pyrethroids.

All CYPs generated by PCR were analyzed by gel electrophoresis. The gel contained 1 % agarose in 1x TAE buffer with 5 µl GeneRuler 1kb DNA ladder from Fermentas as size marker.

Three different CYPs were amplified, called CYP $6B_2$14, CYP $6B_2$15 and CYP 9. The amounts of template DNA used were ≈ 400 ng.
The primer pairs used for PCR can be seen in table 9.
Primer sequences can be found in appendix 1.

Table 9. Primer pairs used to amplify different CYPs.

CYP	Forward primer	Reverse primer
CYP $6B_2$14	CYP $6B_2$14_F	CYP $6B_2$14_R
CYP $6B_2$15	CYP $6B_2$15_F	CYP $6B_2$15_R
CYP 9	CYP 9_F	CYP 9_R

2.3.2. PCR

The CYP genes were amplified by PCR and the reagents used are shown in table 1 and the PCR programme in table 10.

Table 10. PCR programme used.

PCR steps	Temperature, ºC	Time
Initial denaturation	98	30 sec.
Denaturation	98	30 sec.
Annealing	50	30 sec.
Elongation	72	30 sec.
Final elongation	72	10 min.

(Denaturation – Annealing – Elongation) * 30 cycles

The PCR for CYP $6B_2$14 and CYP $6B_2$15 were repeated, this time with 100 – 150 ng DNA as template. Reamplifications in duplicates for CYP $6B_2$14 and CYP $6B_2$14 were also performed. The DNA fragments of the expected size were cut out of the gel and extracted and purified with Pure Link™ Quick Gel Extraction Kit from Invitrogen according to the "Preparing and Dissolving the Gel Slice" and "Purification Procedure using Centrifugation" manual, with two exceptions in the elution step, the incubation time was 4 minutes instead of 1 minute and 30 µl H_2O was added instead of 50 µl. After extraction and purification, the DNA contents were measured with a NanoDrop instrument.

2.3.3. Ligation

Next step was ligation and the condition can be seen in table 11.

Table 11. Reagents used for ligation. The ratio between vector and insert was 1:3.

Reagents	Volume, µl
Ligation buffer	5
25 ng pBluescript SK+ T vector (3.0 kb)	1
11-17 ng insert	5-8
T4 DNA ligase (5U/µl)	1

The ligation mixtures were incubated at 16 ºC over night.

2.3.4. Transformation

The ligation mixtures were mixed together with "home" made competent cells and the transformation procedure was the same as for VSSC, but with two exceptions. The cells were heat-shocked for 2 min. instead of 30 sec., and 200 µl was spread on each plate instead of 50 and 150 µl. The transformations failed and new transformations were made, but this time the competent cells were heat-shocked for 1.5 min and 250 µl LB-medium was added instead of SOC-medium. To the plates, 200 µl of transformation culture were added. Next step was confirmation and it was performed as described earlier.

2.3.5. Plasmid preparation

A 10 µl aliquot of the transformation cultures was added to 5 ml LB + amp (100 µg/ml) and grown over night at 37 ºC. The following day the cultures were centrifuged at 10000 rpm for 10 min. at 4 ºC and the pellets were used for plasmid preparation. The kit that was used was Qiagen Plasmid extraction kit. The plasmid preparations were performed

according to the manual, with one exception, 30 µl MilliQ water was added instead of 50 µl in the elution step. Plasmids were quantified with a NanoDrop.

2.3.6. Colony PCR

From the same transformations, colony PCR was performed, where 10 reactions were prepared with one colony as template. The reagents needed for colony PCR can be seen in table 12 and the PCR programme in table 13.

Table 12. Reagents used for colony PCR.

Reagents	Volumes, µl
H_2O	18.25
10x PCR Buffer	2.5
25 mM $MgCl_2$	1.5
10 mM dNTPs	0.5
10 µM Forward primer	0.5
10 µM Reverse primer	0.5
Template	1 colony
Taq Polymerase (5U/µl)	0.2

Table 13. Colony PCR programme.

PCR steps	Temperature, ºC	Time
Initial denaturation	95	5 min.
Denaturation	95	30 sec.
Annealing	50	30 sec.
Elongation	72	30 sec.
Final elongation	72	10 min.

(Denaturation – Annealing – Elongation) * 30 cycles

Together with colony PCR, PCR was performed to check the plasmids that were purified. The PCR reagents and volumes was the same as the colony PCR, see tables 12 and 13., but 10 ng plasmid DNA as template instead of one colony. The primer pairs were CYP $6B_2$14_F and CYP $6B_2$14_R or CYP $6B_2$15_F and CYP $6B_2$15_R.

2.3.7. New ligation and transformation

The colony PCR did not results in any band on the gel so new ligations were made for CYP $6B_2$14 and CYP $6B_2$15 and the ligation conditions can be seen in table 14.

Table 14. Ligation reagents. Ratio 1:2 between vector and insert.

Reagents	Volume, µl
Ligation buffer	2
25 ng pBluescript SK+ T vector (3.0 kb)	2
11-17 ng insert	4-5
T4 DNA ligase (5U/µl)	1

MilliQ water was added to a final volume of 10 µl.

The ligation mixtures were used for transformation, where 5 µl each was added to 300 µl competent cells. The transformation procedure was the same as described earlier, but with a few changes. The competent cells were heat-shocked for 2 minutes and after LB was added to the cells, they were centrifuged at 2500 rpm for 5 minutes and 300 µl of the supernatant were discarded. This was done to concentrate the samples and 200 µl

were added to LB amp + (100 µg/ml), X-gal +, IPTG + plates that were incubated at 37 ºC over night. The transformations failed and new amplifications and ligations were made for CYP $6B_2$14 and CYP $6B_2$15. The PCR conditions can be seen in table 8. The DNA fragments that had migrated to the expected size were cut out of the gel and extracted and purified with Pure Link™ Quick Gel Extraction Kit from Invitrogen according to the "Preparing and Dissolving the Gel Slice" and "Purification Procedure using Centrifugation" manual, with two exceptions in the elution step, the incubation time was 2 minutes instead of 1 minute and 30 µl MilliQ water was added instead of 50 µl. After extraction and purification the amount of DNA fragments was measured with a NanoDrop instrument. The purified DNA fragments were used for ligation. The ligations were made in two different ratios between vector and insert DNA. The ratios were 1:1 and 1:5. The ligation condition can be seen in table 15 and table 16.

Table 15. Ligation condition with 1:1 ratio between vector and insert DNA.

Reagents	Volume, µl
T4 ligase buffer	2
25 ng pBluescript SK+ T vector (3.0 kb)	2
15 ng insert	2
T4 DNA ligase (5U/µl)	1
50 % PEG 4000 solution	2
H_2O	11

Table 16. Ligation condition with 1:5 ratio between vector and insert DNA.

Reagents	Volume, µl
T4 ligase buffer	2
25 ng pBluescript SK+ T vector (3.0 kb)	2
15 ng PCR product	8
T4 DNA ligase (5U/µl)	1
50 % PEG 4000 solution	2
H_2O	5

The ligation mixtures were incubated at 22 ºC for 1 hour followed by 65 ºC for 10 minutes. The ligation mixtures were analyzed with gel electrophoresis to see if the DNA fragments were correctly inserted into the plasmids.
Next step was transformation and the transformation kit that was used was "Subcloning Efficiency DH5α Competent cells" from Invitrogen and the transformations were made according to the manufacturer's manual. One exception from the manual was made, in step 8, 250 µl LB was added instead of 950 µl. One extra positive control was also made with Bluescript SK+ vector. Transformation failed and a new ligation was made with the same condition as in table 16, but incubation was at 16 ºC over night. Ligation mixture was used in transformation. Transformation failed and a new ligation was made with the same condition as described above. Transformation failed again. New amplifications were made with a sample that gave a good result earlier, the PCR was performed according to table 8 and table 10, but with a denaturation temperature of 95 ºC. The PCR product was run on a 1.5 % low temperature agarose gel and the DNA fragments were purified from the gel and sent for sequencing to Macrogen, Inc Korea. Sequencing was carried out using BigDyeTM terminator cycled sequencing technique and automatic sequencer 3730xl. tblastx at NCBI was used to perform database search

(Altschul et al., 1997). The sequence was also used in VecScreen to detect and remove any sequence left from the vector.

The PCR on CYP 9 did not give any product so two new PCR were made and the reagents and volumes for one of the two samples can be seen in table 8. The other sample had the same reagents, but the volume of $MgCl_2$ was changed to 5 µl instead of 3 µl. The PCR programme can be seen in table 17.

Table 17. Conditions used for PCR programme.

PCR steps	Temperature, ºC	Time
Initial denaturation	95	30 sec.
Denaturation	95	30 sec.
Annealing	45	30 sec.
Elongation	72	30 sec.
Final elongation	72	10 min.

3. RESULTS

3.1. VSSC, domain IV

The first PCR that was performed gave products containing fragments migrating at 1500 bp, which was the expected size (fig. 2).

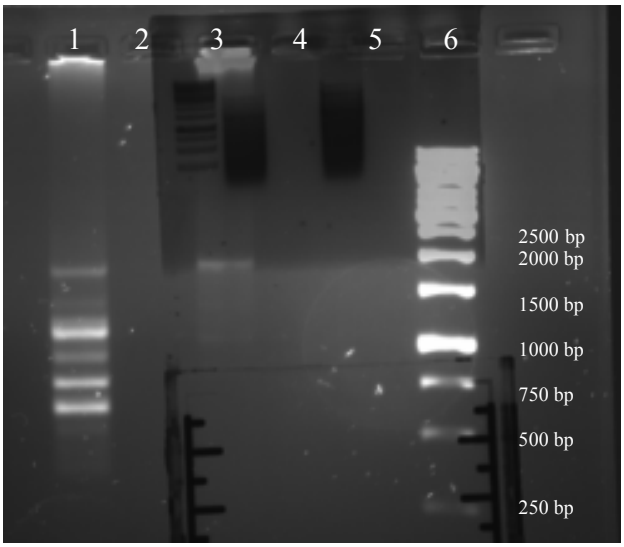

Figure 2. Four samples with genomic DNA as template and a size marker. Lane 1, primer pair VSSC 1-4_F and VSSC 1-4_R; Lane 2, negative control for sample in line 1; Lane 3, primer pair VSSC 3417_F and VSSC 5405_R; Lane 4, negative control for sample in lane 3; Lane 5, empty well; Lane 6, GeneRuler™ 1 kb DNA ladder.

The PCR used for sample in lane 3 in figure 2 was repeated in duplicates and the result for one of the samples, lane 4 in figure 3, gave a weak band at 1,500 bp and the other sample, lane 6 in figure 3, did not contain any DNA fragments. In the reamplifications of samples shown in lanes 1 and 3 in figure 2 there was only one that gave a strong band around 1500 bp (fig. 3).

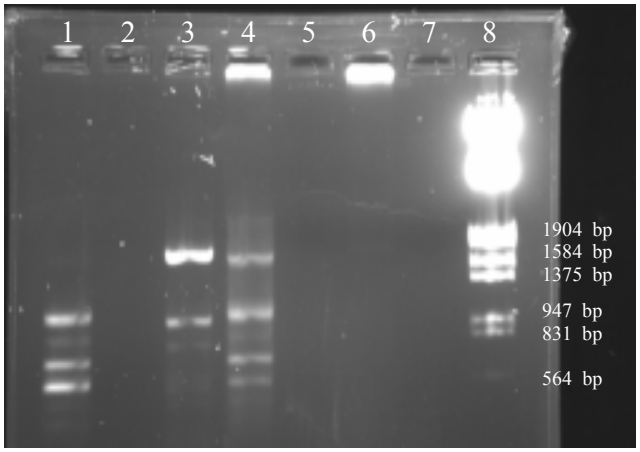

Figure 3. Seven samples and a size marker. Lane 1, reamplification of sample shown in lane 1 in figure 2; Lane 2, negative control; Lane 3, reamplification of sample shown in lane 3 in figure 2; Lane 4, genomic DNA as template, primer pair VSSC 3417_F and VSSC 5405_R; Lane 5, negative control; Lane 6, genomic DNA as template, primer pair VSSC 3417_F and VSSC 5405_R; Lane 7, negative control; Lane 8, λ DNA/*Eco*RI+*Hin*dIII ladder.

The previous analysis of PCR samples did not provide sufficient amount of DNA fragments so reamplifications of the sample shown in lane 3 in figure 2 was made. All four samples contained DNA fragments at 1,500 bp, see lane 1-4 in figure 4.

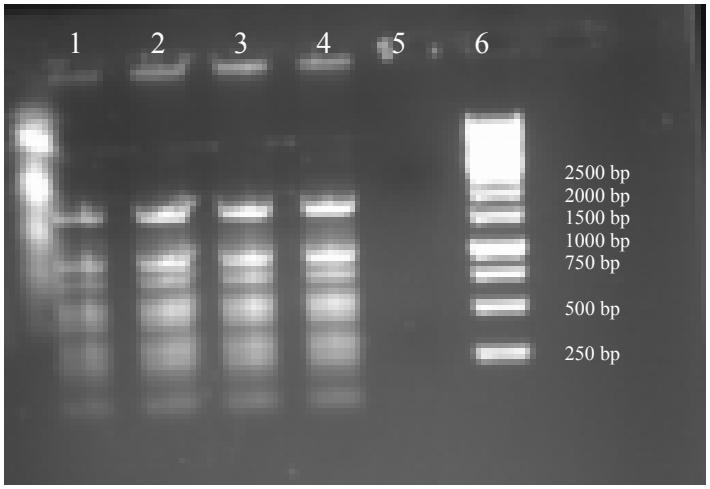

Figure 4. Four samples with reamplifications of sample shown in lane 3 in figure 1- Primer pair was VSSC 3417_F and VSSC 5405_R in lanes 1-4. Lane 5 contains negative control and lane 6 GeneRuler^{TM} 1 kb DNA ladder.

The ligation with the DNA fragments from the band around 1,500 bp shown in figure 4 failed. Transformation did not give any colonies on the plates. The new reamplification of the sample in lane 3 in figure 2 performed in duplicate gave both strong bands on the gel around 1,500 bp, lane 1-2 which was the expected size. The new amplifications with primer pair VSSC 3417_F and VSSC 5405_R with genomic DNA as template in duplicates gave both strong bands around 1,500 bp, line 3-4 (fig. 5) which was the expected size.

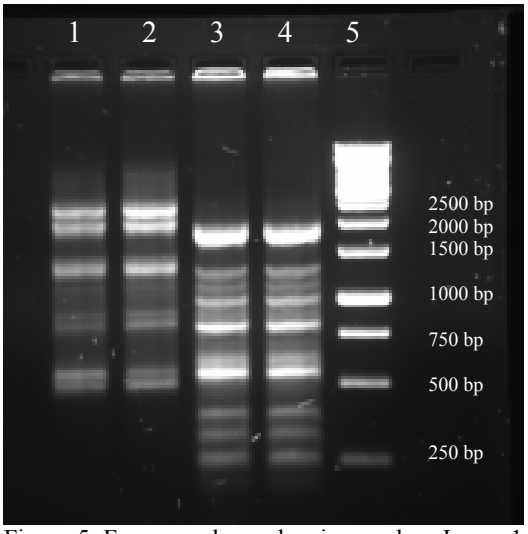

Figure 5. Four samples and a size marker. Lanes 1 and 2, genomic DNA as template and primer pair VSSC 3417_F and VSSC 5405_R; Lane 3 and 4, reamplification of sample shown in lane 3 in figure 2, primer pair VSSC 3417_F and VSSC 5405_R; Lane 5, GeneRuler^{TM} 1 kb DNA ladder.

The ligation with DNA fragments around 1,500 bp purified from the gel shown in figure 5 was successful. Analysis of the plasmid with gel electrophoresis showed that the plasmid contained the inserted DNA fragment. Transformation failed three times. The new amplification with primer pair VSSC 3417_F and VSSC 5405_R gave products around 1,500 bp and can be seen as a bright band in lane 3 (fig. 6) and the sample was used in ligation.

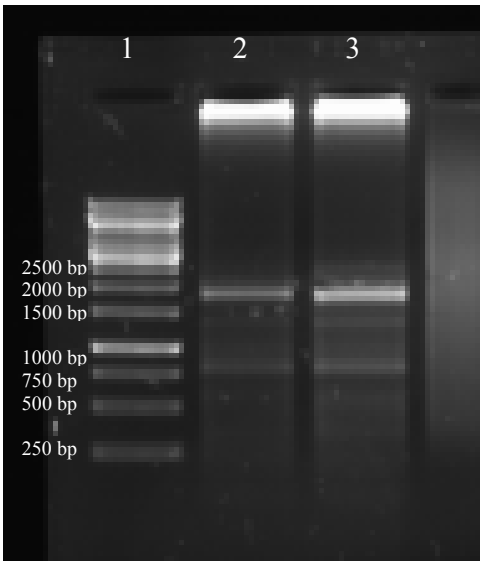

Figure 6. Two samples with primer pair VSSC 3417_F and VSSC 5405_R and genomic DNA as template can be seen in lane 2 and 3. Lane 1 contains GeneRuler^{TM} 1 kb DNA ladder.

Ligation and transformation was successful. The plate with 50 µl transformation culture added had four white colonies and two blue colonies (fig. 7), and the plate with 150 µl transformation culture added had 17 white colonies and 13 blue colonies (fig. 8).

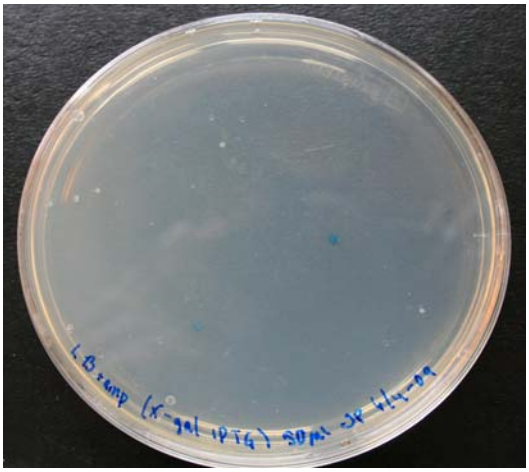

Figure 7. Plate with 50 µl transformation mixture added. On the plate four white colonies and two blue colonies can be seen.

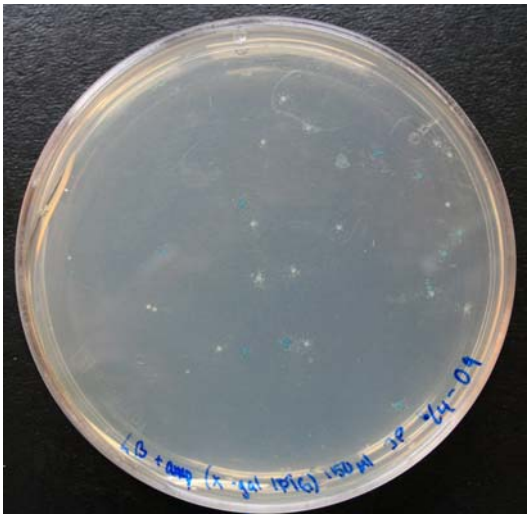

Figure 8. Plate with 150 µl transformation mixture added. On the plate 17 white colonies and 13 blue colonies can be observed.

Confirmation of colonies from transformation did not show any band upon gel analysis suggestion failure of DreamTaq DNA polymerase. Plasmid preparation of colonies from transformation was successful and material was sent for sequencing. tblastx search showed that the sequence probably encodes domain IV of VSSC, e-value was 2e-172 for the sequence encoding a voltage-sensitive sodium channel in *Drosophila melanogaster*, 1e-150 for *Musca domestica* and 1e-149 for *Aedes aegypti*. The sequence for domain IV of VSSC gene and the tblastx search results can be seen in appendix 2.

3.2. VSSC

Two of all PCR conditions tested to obtain the full-length VSSC provided long DNA fragments. DNA fragment around 3,500-4,000 bp can be seen in lane 2 and 3 in figure 9.

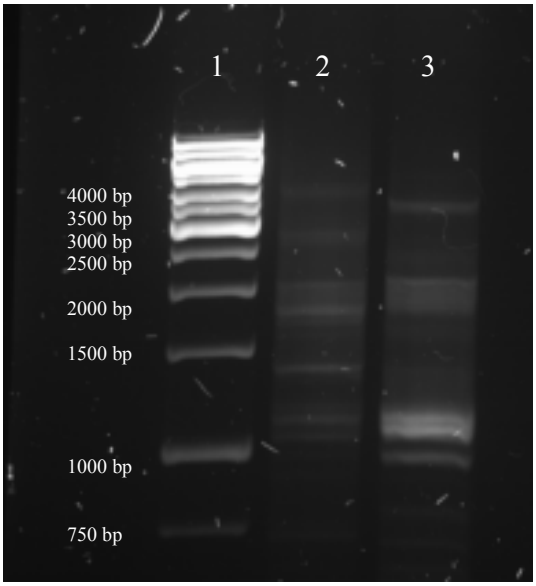

Figure 9. Two samples with genomic DNA as template and a size marker. Lane 1, GeneRuler™ 1 kb DNA ladder; Lane 2, primer pair VSSC 1-4_F and VSSC 1-4_R; Lane 3, primer pair VSSC 1-4_F and VSSC 5405_R.

The results from another PCR gave a weak band on the gel around 6000 bp in two of the samples. The two bands can be seen in lane 4 and 5 in figure 10. The PCR gave a lot of product of different size but no band around 1,500 bp, which was expected for samples in lane 4-5 in figure 10.

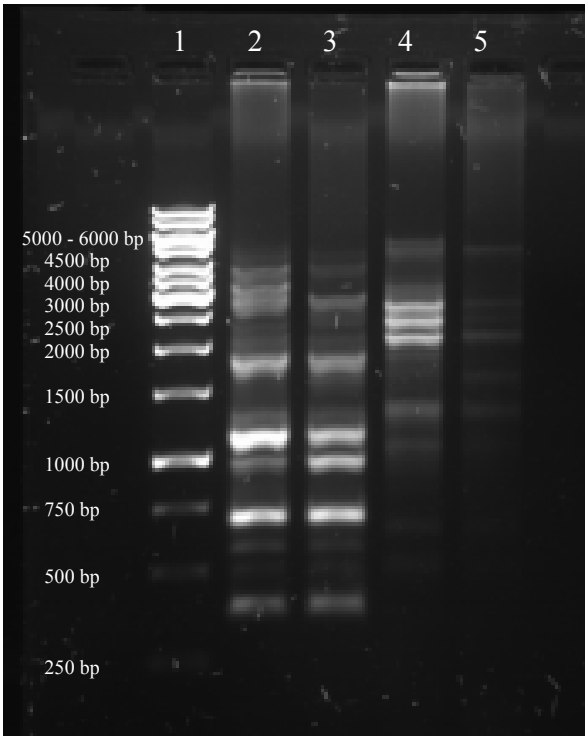

Figure 10. Four samples and a size marker. Lane 1, GeneRuler^{TM} 1 kb DNA ladder; Lane 2, genomic DNA from one of the two DNA extracts as template, primer pair VSSC 1-4_F and VSSC 5405_R; Lane 3, genomic DNA from the other of the two DNA extracts as template, primer pair VSSC 1-4_F and VSSC 5405_R; Lane 4, same template as in lane 2, primer pair VSSC 3417_F and VSSC 5405_R; Lane 5, same template as in lane 3, primer pair VSSC 1-4_F and VSSC 5405_R.

3.3. CYP

By amplifying and studying expression levels of the CYPs in pollen beetles it is possible to observe if there are any differences in expression between resistant and susceptible inserts.

Expected size of CYP fragmnets was around 500 bp. First PCR that was performed yielded DNA fragments at the expected 500 bp size for CYP $6B_2$14 and CYP $6B_2$15. The CYP 9 reaction did not yield any DNA fragments. The bands on the gel at 500 bp were very weak in lane 5 for CYP $6B_2$14 and can be hard to see (Fig.11) and a weak band around 500 bp can be seen in lane 6 for CYP $6B_2$15 in figure 11.

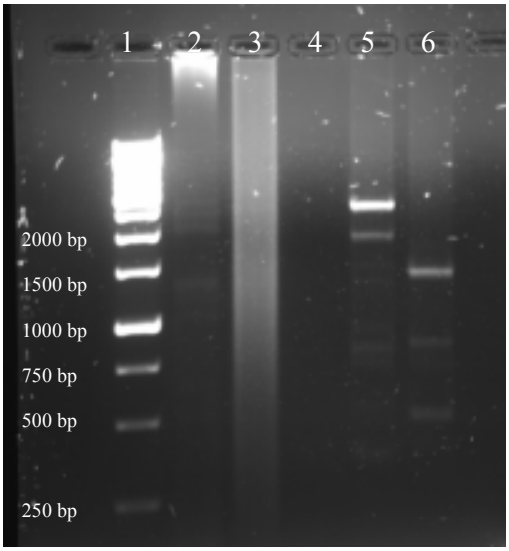

Figure 11. Three samples with genomic DNA as template and a size marker. Lane 1, GeneRuler™ 1 kb DNA ladder; Lane 2, not relevant in this experiment; Lane 3 not relevant in this experiment; Lane 4, CYP 9, Lane 5, CYP $6B_2$14; Lane 6, CYP $6B_2$15.

Reamplification of the samples in lane 5 and 6 in figure 11 gave both strong bands around 500 bp and the repeated PCR gave a weak band at 500 bp (fig. 12)

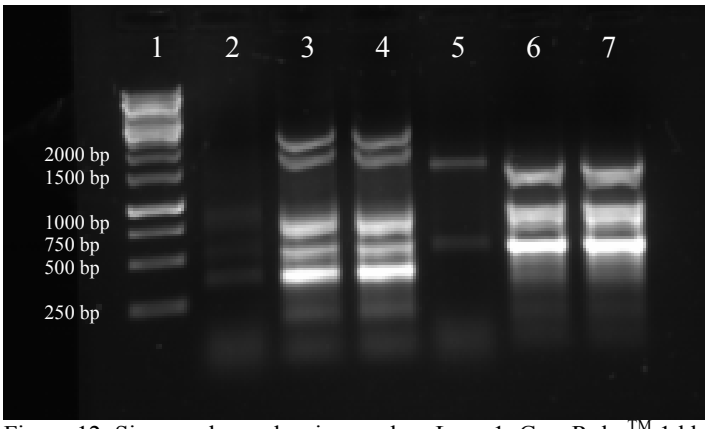

Figure 12. Six samples and a size marker. Lane 1, GeneRuler™ 1 kb DNA ladder; Lane 2, CYP $6B_2$14, genomic DNA as template; Lanes 3 and 4, CYP $6B_2$14, reamplification of sample shown in lane 5 in figure 11; Lane 5, CYP $6B_2$15, genomic DNA as template; Lanes 6 and 7, CYP $6B_2$15, reamplification of sample shown in lane 6 in figure 11.

Ligation and transformation failed once. The second transformation gave 50 colonies on the plate for CYP $6B_2$14, while the CYP $6B_2$15 reaction produced 30 colonies. The positive control showed 35 blue colonies and 40 white colonies. Gel analysis of the plasmid preparations and colony PCR analysis of the colonies were negative. Accordingly, the new ligation and transformation failed.

The new amplification of CYP $6B_2$14 and CYP $6B_2$15 did not give any DNA fragments around 500 bp. The reamplification of CYP $6B_2$14 and CYP $6B_2$15 with purified DNA

fragment from the gel as template gave a strong band around 500 bp for both CYPs (fig. 13).

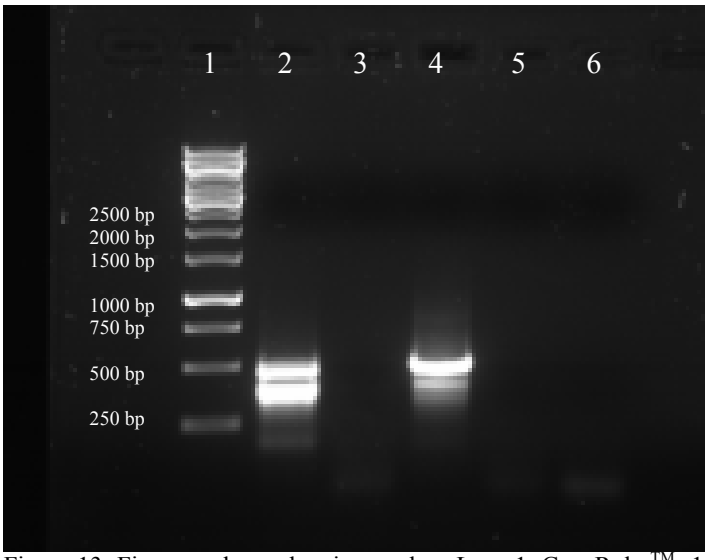

Figure 13. Five samples and a size marker. Lane 1, GeneRuler™ 1 kb DNA ladder ; Lane 2, CYP $6B_2$14, gel purified DNA fragments as template; Lane 3, CYP $6B_2$14, genomic DNA as template; Lane 4, CYP $6B_2$15, gel purified DNA fragments as template; Lane 5, CYP $6B_2$15, genomic DNA as template; Lane 6, negative control.

The reamplification of the reamplified sample shown in lane 2 and 4 in figure 13 in duplicates gave all bands around 500 bp on the gel, see figure 14.

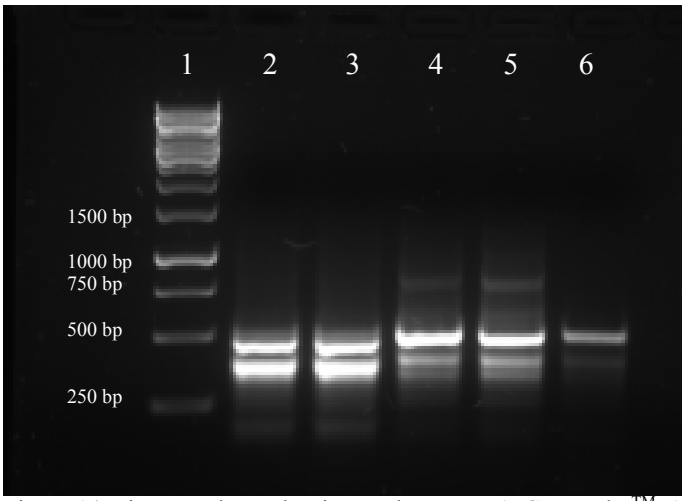

Figure 14. Five samples and a size marker. Lane 1, GeneRuler™ 1 kb DNA ladder; Lanes 2 and 3, CYP $6B_2$14, reamplifications of sample shown in lane 2 in figure 13; Lane 4 and 5, CYP $6B_2$15, reamplifications of sample shown in lane 4 in figure 13; Lane 6, negative control.

Ligation and transformation with the DNA fragments around 500 bp that were purified from the gel failed and the new ligation and transformation also failed. Instead of sending plasmids with the inserted DNA fragment for sequencing, the product from the reamplification of samples shown in lanes 2 and 5 in figure 14 was sent for sequencing.

tblastx search of the sequence for CYP $6B_2$15 gave e-value of 4e-82 for CYP 4G29 in *Leptinotarsa decemlineata*, 1e-80 for CYP 4G14 in *Tribolium castaneum* and 4e-78 for CYP 4G16 in *Anopheles gambiae*. The conclusion from the search is that the fragment obtained after amplification with primers for CYP $6B_2$15 probably encodes CYP and no other gene. The sequencing of CYP $6B_2$14 gave no good results but the cloning of CYP $6B_2$15 was successful and the sequence can together with tblastx search results be seen in appendix 2.

4. DISCUSSION

During the experimental work there were repeatedly problems with ligations and transformations. The condition for ligation was changed several times, both when it comes to incubation time and temperature. The most used method keeps the ligation mixture at 16 ºC over night and most of the ligations were performed in that way. Transformation with these ligation mixtures did not, apart from two transformations, one in the experimental work with VSSC, domain IV and one with CYPs, give any colonies on the plates. Another method to get the insert into the plasmid was to incubate the mixture at 22 ºC for one hour followed by ten minutes in 65 ºC. This ligation reaction was analyzed with gel electrophoresis and it showed that the insert was present in the plasmid, but transformation did not result in any colonies, which was unexpected. Reasons to this are unclear because the transformation procedure was performed according to the manufacturer's manual. One reason can be that the insertion was incomplete so when the cells took up the plasmid it was regarded as foreign and degraded. The insertion could have been incomplete since the insert may not have contained enough A-overhang at the 3'-end and could accordingly not ligate properly into the vector. It could also have been a damage caused to the insert due to prolonged exposure to UV. Another reason can be that the ligase buffer was old and the amount of ATP was decreased. The reaction when the DNA fragment is inserted into the plasmid requires a lot of energy and if the buffer has an insufficient amount of ATP, the reaction can not be completed.
The enzyme used for amplifying the CYPs was Taq DNA polymerase. This enzyme does not have proof-reading capacity and gives the DNA fragments sticky ends with an A-overhang but maybe the enzyme did not add enough A-nucleotides in the ends and the insert, as mentioned above could not ligate into the vector.

The positive control (vector without insert) in the transformation that gave colonies on the plates for CYPs had both white and blue colonies. It should only have blue colonies. The blue and white colonies were located at different places on the plate and not only at the same part of the plate so there should not have been a problem with the spreading of X-gal on the plates. Probably cells were left on the spreader from one of the other colonies when the negative control subsequently was spread on the plate.

One strategy to obtain a gene sequence is to isolate mRNA and from that synthesize cDNA that can be used in PCR amplification of the gene of interest. However, this exam project did not use this strategy, because earlier experiments conducted with mRNA for cloning of domain I-III of the VSSC gene from pollen beetles had failed.

One of the goals with the exam project was to look for mutations in domain IV of the VSSC gene. In order to study such mutations, amplification and sequencing of different pollen beetle individuals are needed for comparative analysis. However, time constraints

did not enable such allelic studies, which means that the possible existence of relevant mutations remains to be established.

The goal with the experimental work with CYPs was to obtain partial DNA sequences encoding the protein and if possible use quantitative PCR (or other transcript level assays) to estimate the relative expression levels of the CYPs in resistant and susceptible individuals. Lack of time did not allow expression analysis of the CYPs transcripts.

What is left to do with respect to VSSC gene is to amplify the full-length cDNA encoding the protein and use *Xenopus* oocytes as vector to study the potential role of mutant alleles in resistance for pyrethroids.

5. REFERENCES

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Appendix 1

Primer sequences (5´- 3´) used for amplification of VSSC:
VSSC 1-4_F ATG ACA GAA GAT TCC GAC TCG ATA
VSSC 1-4_R TCA GAC ATC CGC CGT GCG CG
VSSC 3417_F CCT CAT CAA GAA GGG GAT CA
VSSC 5405_R GCT GAC GTC GAC ATC TGA AA

Primer sequences (5´- 3´) used for amplification of CYPs:
CYP6$B_2$14_F: GAG GTI GAY ACI TTC ATG TTC GAR GGI CAC GAY AC
CYP6B214_R: CTG ICC GAT RCA GTT CGB GGI CCI GCS IWG AAB GG
CYP6$B_2$15_F: TTY ATG TTY RAR GGH CAY GAY AC
CYP6$B_2$15_R: ATR CAR TTI CKI GGD CCN GC
CYP9_F : TAC GAT YIG CIR WIA AYC CYG A
CYP9_R : CCI AKR CAR TTI CKI GGI CC

Explanation of the letters in the CYP primer sequences.
V A+C+G
N A+C+G+T
D A+T+G
B T+C+G
H A+T+C
W A+T
S C+G
K T+G
M A+C
Y C+T
R A+G

Appendix 2

Nucleotide sequence encoding for VSSC, domain four:

GGAACAGCCTAACGTTCTTCAACATCTGTCGAGTGGAATCTTCTGTCATGTTCATCTTTGCCATTTTCGGCATGT
CGTTCTTCATGCACGTGAAGGACAAGAGCGGATTTAACGACGTCTACAACTTCAAAACCTTTGCCCAGAGCAT
GATCCTGCTATTTCAGATGTCGACGTCGGCCGGGTGGGATGGTGTTCTGGACGCCATTATCAATGAGGAAGCAT
GCGAGCTGCCCGACAACGACAAAGGAGATCCGGGGAATTGTGGGTGAGCGACAGTTGGAATCACGTTTTTCGT
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CACGGAGGACGTGCAAGAGGGTCTAACCGACGACGACTACGACATGTACTATGAGATCTGGCAGCAATTCGAT
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TCCACAAACCGAACAAGTACAAGATAATATCCATGGACATCACCATCTGTCGCGGTGACCTCATGTACTGCGT
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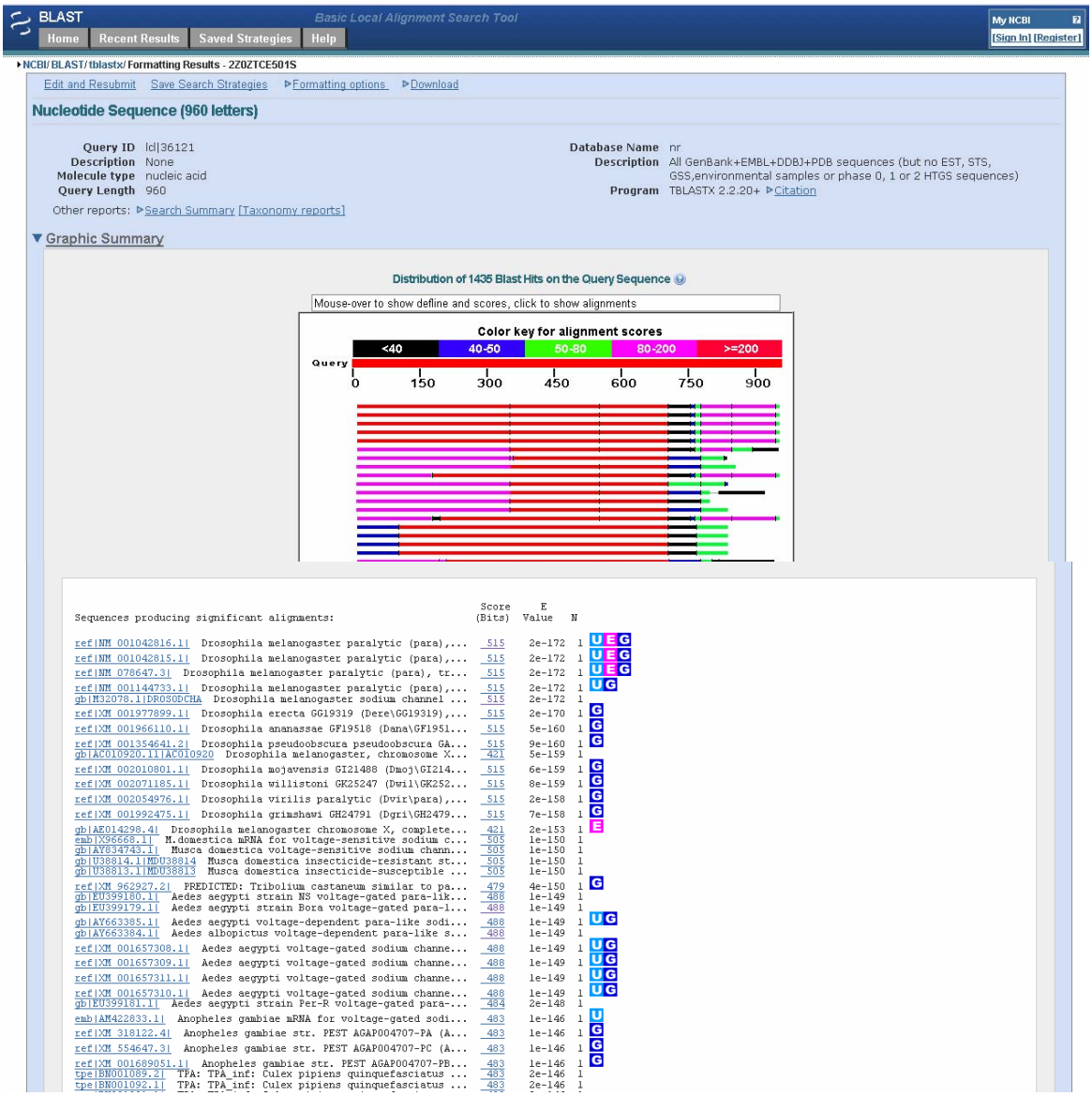
GATGTATGA

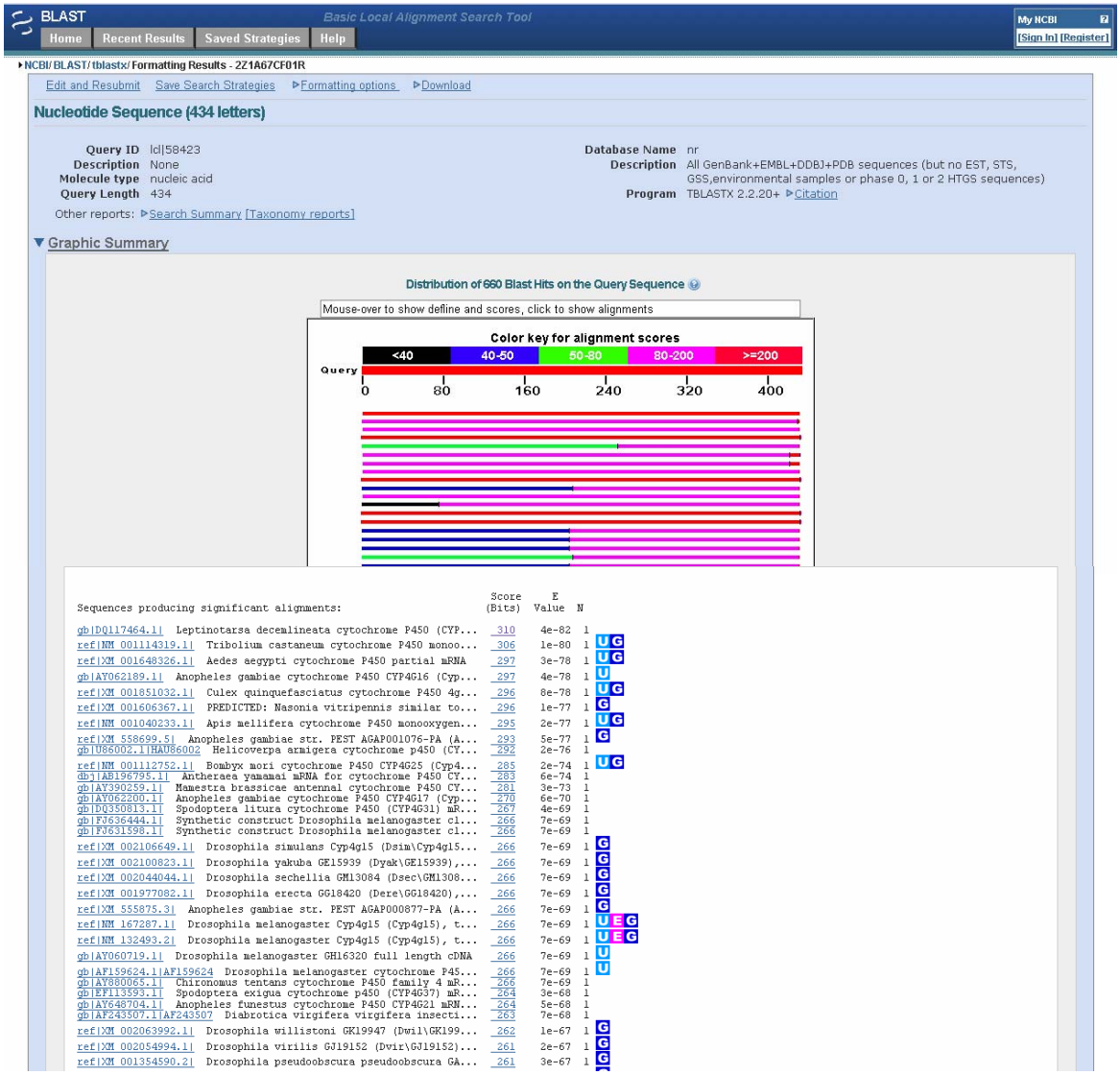
Results from tblastx search for VSSC, domain IV



Nucleotide sequence for CYP6B$_2$15:

TTTATGTTCGAAGGCCATGACACCACCGCTGCCGGTAGCAGTTTCTTCCTCTCCATGATGGGTATCCATCCACA
CATCCAACAGAAGGTCGTGCAAGAATTGCACGAAATCTTTGGTGATTCCGACAGACCAGCAACCTTCGCCGAC
ACCTTGGAAATGAAATATTTGGAAAGATGTTTGATGGAGACTTTGAGAATGTACCCACCAGTACCCATTATTGC
CAGACAAATCAACCAGGACATTAAATTGGCTTCCTCCAACCTGACCGTACCCGCTGGTGCTACTGTGGTAATCG
GTACCTTCAAGATCCACCGTCAAGAAAAGATCTACCCCAACCCCGAAAAATTCGACCCTGACAACTTCTTACC
AGAAAAATCCGCAAACAGGCATTACTATGCATTTATTCCATTCAGCGCAGGTCCCAGCAATTGCAT

Results from tblastx search for CYP6B$_2$15



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