Genotype Analysis and Studies of Pyrethroid Resistance of the Oilseed Rape (*Brassica napus*) Insect Pest - Pollen Beetle (*Meligethes aeneus*)

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2007

Acta Universitatis Agriculturae Sueciae
2007: 11

ISSN 1652-6880
ISBN 978-91-576-7310-7
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Я в серці маю те, що не вмирає

Л. Українка

Светлой памяти моей дорогой бабушки Маши посвящается

To the memory of my dear granny Masha

Abstract

Kazachkova, N. I. 2007. Genotype analysis and studies of pyrethroid resistance of the oilseed rape (*Brassica napus*) insect pest - pollen beetle (*Meligethes aeneus*).
Doctor's dissertation

ISSN: 1652-6880, ISBN: 978-91-576-7310-7

Oilseed *Brassicas* are vulnerable to attack from many insects and pathogens, calling for an extensive use of pesticides to secure crop yields; this can cause increased resistance in pests. During recent years, one of the main oilseed insect pests—the pollen beetle (*Meligethes aeneus*), resistant to pyrethroid insecticides—has emerged in southern Sweden. This, because of its frequency and geographic range, provides an excellent source of material for analysis of genetic variation among pollen beetle populations, for study of insecticide resistance and for testing new sources of plant protection.

For genotyping pollen beetles, we modified the amplified fragment length polymorphism (AFLP) technique (chosen because it does not depend on prior sequence information when no genome information is available for pollen beetles), and applied it to 133 Swedish populations (susceptible and resistant), collected in different years, and to 14 European populations. AMOVA showed high levels of genetic variation within populations and gene flow among populations, and no evidence of expected regional and resistance-susceptibility to insecticide diversification (clear diversification by time and generations instead) for Swedish populations. European populations showed a clear pattern of regional diversification and a low level of gene flow.

To identify possible point mutations associated with pollen beetles resistance to pyrethroids, the primary target sites for pyrethroids—voltage-sensitive sodium channels (VSSC) and metabolic resistance sites—Cytochrome P450, were studied using RT-PCR in resistant and susceptible insects. Two CYP450 partial cDNAs and four cDNA fragments composing VSSC domains I and II were amplified (using primers designed for homologue sequences) and sequenced showing point mutations, which can confer pyrethroid resistance.

Key words: AFLP, genotyping, insect pest, genetic variation, insecticide resistance, VSSC, pyrethroid, CYP450.

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Абстракт

Казачкова Н.І. 2007. Аналіз генотипу та вивчення піретроїдної стійкості комахи-шкідника рапсу (*Brassica napus*) – рапсового пилкоїда (*Meligethes aeneus*).
Докторська дисертація
ISSN: 1652-6880, ISBN: 978-91-576-7310-7

Рослини роду *Brassica* дуже чутливі до впливу різних патогенів, що зумовлює надмірне використання різноманітних інсектицидів заради збереження врожаю, а це призводить до зростання стійкості шкідників до інсектицидів. На протязі останніх років один з головних шкідників рапсу – рапсовий пилкоїд (*Meligethes aeneus*), стійкий до інсектицидів класу пиретроїди, з'явився на півдні Швеції. Ця проблема, завдяки своїй частоті та поширеності, є чудовим джерелом матеріалу для аналізу генетичної варіації між популяціями рапсового пилкоїда, вивчення його стійкості до інсектицидів та випробування нових джерел захисту рослин.

Для аналізу генотипу рапсового пилкоїда у дисертаційній роботі модифікували метод «поліморфізм довжини ампліфікованих фрагментів, ПДАФ» (обраний тому, що він не залежить від попередньої інформації з послідовності ДНК в той час, коли немає ніякої інформації щодо геному рапсового пилкоїда) та застосували його до 133 шведських популяцій рапсового пилкоїда (чутливих та стійких до інсектицидів), зібраних на протязі декількох років, та 14 європейських популяцій. АМОВА-аналіз виявив високий рівень генетичної варіації всередені популяцій та високий рівень потоку генів між популяціями й ніякого доказу очікуваного регіонального та стійкісно-чутливого до інсектицидів поділу (замість цього – чіткий поділ за часом та поколіннями) для шведських популяцій. В той же час, європейські популяції виявили чіткий поділ за регіонами та низький рівень потоку генів між популяціями.

Щоб виявити можливі крапкові мутації, пов'язані зі стійкістю рапсового пилкоїда до пиретроїдів, первинні сайти-мішені пиретроїдів – натрієві канали та сайти метаболічної стійкості – цитохроми P450 було вивчено методом RT-PCR для стійких та чутливих до інсектицидів комах. Два CYP450 гена та чотири кДНК-фрагменти натрієвих каналів було ампліфіковано, використовуючи праймери до гомологічних нуклеотидних послідовностей, та сиквенсовано. Виявлено крапкові мутації, які можуть бутти пов'язані зі стійкістю рапсового пилкоїда до пиретроїдів.

Ключові слова: ПДАФ, CYP450, аналіз генотипу, комаха-шкідник, генна варіація, стійкість до інсектицидів, натрієві канали.

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Appendices

Papers I-V

This thesis is based on the following papers, referred to by their roman numerals.

- **I.** Kazachkova, N., Fahleson, J., & Meijer, J. (2004) Establishment of the Amplified Fragment Length Polymorphism (AFLP) technique for genotyping of pollen beetle (*Meligethes aeneus*) - a noxious insect pest on oilseed rape (*Brassica napus*). *Molecular Biology Reports.* 31: 37-42.
- **II.** Kazachkova, N., Meijer, J. & Ekbom, B. (2006). Genetic diversity in pollen beetles (*Meligethes aeneus*) in Sweden: role of spatial, temporal and insecticide resistance factors. (Submitted).
- **III.** Kazachkova, N., Meijer, J. & Ekbom, B. (2006). Genetic diversity in European pollen beetle (*Meligethes aeneus*) populations. (Submitted).
- **IV.** Kazachkova, N., Meijer, J. & Ekbom, B. (2006). Analysis of common insecticide resistance gene (Voltage sensitive sodium channel gene and CYP450 genes) in pollen beetles (*Meligethes aeneus*): point mutations associated with resistance to pyrethroids. (Manuscript).

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Additional publication:

- Åhman, I., Kazachkova, N., Kamnert, I., Hagberg, P., Dayteg, C., Eklund, M., Meijer, J. & Ekbom, B. (2006) Characterisation of transgenic oilseed rape expressing pea lectin in anthers for improved resistance to pollen beetle. (*Euphytica*, 151: 321-330).

Abbreviations

List for abbreviations used in the text:

AMOVA - Analysis of Molecular Variance
AChE – acetyl cholinesterase
AFLP - amplified fragment length polymorphism
CNS - central nervous system
cpDNA – chloroplast DNA
CYP450 - cytochrome P450
DDT - dichlorodiphenyltrichloroethane
F_{ST} – the fixation index
GABA - γ-amino butyric acid
GSPs - gene-specific primers
GST - glutathione S-transferase
kdr - knockdown resistance
mtDNA – mitochondrial DNA
nDNA – nuclear DNA
N_m - the number of migrants per generation
OPs – organophosphorus
RACE-PCR - rapid amplification of cDNA-ends polymerase chain reaction
RAPD - randomly amplified polymorphic DNA
RFLP - restriction fragment length polymorphisms
RT-PCR - reverse transcription polymerase chain reaction
SNPs - single nucleotide polymorphisms
SSRs - simple sequence repeats
STRs - short tandem repeats
VNTR - variable number of tandem repeats
VSSC - voltage-sensitive sodium channel

Introduction

Crop plants are vulnerable to attack from a number of pathogens and insect pests, calling for extensive use of pesticides to secure crop yields. Although they provide protection, insecticides also have a number of negative effects, and with time the pests may become resistant. The pollen beetle (*Meligethes aeneus,* Coleoptera: Nitidulidae) is a pest with a great economic impact on oilseed *Brassicas*. In the absence of a control crop, losses can reach 70 %. During recent years, pollen beetles resistant to pyrethroid insecticides have emerged in some areas of southern Sweden. Because of its frequency and geographic range, the problem provides an excellent source of material for analysing genetic variation among pollen beetle populations, for studying insecticide resistance and for testing new sources of plant protection. Thus, oilseed rape (*Brassica napus*) plants can be protected against pollen beetles by insecticide spraying, by activation of their own defence system and by use of other plants' natural resources.

Pollen beetles' adults and larvae, by feeding on buds and flowers from spring to late summer, damage plants, thus preventing seed development. In consequence, insecticides are commonly applied to control pest infestation; to secure crop yields, several applications usually are necessary throughout the cultivation season. Therefore, it is of great importance to analyse the genetic status of different pollen beetle populations, to understand their variability, especially with respect to insecticide resistance.

Oilseed rape (*Brassica napus*) in worldwide agriculture

Oilseed Rape (*Brassica napus*), also known as Rapeseed, Rape, Rapa, Rapaseed and Canola, is a bright yellow-flowering member of the family Brassicaceae. It is an annual (spring) or biennial (winter) plant, when sown late and flowering the following spring. A plant flowers in late spring to fall, producing fruits in early summer to fall (Duke, 1983). Rapeseed is very widely cultivated throughout the world for the production of animal feed (due to its high lipid and medium protein content)., vegetable oil for human consumption, and biodiesel. Leading producers include the European Union, Canada, the United States, Australia, China and India. According to the United States Department of Agriculture, rapeseed was the third leading source of vegetable oil in the world in 2000, after soybean and oil palm, and also the world's second leading source of protein meal, although it reached only one-fifth of the production of the leading soybean meal. Rapeseed is the most important oil seed crop in Western Europe. World production is growing rapidly; FAO reported that 36 million tonnes of rapeseed was produced in the 2003–2004 season, and 46 million tonnes in 2004–2005. A reason for the increase is the manufacture of biodiesel for powering motor vehicles. Rapeseed oil has also

a potential market in detergent lubrication oils, emulsifying agents, resins, and waxes.

Insect pests and pathogens

Several factors cause disease or damage in rape: fungi, viruses, bacteria and major pests—insects. Control includes the use of chemicals, crop rotation, seed treatments and the use of transgenic resistant plants (Rimmer & Buchwaldt, 1995).

One of the most important limiting factors for production of *Brassica* oilseeds is the complex of insect pests associated with these plants. The insect pests of *Brassica* oilseeds are primarily crucifer specialists. Most economically important herbivores–crucifer specialists of *Brassica* oilseeds use a group of secondary compounds, the glucosinolates, as attractants, feeding stimuli or oviposition stimuli, while for non-crucifer specialists the same compounds act as feeding detergents or toxins. Different groups of insect pests cause damage to seedlings, pods or seeds (Ekbom, 1995; Ekbom & Borg, 1996).

The pollen beetle attacks the buds and flowers of the plant from spring to late summer, causing severe damage. Flea beetles (Phyllotreta spp.) attack seedlings, and these, along with Diamondback moth (*Plutella xylostella*); attack from the bud stage until maturity. Aphids (e.g. *Brevicoryne brassicae*) damage seedlings, leaves, stems. Nematodes (e.g. *Heterodera schactii*) damage all parts of a plant. The pod midge (*Dasineura brassicae*) damages pods.

Pollen beetle (*Meligethes aeneus*) as a serious pest of *Brassica napus*

The pollen beetle (*Meligethes aeneus*, *Coleoptera: Nitidulidae*) is a pest of great economic importance, attacking oilseed *Brassicas.* It feeds on pollen from a large number of plant families, especially the Brassicaceae. Adults and larvae feed on buds and flowers of the plants from spring to late summer, and cause extensive damage to plants.

Description and biology

Pollen beetles have long been the most important insect pest of oilseed rape in Scandinavia (Nilsson, 1987; Hokkanen, 1989). They have been less important in the rest of Europe, but have become more significant with the higher proportion of spring crops grown in recent years (Ekbom, 1995).

Pollen beetle adults are small and black, 2–2.5 mm in size. The upper part of the body is punctured in a regular manner and has a metallic lustre. Eggs are elongated, with a glassy appearance verging on the milky-white. The larva is 4 mm long. It is elongated, much flattened, yellow-white, covered with light brown dots; the head and legs are brown; it has two instars.

Life cycle

Pollen beetles have one generation per year. The adult overwinters in the ground. It emerges when the temperature reaches 11 °C, and begins to feed on the pollen and nectar of various plants, preferably on *Brassicas*, when the temperature reaches 15 °C (late March–May). The female bites a small hole at the base of a flower bud and deposits eggs there. The eggs hatch within 4–9 days, and the larvae remain in the flower bud, feeding on pollen until the flower opens. When population levels are high, larvae will also attack the stem of the plant. After feeding for 25–30 days the fully-grown larvae drop to the soil, where they pupate in earthen cells. Young beetles emerge 2–3 weeks later. The new generation of adults appears between the end of June and the end of July (Fig. 1; Ekbom & Borg, 1996).

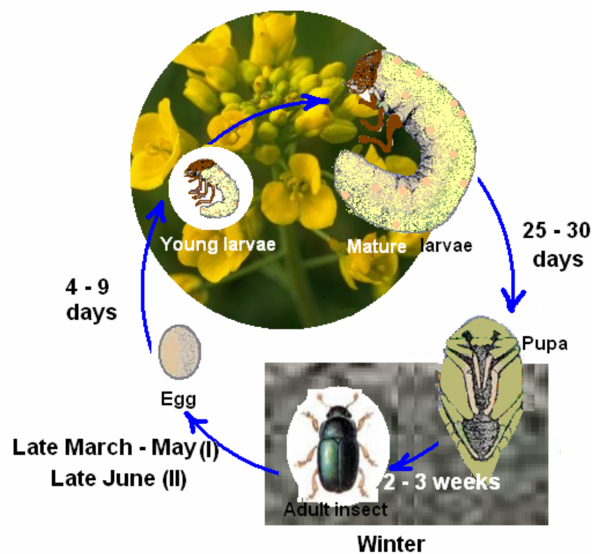


Fig. 1. The life cycle of pollen beetles

Damage and control

When pollen beetle numbers are low, damage may be confined to bud and flower abortion, but as plants may abort up to 50 or 60% of their buds; without insect attack, small or moderate loss of buds and flowers due to insect damage will not necessarily severely affect yield (Williams & Free, 1979). Development of more side shoots may compensate for serious damage to the main shoot. Damaged plants will have an extended flowering period, and maturation will be uneven and delayed. Fewer pods per stalk and blind stalks will also occur.

Chemical control of pollen beetles is often necessary to ensure yields. Economic thresholds for both winter and spring varieties are in use in Scandinavia (Nilsson,

1987), and the U.K. (Lane & Walters, 1993). Winter varieties have often come so far in their development that they can tolerate more beetles than spring varieties, and the threshold also increases as the plants mature. Pyrethroids are the most commonly used chemicals. A potential for alternative control measures does exist (Ekbom, 1995). Several natural enemies (parasitoids) are common, and cultivation methods, such as avoidance of ploughing, can increase parasitoid numbers (Nilsson, 1985). An insect-pathogenic protozoan (*Nosema meligethi*). and the fungus *Beuveria bassiana*, have been studied as potential control methods. The impact of natural enemies is, however, very marginal. This probably is a consequence of the intensive use of insecticides against pollen beetles, which can also destroy many potential biological control agents. One of the most promising alternatives to chemical control, is the possibility of developing crop varieties with resistance or tolerance to insect pests where a transgenic lectin has proven to be effetice to pollen beetle larvae (Melander et al. 2003). Rational use of chemicals within integrated pest management systems can result in effective control of pests and minimize harmful insecticide effects to the environment (Rimmer & Buchwaldt, 1995; Ekbom, 1995).

Genetic diversity and differentiation

Variation is present in natural populations of all organisms. The observed variation, the phenotype, can be reflected in genetic variation, the genotype. However, the genotype interacts with the environment to produce the phenotype. Genetic variation, the raw material upon which natural selection acts, is continuously being created by mutation and at the same time eroded by selection and drift. If genetic variation is present within a species, any alterations in selective pressures due to environmental changes will allow certain individuals to survive and reproduce.

Genetic variation within a species has three components: genetic diversity (the amount of genetic variation); genetic differentiation (the distribution of genetic variation among populations); and genetic distance (the amount of genetic variation between pairs of populations). Molecular markers are used to describe and estimate genetic variation (Lowe *et al.,* 2004; Felsenstein, 1997).

Factors influencing genetic diversity and differentiation

Organism and its environment considerations

Population size

Random changes in allele frequency are related to population size: the smaller the population, the more likely chance events are to change allele frequencies. This random process of allele frequency change is called genetic drift, and is a result of random sampling of gametes. It can lead to the extinction of alleles and the loss of polymorphism, such that a locus becomes fixed for a single allele. Thus, in order

to eliminate the effects of drift, populations should be large. Importantly, drift is independent of natural selection (Lowe *et al.,* 2004).

Genetic drift

Genetic drift has two important consequences: every population loses genetic variability with a speed inversely proportional to its number; as a result, it can lose some alleles. If a population is divided into two or more new independent populations, genetic drift will increase differentiations between them, which can be interrupted by mutation and migration (Timofeev-Ressovsky *et al.,* 1973).

Gene flow

Gene flow is the proportion of newly immigrant genes moving into a population. Populations of the same species are not isolated from each other, there is always an exchange of individuals—migration. Migrant individuals exchange alleles, which could be not present at all in a definite population, but for this exchange. Such patterns of allele movement can have profound impacts on the structure of genetic diversity. The extent of gene flow is determined by the mobility of the species, the dispersal ability of gametes, and the degree of isolation of populations, whether that is physical, ecological or temporal (Felsenstein, 1997).

Breeding preferences

Any single allele carried by a gamete is equally likely to fuse with any other allele, thus alleles fuse at random (panmixia or non-assortative mating). If individuals chose a mating partner of similar phenotype (same individual characteristics), positive assortative breeding (or mating), it leads to a reduction in the expected proportion of heterozygous loci relative to panmixia. If individuals choose a mating partner of opposite phenotype, negative assortative breeding, heterozygosity will increase (Timofeev-Ressovsky *et al.,* 1973; Felsenstein, 1997; Lowe *et al.,* 2004).

Natural selection

Natural selection is based on the concept of survival of the fittest. It means that those individuals best suited to their environment will survive to reproduce and pass on their genes to subsequent generations. Those less suited will die without passing on their genes. Over time, certain genes survive and other genes are weeded out of the population. This is a never ending process (Felsenstein, 1997).

Genome considerations

Mutations

Mutations are rare. and the rate of mutation of different genes is considered to be of the order 1×10^{-4} to 1×10^{-7} per generation. Mutations are the original source of all genetic diversity and increase genetic differentiation between populations. This is contradictory to the effect of gene flow (Timofeev-Ressovsky *et al.,* 1973). Mutations are harmful, neutral or helpful. Harmful mutations hinder the survival of the individual or cause death. If the individual dies before it can reproduce, the mutated allele is eliminated. Neutral mutations neither help nor hinder the individual and is most likely reproduced. Helpful mutation improve survival and will pass on to future generations (Felsenstein, 1997).

Polyploidy

Polyploidy is the occurrence of more than two copies of an entire nuclear genome within a cell. This can affect genetic diversity statistics. At its simplest, polyploidy arises through multiplication of genomes within a species (autopolyploidy) or by genome multiplication of interspecific crosses (allopolyploidy).

Linkage

Linked genes are genes that are found on the same chromosome. When large numbers of loci are utilized, it is inevitable that linked genes will be present in the data set. However, linkage only becomes a consideration in the analysis of genetic data, if the genes are very close together or if recombination rates are very low. In such situations, linkage disequilibrium occurs, whereby an allele from one locus is found to be associated with an allele from another locus, more frequently than would be expected under random association (Lowe *et al.,* 2004; Felsenstein, 1997).

Genetic distance

Genetic distance measures the amount of genetic variation between pairs of populations. Populations differ from each other in allele frequencies. Ideally, a genetic distance method should produce values that vary between zero (when all markers are shared between two individuals or populations) and unity (when no markers are shared between two individuals or populations). Many genetic distance measures (for example, Nei's genetic distance) are calculated on the basis of allele frequencies, and displayed as dendrograms (Lowe *et al.,* 2004).

Use of genetic diversity statistics

Different population-genetic processes influence the genetic parameters of populations: inbreeding leads to a decrease in the number of heterozygous individuals; mutations and migrations increase, while genetic drift decreases genetic diversity of populations; natural selection changes the frequencies of genes and genotypes; genetic drift increases, and migrations decrease, genetic distance *etc*. Knowing all these regularities one can study the genetic structure of populations and predict their possible changes. This is supported by the statistical-theoretical basis of population genetics (Falcorner & Mackay, 1996).

Estimating gene flow

Gene flow is a central parameter, offsetting the combined effects of mutation and genetic drift that prevent populations from differentiating over time. Using fixation index (F_{ST}) as a measure of population subdivision, and noting that populations with high differentiation should have lower levels of gene flow between them than those with low differentiation, Wright (1931) derived a parameter for gene flow, the number of migrants per generation, N_m (N_m = (1-F_{ST})/4F_{ST}). The relationship between N_m and F_{ST} is such that, with N_m values of less than one (F_{ST} = 0.2), populations are expected to diverge genetically over

time, but where N_m is greater than one, populations are expected to retain genetic connectivity (Quinn & Keough, 2002; Lowe *et al.,* 2004).

Spatial structuring of genetic diversity

In the situation where all populations of a species are not completely panmictic, there will be genetic differentiation over some spatial scale, owing to a lack of gene flow. If there is lower gene flow between more distantly separated populations, which consequently exhibit higher differentiation, this effect is termed 'isolation by distance' (Wright, 1943; Wright, 1946).

At the spatial scale, where an effect is suspected between widely spaced, discrete populations, a correlation between pairwise measures of geographic distance and genetic distance or differentiation can be plotted, and the closeness of fit estimated using a Mantel test (1967).

Genetic bottlenecks

The term 'genetic bottleneck' refers to the process by which genetic variation is lost following a population crash. While a population may rapidly recover its numbers following a crash, the level of genetic variation does not recover its previous value, until restored by mutation or gene flow. Comparisons between populations that have experienced bottlenecks have shown that both allelic richness and heterozygosity decline with reduction in population size (Lowe *et al.,* 2004).

Conservation biology

Conservation biology has a fundamental basis in genetic diversity. There are several important issues to the utilization of genetic diversity statistics, which include: comparison of the level of genetic diversity in rare species with that in more widespread ones; examination of the portion of genetic variation within and among populations as a guide to sampling stratageis for *ex situ* conservation; investigation of the effect of a population bottleneck on genetic variation; assessment of the relationship between genetic variation and fitness components; measurement of the level of gene flow between populations, and identification of unique allele units in a population (Lowe *et al.,* 2004).

Historical processes

The use of markers which can be interpreted phylogenetically, allows application of coalescent approaches to assess the historical dynamics of populations. The approximate age of populations, their historical size, whether they have been expanding or contracting, and even the influence of selection at linked loci, can be determined by using such techniques (Lowe *et al.,* 2004).

Adaptation

Comparison of the distribution of adaptive gene variation with neutral locus markers within the same individuals and populations permits testing for the action of selection, and should prove to be a developing area of ecological genetics study in the future (Lowe *et al.,* 2004).

Methods for studying genetic diversity among pollen beetle populations

Suitability of molecular markers

Knowledge of the level, structure and origin of genetic variation within and between populations is important for the effective utilization and conservation of species. Factors that influence genetic diversity and differentiation, together with morphological characters, have been used traditionally to characterize levels and patterns of diversity (Dawson & Chamberlain, 1996).

An ideal genetic marker for ecological genetic studies has several important characteristics: it can detect qualitative or quantitative variation; shows no environmental or developmental influences; shows simple codominant inheritance; detects silent nucleotide changes; detects changes in coding and non-coding portions of the genome; detects evolutionary homologous changes. None of the marker systems currently used in ecological genetics has all of these characteristics. The choice of a marker system is a compromise between the properties of the marker system and its availability and the available resources (Lowe *et al.,* 2004).

Types of marker

The six most commonly used types of protein and DNA markers are; allozymes, restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), micro- and minisatellites, and sequence analysis (Gibson & Muse, 2002; Lowe *et al.,* 2004).. However, other types of marker have been proposed, such as single nucleotide polymorphisms (SNPs). DNA markers are based on PCR analysis; which can either be targeted to specific regions of the genome, or alternatively, chosen at random to amplify unspecified regions. Marker systems can be classified according to their modes of inheritance, that is, dominant (*e.g.*, AFLP) vs. codominant (*e.g.*, RFLP); the number of putative loci that they detect: that is (*e.g.*, allozymes) vs. many loci (*e.g.*, RAPDs); the numbers of alleles that they detect at a locus, that is diallelic (*e.g.*, RAPDs) vs. multiallelic (*e.g.*, SSRs); or their ease of use, that is, simple (*e.g.*, RAPDs) vs. complex (*e.g.*, AFLPs), (Dawson & Chamberlain, 1996; Lowe *et al.,* 2004).

Allozymes

Allozymes (protein isoforms due to different alleles, usually detected by electrophoresis) was earlier the most widely used marker system in ecological genetic studies. The codominant expression, cost effectiveness, and simplicity of allozyme detection made these markers widely used, although their use is declining because of the low number of alleles detected per locus, the absence of phylogenetic information, and the need to have access to suitable fresh material.

RAPD markers

Of the approaches based on PCR analysis, RAPD analysis was earlier the most common among those based on unspecified targeting. It relies on primers of arbitrary sequence to detect different forms of polymorphism of the DNA. Polymorphism is due to the matching of the primer sequence to a complementary sequence on the target DNA; without a match no amplification of the DNA will occur. Polymorphisms are detected by the presence or absence of DNA products. This technique is cheap, simple, requires no sequence information, and a large number of putative loci can be screened. The criticism of this technique includes its poor reproducibility, marker dominance, product homology, allelic variation, *etc*.

RFLP markers

In RFLP analysis, restriction enzymes are used to detect variation in DNA sequence. The number of bases in the restriction site and the genome base composition determine the number of restriction sites in a genome. RFLP markers are codominant, and it is possible to detect nDNA and organelle DNA polymorphisms in total DNA extracts. RFLP requires a large amount of DNA, and is an expensive, time-consuming technique. By means of RFLP, one can investigate gene diversity and population structure, hybridisation, introgression, gene flow, and autopolyploidy. RFLP markers can be valuable phylogenetic and phylogeographic markers.

Micro- and minisatellites

Microsatellites (*e.g.*, SSRs—simple sequence repeats, STRs—short tandem repeats) are short (10–50 copies) tandem repeats of mono- to tetra-nucleotide repeats, whereas longer repeats give rise to minisatellites (*e.g.*, VNTR—variable number of tandem repeats), which are assumed to be randomly distributed throughout the nDNA, cpDNA, and mtDNA. These markers are codominant, and it is possible to detect both nDNA and organelle DNA polymorphism in total DNA extracts. Mutation rates are high in these markers compared to other markers, making them useful for intrapopulation studies. Although the initial identification of micro- and minisatellites is expensive, and requires cloning and sequencing; and homoplasy (identical characters that have evolved separately in independent evolutionary lineages) between alleles may be high. The applications of these markers include estimation of gene diversity and population structure. They are ideally suited for analysis of gene flow, having high number of alleles per locus.

DNA sequencing

Specific DNA regions are amplified by PCR and then subjected to sequencing. Direct sequencing of DNA produces easily scored, high-quality information, and high capacity facilities allow large amounts of data to be generated, and comparisons between taxa can be quickly and easily made. Meanwhile, DNA sequencing is expensive, since loci are screened one at a time; and some DNA samples may be very difficult to sequence. This technique can be used in applications that include estimation of gene diversity and population structure, investigation of hybridisation, introgression and gene flow. The approach has found its greatest value for phylogenetic and phylogeographic analyses.

AFLP markers

AFLP technology is based on selective amplification of a subset of genomic restriction fragments. The amplification primers, known as AFLP primers, are generally 17–21 nucleotides in length, and anneal perfectly to their target sequences; *i.e.* the adapter and restriction sites, and a small number of nucleotides adjacent to the restriction sites. The high marker densities that can be obtained with AFLP are an essential characteristic of the technology: a typical AFLP fingerprint contains between 50 and 300 amplified fragments, of which up to 80% may serve as genetic markers. Moreover, AFLP technology requires no sequence information or probe collections prior to the generation of AFLP fingerprints. This is of particular benefit when studying organisms for which very little DNA marker information is available. The AFLP technique provides a novel and powerful DNA fingerprinting technique for DNAs of any origin or complexity. However, the AFLP technique requires technical skills and DNA of high quality. The majority of AFLP applications have been for genome mapping and breeding studies, although it is coming to be used widely in ecological genetics for studies of gene diversity, population structure, and in phylogenetic and phylogeographic studies.

AFLP analysis

Analysis of the genetic variation between populations and individuals of a given species depends on the successful detection of basic variation between different samples. Several techniques have been developed to identify and estimate genetic variability, most often as DNA sequence variations (polymorphism), which are described above (Donini *et al*., 1997; Gibson & Muse, 2002; Linstedt *et al*., 2000; Lowe *et al.,* 2004; Savelkoul *et al*., 1999; Vos *et al.,* 1995).

AFLP analysis involves the selective amplification of an arbitrary subset of restriction fragments, generated by double digestion of DNA with two restriction enzymes, preferably six-cutter and four-cutter. Fragment ends are modified by the addition of double-stranded adapters, which provide the primer site for subsequent PCR amplification. Two phases of PCR amplification are involved. In the preselective amplification, primers are used, which are complementary to the adaptors but have an additional base pair. Selective amplification uses the preselective PCR product as a template for amplification with selective primers that are identical to the preselective primers, except for the addition of one to three

preselective amplification, primers are used, which are complementary to the adaptors but have an additional base pair. Selective amplification uses the preselective PCR product as a template for amplification with selective primers that are identical to the preselective primers, except for the addition of one to three additional selective bases, which are either radioactively or fluorescently labelled. The resulting fragments are then separated by denaturing polyacrylamide gel electrophoresis, and analysed, *e.g.* in a DNA sequencer (Fig. 2). By increasing the number of primer combinations, a large number of loci can be screened, whereby the chance of detecting polymorphisms is greatly enhanced. As a consequence, genetic variation of strains or closely related species can be revealed, and phenetic relationships can be established (Mueller & Wolfenbarger, 1999). The raw data are processed using specific softwares (e.g. GeneScan, Perkin Elmer/Applied Biosystems, Foster City, USA). Thereafter, data are imported into a genotyping analysis software (e.g. Genotyper), and only peaks that can be unambiguously scored are selected for further analysis. A dendrogram is constructed using Treecon or another tree-building program. Bootstrap analysis is usually based on 100–1,000 replicates.

Patterns differ in the presence or absence of a restriction site (particular band), which enabled the construction of a binary data matrix. Thus, two basic profile changes may occur, gain or loss of a band (peak on a chromatogram profile). Such changes could be produced by an insertion, deletion or duplication event. In addition, a change can be caused by a point mutation in the restriction enzyme recognition sequence (the loss) and by a point mutation changing a potential site into a recognisable site (the gain; Robinson & Harris, 1999).

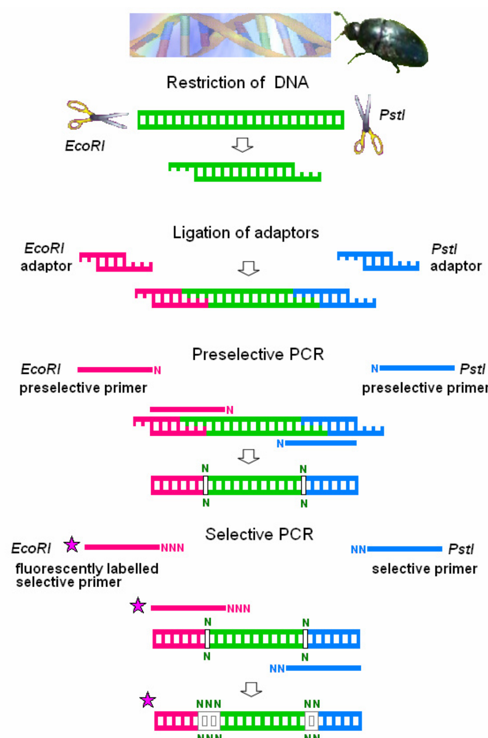


Fig. 2. Schematic description of the AFLP technique.

Advantages and disadvantages

AFLP has in several cases been adopted for studies of genetic variability of different organisms, since it can generate a large amount of data in a short time. Furthermore, it is a highly reproducible method, in which very little DNA is required, and no prior sequence information is needed.

Because AFLP gels are typically complex, containing many polymorphic sites, it is rarely possible to find the alternative allele, unless segregation analyses of family data are conducted. The introduced bias is, however, assumed to be negligible as long as AFLP-length codominance is rare (less than 10%), and a large number of informative bands (more than 100) are studied. Another problem is size homoplasy, *i.e.* that bands of the same length are not homologous, and thus represent two or more different AFLP loci; this is of particular concern in studies of genetic diversity and phylogenetic reconstructions. To make more out of the data, it has been shown that with the use of special software, it might be possible to score AFLP data for codominance. This procedure assumes that strong bands indicate homozygous (1/1) individuals, and weaker bands, about 50% of the strength of the homozygote band, indicate heterozygous (1/0) individuals. However, there is an overlap between the band intensities, and unless family data are available to confirm a Mendelian inheritance pattern, it is not recommended to employ codominance scoring of AFLP data (Bensch & Åkesson, 2005; Mueller & Wolfenbarger, 1999).

History of application

In the study by Vos *et al*. (1995), the AFLP method was evaluated by using organisms with genomes widely differing in complexity (bacteria, yeast, plants and humans) demonstrating its broad applicability. Plant researchers rapidly embraced the AFLP method, especially for genomic studies of crop species. Of all plant studies using AFLP up to 2003 (n = 223), 72% were conducted on crop species or other species of economic importance. Most studies of fungi involve parasitic species that are pathogens which affect crop production. Typically, these studies of plants and fungi have used AFLP to determine the genetic architecture of economically important traits such as productivity, disease resistance, and in animals—the history of domestication, in insect pests—resistance to insecticides. There are rather few AFLP studies of mammals, birds, fish and insects. Techniques such as microsatellite technology, RAPD, *etc*., are more often used instead (Bensch & Åkesson, 2005).

Examples of application in molecular ecology

Parentage analysis and individual genetic similarity

The major limitation of AFLP is its dominant nature. Thus the only scenario in which a parent or (parent pair) can be excluded, is when both parents are homozygous for the absence allele (0/0), and the offspring shows a presence allele. It is thus not possible to exclude individual parents when the other parent is unknown. The other limitation of AFLP is its low level of polymorphism (only

two alleles per locus), (Bensch & Åkesson, 2005; Gerber *et al*., 2000; Mueller & Wolfenbarger, 1999).

Genetic diversity of species or populations

The level of genetic diversity may reveal information about historical population sizes and structure. The traditional ways to measure genetic diversity, *e.g*. as the average level of heterozygosity at codominant markers, such as allozymes or microsatellites, are problematic in this respect. The mutation rate at the studied loci will affect the heterozygosity estimate, and microsatellites are particularly sensitive to this sort of bias. Also these methods normally restrict the user to examining less than a few dozen loci, for most species corresponding to less than one marker per chromosome (Bensch & Åkesson, 2005; McMichael & Prowell, 1999; Ravel *et al*., 2001).

Population structure

For population structure, study data from many loci and individuals are required. AFLP is very suitable for such cases, as was shown by several studies of birds, fish, insects and molluscs. In populations continuously distributed over a larger area, where gene flow is mainly between nearby locations, we expect to see a pattern of genetic isolation by distance. However, single markers may behave quite stochastically in terms of differentiation between sites, even if gene flow is constant and continuous (Bensch & Åkesson, 2005; Samils *et al.,* 2001; Yan *et al.,*1998).

Assignment of individuals

With multiple genetic markers, it is possible to investigate the affinity of each genotype to presumed populations of origin by employing assignment tests. AFLP-based genotyping holds a lot of potential for such studies, but migratory species may, however, show quite low levels of spatial differentiation; consequently, one has to use several hundreds of loci before successful assignments can be made (Bensch & Åkesson, 2005; Campbell *et al.,* 2003; Dearborn *et al.,* 2003).

Finding genes that affect phenotypes

AFLP can be successfully used (because it scans many polymorphic loci at the same time for a short period of time) for finding genes that matter, or rather markers for such genes, following a strategy called 'genome scans'—scans for loci involved in adaptive population divergence (Bensch & Åkesson, 2005; Campbell *et al.,* 2004).

Hybridization and hybrid zones

AFLP has proved to be very useful when identifying hybrid individuals (interspecific or intraspecific), even in systems where microsatellites have failed to do so. It is the possibility to generate many polymorphic markers in a short time that makes AFLP preferable for identifying hybrids (Bensch *et al.,* 2002; Bensch & Åkesson, 2005).

Gene mapping and linkage

AFLP provides fast and easily developed markers that can be positioned throughout the genome in any organism. It has been used in the construction of

such linkage maps in various plant fungal species, and now also many animal species. AFLP, together with microsatellites, is the most common marker used when developing new linkage maps (Bensch & Åkesson, 2005).

Species phylogenies

Although DNA sequencing produces data of much higher quality than AFLP, the latter allow data to be collected at more than 100 times as many loci for the same cost. A major concern with using dominant multilocus DNA profiles (such as AFLP data) for phylogenetic reconstructions, is that bands of the same length seen in two species, may not be homologous. If such artificial similarities are common, they may contribute to spurious phylogenetic relationships, and on average, this should be more of a problem when the studied species are distantly related. (Bensch & Åkesson, 2005; Parsons and Shaw, 2002; Vos *et al.,* 1995).

Useful extension of the basic protocol

Microsatellites from AFLP

Microsatellite markers often exhibit high levels of polymorphism, and are most often codominant. In order to circumvent the time-consuming procedures often involved when developing microsatellite markers, several attempts have been made to amplify microsatellites in AFLP experiments. Microsatellite-AFLP is an AFLP-based fingerprinting method for simultaneous amplification of microsatellite- and AFLP markers. It uses the combination of a randomly amplified microsatellite polymorphism (RAMP) primer and a selective AFLP primer to amplify restriction fragments containing simple sequence repeat (SSR) motif sequences. Microsatellite-AFLP can be used as a fingerprinting technique and as discovery tool for highly informative SSRs. (Bensch & Åkesson, 2005; Robinson & Harris, 1999; Vos *et al.,* 1995; Witsenboer *et al.,* 1997).

cDNA-AFLP

It has been shown that the level of gene expression can be very different also in genetically very similar organisms. The state-of-the-art method to compare gene expression is based on the microarray technique. cDNA-AFLP was found to be a fast and robust alternative to explore variation in gene expression between individuals and groups of phenotypes (Bachem *et al.,* 1999; Bensch & Åkesson, 2005).

DNA methylation

The methylation patterns of DNA are relatively stable over cell generations, but can also be modified by intrinsic and external influences. Variation in methylation has been found to influence, *e.g.* gene expression and genomic imprinting. Patterns of DNA methylation can be retrieved by a slight modification of the original AFLP protocol. The method makes use of two isoschizomeric restriction enzymes with differential sensitivity to DNA methylation, and by comparing different groups of phenotypes or tissue, DNA methylation differences can be identified and quantified (Bensch & Åkesson, 2005; Xu *et al.,* 2000).

Phylogenies and phylogeography

Phylogenies, or phylogenetic relationships, are in general patterns of shared history between biological replicators, such as species or genes. The aim of *phylogenetic inference* is to propose a well-corroborated hypothesis of this shared history. Phylogenetic analyses are useful in many different contexts, either directly (*e.g.* to infer the evolutionary history of the molecule used, to infer the temporal order of other events mapped on the phylogeny, such as gene transfers, or to study epidemiology) or indirectly. The indirect use stems from the fact that, since all species and genes share more or less of a common history, these are not independent observations.

Neighbours on the tree share the same ancestor. Characters derived from this common ancestry are called homologous (Holmes, 1999).

With the advent of molecular methods that allow the phylogenetic analysis of mutations that differ among genetic variants, it has become possible to trace evolutionary relationships, not only among species but also among combinations of genetic markers within and among populations.

With appropriate data sampling and analysis, it is possible to investigate the impacts of selection, changes in population size, and population substructuring on genealogical relationships among these alleles. Such investigations have been described as phylogeography (Avise, 2000; Lowe *et al.,* 2004).

General principles

The phylogenetic relationships between a group of replicators (species or genes), are commonly modelled as a *tree*. A tree is a mathematical structure that consists of *nodes* (or *vertices*) that are connected by *branches* (or *edges*). An edge may have a *weight* (*branch length*) associated with it. The number of adjacent edges connected to a vertex is the degree. If an internal vertex has a degree other than three, the node is a *poly(cho)tomy*; a tree without polychotomies is fully resolved or a *dichotomous* (meaning bifurcating) tree.

Trees may be *rooted* or *unrooted* (Fig. 3). A rooted tree has an internal vertex designated as an ancestral state of the replicator, and the tree thus has a direction corresponding to evolutionary time. This information is necessary to tell which terminal nodes are more closely related (*i.e.* share a history not shared with any of the other terminals). An unrooted tree lacks this information

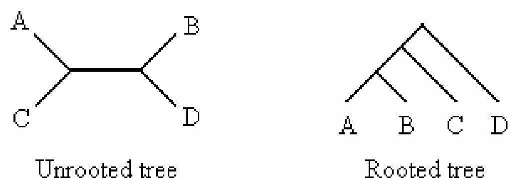


Fig. 3. Types of phylogenetic tree.

A cladogram (Fig. 4, I) is simple tree depicting only relationships between terminal nodes (an *n*-tree in mathematical parlance). An *additive tree* (or phylogram, Fig. 4, II) has additional information in that edge lengths are drawn proportional to some attribute such as amount of change. An *ultrametric tree* (or *dendrogram*, Fig. 4, III) is a special kind of additive tree, where all pendant vertices (the "tips" or terminal nodes) are equidistant from the root. Ultrametric trees can thus depict evolutionary time (directly or as divergence with a molecular clock).

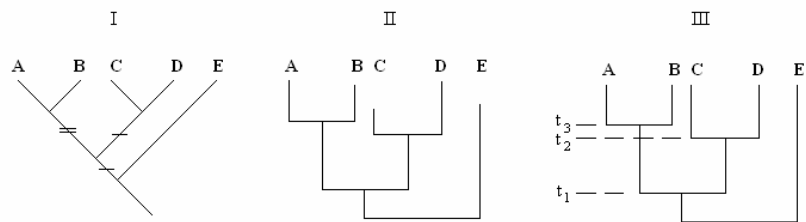


Fig. 4. Cladogram (I), Phylogram (II), Dendrogram (III).

The leaves of these phylogenetic trees are called Operational Taxonomic Units or OUTs. They can be genes, individuals, populations, species, families or larger classes of species (Holmes, 1999).

Methods of phylogeny reconstruction

The primary methods of phylogeny reconstruction are parsimony, distance and likelihood. There are many variants within each of the three broad classifications. The shared thread among all of the methods is an attempt to identify the topology that is most congruent with the observed data. The methods differ in their mechanisms for measuring this congruence. Some methods define a metric between topology and data, and require an exhaustive search through all possible tree topologies (Gibson & Muse, 2002).

Parsimony methods

Parsimony, or *maximum parsimony*, scores the number of changes between different character states that at minimum are necessary to explain the observed data given the tree. The best hypothesis is the tree requiring the fewest changes. The changes may be restricted in what kind of changes that are allowed. This score, often referred to as the tree's *length*, is the minimum number of changes for the tree.

Maximum likelihood methods

Maximum likelihood is a kind of estimate that is very common in statistics. For example, estimation of the population mean by the average of a sample is a maximum likelihood (or ML) estimate. ML is different from parsimony, in that an explicit model is used to calculate the score. The model in phylogenetic contexts consists of two parts: a model of how the character state changes occur (probabilities of change), and a tree with branch lengths.

Distance methods (minimum evolution)

The minimum evolution criterion differs from the two previous criteria, in that the observations are not used directly to calculate the tree score (which is called length also for ME). Instead, the data are transformed to pair-wise distances, and the score is calculated from those. The use of pair-wise distances is an advantage for some kinds of data (*e.g.* DNA-DNA hybridization), where the data from the experiments are pair-wise differences.

The neighbour-joining method proposed by Saitou & Nei (1987) is a good approximation of the best tree, and uses minimum evolution as a criterion.

Evaluation of trees

Data support is measured for a particular grouping, or clades, in an estimated tree. The most common approach in measuring support is through the use of bootstrapping, as introduced by Felsenstein (1985). Numerical resampling techniques are used to compute bootstrap support levels for every node in the tree topology. Bootstrap values near 100% indicate clades that are strongly supported by the data, while lower levels indicate reduced support. Values greater that 70–80% are often taken to indicate fairly strong support for the clade (Gibson & Muse, 2002).

Insecticide resistance

Insecticide resistance is the result of an increase in the ability of individuals of an insect species to survive insecticide application. It is a shift in response to insecticide exposure; a population-level trait, not a species-level trait; it is not the same as tolerance, because low-level resistance is still resistance, not tolerance, whereas species-wide abilities to survive particular insecticides are tolerance, not resistance.

The scale of the problem

All chemical insecticides exert a selective evolutionary pressure upon the insect pests they are intended to control. Therefore, over a period of time, resistant strains of insect are certain to emerge. The time to resistance depends on a number of factors, including the frequency and nature of resistance genes, pest-management strategies, and the relative fitness of the resistance strains relative to the wild type. Resistance causes pesticide failures that lead to loss of human life, crop failures, cosmetic damage, and nuisance. Resistance has been documented to every type of insecticide, it is most common in multivoltine pests; pests exposed to multiple sprays each season or extended-release applications; plant-eating pests and some animal ectoparasites instead of natural enemies.

Currently, *ca*. 500 species of insect pest are resistant to one or more common insecticides. 56% are crop pests, 39% are medical/veterinary pests; 5% are

beneficial species. Resistance is identified or measured in bioassays, when insects are treated with a range of doses or concentrations or held for a range of times. Bioassays may use F1 or F2 generations, backcrosses, or other steps to characterize the nature of resistance (dominant or recessive, single or multiple-gene based, *etc*.). After the linear relationship between dose and mortality is known, a diagnostic dose may be used to detect the presence of resistance in the field.

Resistance mechanisms

Behavioural resistance

It is not a common mechanism. A shift in behaviour avoids exposure to insecticide; examples are controversial, as they often involve metabolic or target-site resistance as well. It is not clear whether they really represent heritable shifts in behaviour or simply survival for a long enough period (as a result of metabolic or target-site resistance) to exhibit avoidance behaviours.

Reduced penetration

This usually provides low levels of resistance, most useful where increased metabolism provides internal detoxication. Examples include *pen* in the housefly, a gene that confers cross-resistance to different insecticides. Similar genes seem to occur in other species.

Detoxification enzyme-based (metabolic) resistance

This occurs when increased levels or modified activities of esterases, oxidases, or glutathione S-transferases (GST) prevent the insecticide from reaching its site of action.

Esterases

Perhaps the most common resistance mechanisms in insects are modified levels or activities of esterase detoxification enzymes that metabolize a wide range of insecticides. These esterases comprise six families of proteins belonging to the α/ß-hydrolase fold superfamily. In Diptera, they occur as a gene cluster on the same chromosome. Individual members of the gene cluster may be modified in instances of insecticide resistance, for example, by changing a single amino acid that converts the specificity of an esterase to an insecticide hydrolase or by existing as multiple-gene copies that are amplified in resistant insects (Brogdon & McAllister, 1998).

Oxidases (cytochrome P450)

The cytochrome P450 oxidases (also termed oxygenases) metabolize insecticides through hydroxylation or oxidation. The cytochrome P450s belong to a vast superfamily. Of the 62 families of P450s recognized in animals and plants, at least four (families 4, 6, 9, 18) have been isolated from insects. The insect P450 oxidases responsible for resistance have belonged to family 6, which, like the esterases, occur in Diptera as a cluster of genes. Members of the cluster may be

expressed as multiple (up to five) alleles. Enhanced levels of oxidases in resistant insects result from constitutive overexpression rather than gene amplification. The mechanisms of oxidase overproduction in resistance are under extensive investigation, and appear to result from both cis- and trans-acting factors (Brogdon & McAllister, 1998; Gong *et al.,* 2005; Pittendrigh *et al.,* 1997).

Glutathione S-transferases (GST)

Most organisms possess multiple GSTs from two or more classes. GSTs implicated in DDT insecticide resistance exist as clusters of genes that have been further shuffled through the genome by recombination. GSTs can cause resistance to insecticides by conjugating reduced glutathione to the insecticide or its metabolites. Most reports on GST-mediated resistance involve organophosphate resistance in houseflies. Some GSTs are able to dehydrochlorinate DDT and recently, GSTs were shown to be involved in pyrethroid resistance in other insect species (Kristensen, 2005; Brogdon & McAllister, 1998).

Target-site resistance

This resistance occurs when the insecticide no longer binds to its target.

Ligand-gated ion channels

They receive chemical signals, neurotransmitters, such as acetylcholine or γ-amino butyric acid (GABA), which they then convert into electrical signals via the opening of their integral ion channels. The insect GABA receptor is the site of action of cyclodiene insecticides and phenylpyrazoles such as fipronil (Ffrench-Constant *et al.,* 2004).

Acetylcholine esterases

They are target sites of organophosphorus (OPs) and carbamate insecticides, located in nerve synapses. Acetylcholine esterase (AChE) is a key enzyme of the cholinergic system, because it regulates the level of acetylcholine and terminates nerve impulses by catalyzing the hydrolysis of acetylcholine. Its inhibition causes death, leading to an accumulation of acetylcholine in the synapses, which in turn leaves the acetylcholine receptors permanently open (Brogdon & McAllister, 1998; Ffrench-Constant *et al.,* 2004; Fournier, 2005).

Voltage-sensitive sodium channel (VSSC)

VSSC or voltage-gated ion channels are target sites of organochlorines (DDT) and synthetic pyrethroids, located in the nerve sheath. Unlike ligand-gated channels, voltage-gated channels are triggered by changes in membrane voltage rather than changes in the concentation of a neurotransmitter. Target-site resistance to pyrethroids was first characterized as knockdown resistance (kdr) in houseflies. Subsequently, a single amino acid replacement (point mutation) was found to be associated with kdr and the addition of a second replacement was associated with an enhanced allele, super-kdr (Ffrench-Constant *et al.,* 2004; Brogdon & McAllister, 1998).

Voltage-sensitive sodium channel (VSSC) as a target site for pyrethroids

Voltage-sensitive sodium channels are the primary target sites of pyrethroid insecticides. A number of studies have shown that resistance to pyrethroid insecticides is associated with the para homologous sodium channel genes. In insect pests, such as housefly and cockroach, point mutations in the para homologous sodium channel gene are responsible for kdr and super-kdr to pyrethroids (Wang *et al.*, manuscript; Brogdon & McAllister, 1998).

VSSC structure and functions

An ion channel is a transmembrane protein complex that forms a water-filled pore across the lipid bilayer, through which specific inorganic ions can diffuse down their electrochemical gradients. The membranes of electrically excitable cells possess voltage-gated ion channels in which the electrical conductance is operated through a gating process, induced by small voltage-driven changes in the conformation of the channel protein, expressed in the opening and closing of the ion pores (Zlotkin, 1999).

Separate pathways are involved in increases in sodium and potassium permeability within an action potential. The change in sodium permeability during a voltage clamp-maintained depolarization is biphasic. It increases for a few milliseconds and then spontaneously returns to its resting level. These changes have been described in terms of two voltage-dependent processes: activation, which controls the initial increase in sodium permeability after depolarization, and inactivation, which controls the subsequent return of sodium permeability to the resting level during a maintained depolarization. These processes allow the voltage-gated sodium channel to exist in any one of three distinct functional states: resting (closed), open (permeable), and inactivated (closed). Although both the resting and the inactive channels are non-conducting, they differ in their voltage dependence for activation. An inactivated channel is refractory to depolarization and must first return to its resting state by repolarization before being activated (opened by depolarization), see Fig. 5. Ion selectivity, activation, and inactivation of the voltage-gated sodium channel can be modified by the selective pharmacology of several groups of sodium channel neurotoxins (Zlotkin, 1999; Shafer *et al.,* 2005).

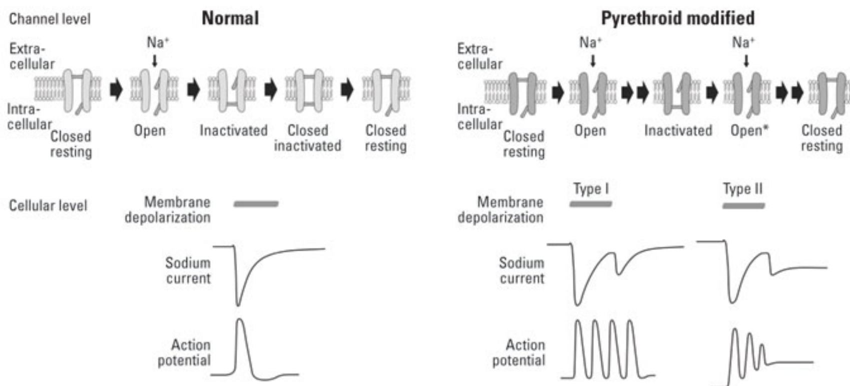


Fig. 5. Pyrethroid effects on neuronal excitability. This schematic depicts pyrethroid effects on individual channels, whole-cell sodium currents, and action potentials (Shafer, 2005).

The primary structure of sodium channels contains a large glycoprotein α subunit of 240–280 kDa. In insects, the α subunit is coded by the para locus, first identified in *Drosophila melanogaster*. The sodium channel α subunit has four homologous repeated domains (I–IV), with a circular radial arrangement in which a central ion pore is formed (Fig. 6). This brings domains I and IV into close proximity. Each domain consists of six putative transmembrane helical segments. The most conserved segment is S4, present in each repeated domain, which contains a unique motif of a positively charged amino acid residue, followed by two nonpolar residues that repeat four to eight times in each helix. The S4 structures are suggested to participate in the voltage-sensing mechanism. Restoration assays with mutated or otherwise inactivation-deficient sodium channels and subunits coupled with synthetic peptides, led to the conclusion that a hydrophobic sequence (IFM) in the intracellular segment connecting domains III and IV of the α subunit is required for fast inactivation, and serves as an inactivation particle of the sodium channel. The short segments SS1 and SS2, which are part of the extracellular amino acid loop between transmembrane segments S5 and S6, are supposed to form a hairpin structure inside the membrane and to serve as part of the ion-conductive pathway.

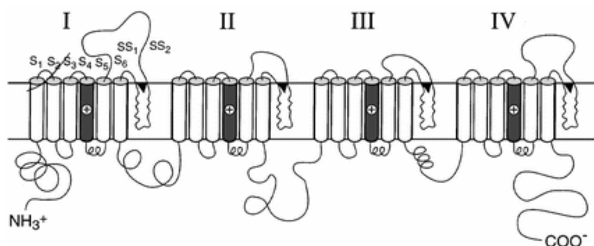


Fig. 6. Drosophila *para* voltage-gated sodium channel. Schematic presentation of the transmembrane arrangement of the main subunit (α) of the sodium channel adopted as the general convention in most sodium channel gene descriptions (see text). The S4 segments indicated by (+) are suggested to participate in the voltage sensing mechanism. The

intramembrane short segments SS1 and SS2 are referred to as the pore region. The black triangles represent the entrance of the pore. The loop connecting domains III and IV is the region suggested to participate in the fast inactivation (Zlotkin, 1999).

Pyrethroid structure and mode of action

For several decades, pyrethroid insecticides have been widely used to control many insect pests. Because of the intensive use of pyrethroids, many pest populations have developed resistance to these compounds. Many of these resistant insects carry specific point mutations in the sodium channel gene. Pyrethroids slow the kinetics of sodium channel activation and inactivation, resulting in the prolonged opening of individual channels, and leading to paralysis and death of poisoned insects (Liu *et al.,* 2000; Soderlund & Knippe, 2003).

Chemistry and mode of action

Pyrethrum is a naturally occurring mixture of chemicals found in certain chrysanthemum flowers. Six individual chemicals have active insecticidal properties in the pyrethrum extract, and these compounds are called pyrethrins. They break down quickly in the environment, especially when exposed to sunlight (Agency for Toxic Substances and Disease Registry, 2003).

Pyrethroids are manufactured chemicals that are very similar in structure to the pyrethrins, but are often more toxic to insects, as well as to mammals, and last longer in the environment than pyrethrins. More than 1,000 synthetic pyrethroids have been developed. Most commercial pyrethroids are a mixture of stereoisomers with different insecticidal properties and different toxicities.

Molecular mechanisms of knockdown resistance (kdr)

In insects, the effects of pyrethroids can develop within 1–2 minutes after treatment and can result in knockdown, which is a loss of normal posture and locomotion.

The signs of intoxication by pyrethroids develop rapidly, and there exist different poisoning syndromes. Typical signs of insect intoxication by pyrethroids include hyperexcitability and convulsions or predominantly ataxia and incoordination.

Pyrethroid intoxication results from their potent effects on nerve impulse generation within both the central and peripheral nervous systems. Pyrethroids modify neuronal sodium channels by slowing the kinetics of their activation and inactivation resulting in the prolonged opening of individual channels leading to paralysis and death (Shafer *et al.,* 2005; Bloomquist, manuscript).

Point mutations in VSSC associated with kdr to pyrethroids

One class of the most important resistance mechanisms is knockdown resistance (kdr): both knockdown (rapid paralysis) and killing by pyrethroids and dichloro diphenyl trichloroethane (DDT) occur through reduced neuronal sensitivity to these compounds. The primary target site for pyrethroids is a voltage-sensitive sodium channel in the nervous system. An insect sodium channel gene, *para*, was

first identified in *Drosophila*. Recent studies show that point mutations in the Para sodium channel protein are responsible for *kdr* and super-*kdr* resistance in insects. The *kdr* resistance in the housefly and German cockroach is associated with a leucine (L) to phenylalanine (F) mutation in segment 6 of domain II (IIS6) of VSSC (L1014F), also detected in horn flies, mosquitoes and aphids. The super-*kdr* resistance in housefly is associated with an additional methionine (M) to threonine (T) mutation in the linker region between S4 and S5 of domain II (M918T), also detected in horn fly (Liu *et al.,* 2000; Lee *et al.,* 1999; Wang *et al.,* 2003).

Among the 20 unique sodium-channel point mutations associated with pyrethroid resistance, those occurring at four sites have been found as single mutations in resistant populations: Val410 (V410M in *H. virescens*), Met918 (M918V in *B. tabaci*); Leu1014 (L1014F in several species, L1014H in *H. virescens*, and L1014S in *C. pipiens* and *A. gambiae*); and Phe1538 (F1538I in *B. microplus*). Mutations at 6 sites (M918T in *M. domestica* and *H. irritans*; T929I in *P. xylostella*; D59G, E435K, C785R, and P1999L in *B. germanica*) have been found in combination with the L1014F mutation in highly resistant strains, and have therefore been hypothesized to function as second-site mutations that produce additive or synergistic enhancement resistance, caused by the L1014F mutation. The status of the remaining resistance-associated mutations is more ambiguous. The L932F mutation has been found only in combination with the T929I mutation (a putative second-site mutation) in *Pediculus capitis*, whereas the D1549V and E1553G have been found only together in resistant strains of *Helicoverpa virescens* and *Helicoverpa armigera*. Finally, the four mutations identified in temperature-sensitive *para* mutants of *D. melanogaster* were selected on the basis of a behavioral rather than toxicological phenotype (Fig. 7; Soderlund & Knipple, 2003).

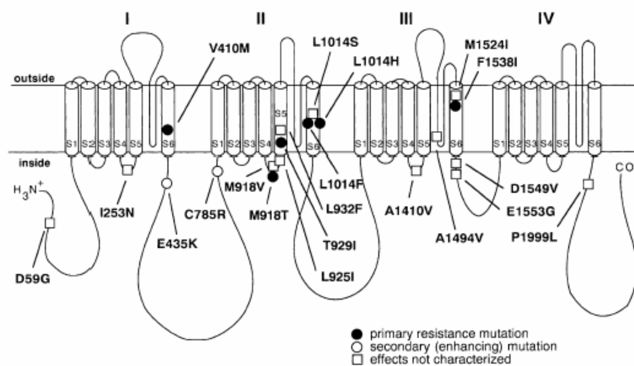


Fig.7. Diagram of the extended transmembrane structure of voltage-sensitive sodium channel α subunits, showing the four internally homologous domains (labeled I–IV), each having six transmembrane helices (labeled S1–S6 in each homology domain), and the identities and locations of mutations associated with knockdown resistance. The symbols used to identify mutations indicate their functional impact as determined in expression assays with *X. laevis* oocytes (Soderlund & Knipple, 2003) .

Resistance management and control

Among many tools for managing resistance, such as new insecticide chemistries and transgenic plants, single strategies all have the potential for failure. The best option for saving crops and safeguarding health is in adopting integrated pest management strategies. Modern pest control strategies include not only the methods that cause direct mortality, but also a variety of other tactics that reduce the reproductive potential of a pest population or modify its behavior.

Most of the control tactics that are commonly used today can be grouped into two broad categories: natural control or artificial control. By definition, a natural control may be any environmental factor that keeps a pest population below its economic injury level. Examples might include geographic barriers, cold temperatures, natural enemies (biological control, physical control). Artificial controls are products or processes of human origin to modify a pest's distribution, behaviour or physiology (mechanical, cultural, chemical control) (Ware, 1994; Gullan & Cranston, 2000).

So, what can be done in resistance management? There are several pest management control strategies, such as biological, cultural, physical and mechanical control and chemical control.

Biological pest control is a pest control by other species (parasites, predators, and pathogens). These species are important control agents, e.g. *Rodolia cardinalis*, a ladybeetle is a control agent for cottony cushion scale (*Icerya purchasi*). Very often instead of finding a natural enemy for a pest, breeders look for genetic traits that reduce an organism's susceptibility to attack or injury by its insect pests. Another form of biological control is eugenic or genetic control, which works by causing (inducing) reproductive sterility, or by incorporating new and potentially deleterious genes (or alleles) into the genome of a pest population (Ware, 1994; Dawson & Chamberlain, 1996; Gullan & Cranston, 2000).

The main aim of cultural control is to make the environment less favourable to insect pests. It can be achieved by crop rotation, cultivation of alternate hosts (e.g., weeds), introducing of trap crops, adjusting the timing of planting or harvest. At conditions of crop rotation insects emerging after overwintering will not find suitable food and they will starve. Trap crops will attract pest insects where they can be managed more efficiently to prevent or reduce their movement onto valuable crops (Ferro, 1987; Gullan & Cranston, 2000). Some pest populations can be affected by water or nutrient management (some are enhanced by poor crop growth, while others – by succulent crop growth), (Ferro, 1987).

The use of physical barriers such as row covers or trenches prevents insects from reaching the crop. Other methods include hand picking of pests, sticky boards or tapes for control of flying insects. Physical controls can be classified as passive (e.g., trenches, fences, organic mulch, particle films, inert dusts, and oils), active (e.g., mechanical, polishing, pneumatic, impact, and thermal) or miscellaneous (e.g., cold storage, heated air, flaming, hot-water immersion) (Ferro, 1987; Vincent et al., 2003).

The most widespread control strategy is chemical control. If all other tactics are unable to keep an insect pest population below an economic threshold, then use of insecticides to prevent economic loss is justified. Chemical control tactics involve a wide variety of substances that cause direct mortality (toxicants), disrupt developmental processes (growth regulators), prevent reproduction (sterilants), or modify insect behaviour (semiochemicals, e.g. pheromones) (Ware, 1994; Gullan & Cranston, 2000). It is important to note that while using insecticides it is necessary to control selection pressure to keep susceptible insects alive. It can be achieved by using the following conditions: no unnecessary treatments; lowest possible effective rates; shortest effective residual; local instead of area-wide treatments (to include spot treatment); preserve untreated refugia (refuges); use other controls whenever possible (cultural practices, host plant resistance, etc.). Also, it is necessary to kill the developing resistant population, which is achieved by using a high-dose strategy (a well-chosen dose to kill rare heterozygotes); synergists to neutralize resistance (for metabolic resistance); mixtures or rotations of insecticides (to kill those insects that are developing resistance to one compound by using a different one), (Wu & Guo, 2004).

So far, minimizing insecticide usage is the only strategy really demonstrated to work satisfactorily. Rotations involve no extra cost, so they represent a good idea, even if they are unproved. Mallet (1989, handout) makes a case for mixtures, but practical considerations make most mixture strategies undesirable (cost, environmental risk, residues) or ineffective (differential persistence of components of the mixture). Despite their popularity with pesticide manufacturers and distributors, mixtures and high doses usually involve too many negative consequences to be practical (Regulatory Directive, 1999).

Objectives of this study

The main aims of this thesis were:

- to establish a genotyping method for pollen beetles;
- to characterize the basic variation between different populations of Swedish and European pollen beetles;
- to elucidate the basis for pesticide resistance, by using different approaches for identification of "resistance genes", such as AFLP analysis and RT-PCR.

Thus, the project was divided into three parts: (I) Establishment of the AFLP technique for genotyping pollen beetles; (II) Characterising the basic variation between different populations of Swedish and European pollen beetles by AFLP technique; (III) Analysis of common insecticide resistance genes in pollen beetles (Voltage-sensitive sodium channel and CYP450 genes).

Materials and methods

Study I

In order to conduct studies concerning the genetic variability of pollen beetles, a genotyping protocol was established. No genome information is available for pollen beetles; hence the amplified fragment length polymorphism (AFLP) technique was chosen, since it does not depend on any prior sequence information of the samples, and is also a sensitive and robust technique. However, several modifications were needed to adapt the method for analysis of pollen beetles. Basic modifications included (i) alterations to DNA purification, (ii) use of two six-cutter restriction enzymes, and (iii) modified PCR conditions. This protocol resulted in a favourable number of fragments of an appropriate size range for standard gel analysis by a DNA sequencer applicable to a single insect and even body parts enabling different assays to be conducted on a single specimen. Pollen beetles from different areas of Sweden were analysed to verify the reproducibility and efficacy of the protocol as well as for phenetic analysis. The high reproducibility of the modified AFLP protocol allows it to be used as a reliable tool for genotype analysis of pollen beetles.

Study II

The modified AFLP protocol was applied for studies of genetic differentiation of pollen beetle populations from different areas of Sweden and Europe: AFLP analysis, with the restriction endonuclease combination *Eco*RI and *Pst*I (four primer combinations), was performed on 133 samples of pollen beetles, both susceptible and resistant to pyrethroid insecticides, collected from 2001–2004 from five different provinces of Sweden. A subsample of 59 single beetles was analysed, using one primer combination. AFLP profiles were analysed by similarity measures, using the Nei and Li coefficient, and dendrograms were generated by means of TREECON software. Statistical analysis of single beetle samples by AMOVA was performed.

Study III

DNA polymorphism was also studied by the modified AFLP technique (one primer combination) in 14 populations of pollen beetles, collected during 2004 in six European countries (Denmark, France, Finland, Germany, Sweden, and UK). Using one primer combination, 410 polymorphic DNA fragments were obtained for analysis of single beetles. AFLP profiles were analysed by similarity measures using the Nei and Li coefficient and dendrograms were generated. Statistical analysis (AMOVA) was performed.

Study IV

To identify possible point mutations associated with pollen beetles resistance to pyrethroids, the primary target site for pyrethroids—voltage-sensitive sodium

channels (VSSC, *para* sodium channels in insects)—were studied by means of the RT-PCR technique. Two susceptible pollen beetle populations, and three resistant to pyrethroid insecticides, collected from the Swedish province Östergötland, were used in the experiments. First-strand cDNA was used as a template to amplify *para* cDNA fragments, using gene-specific primers (GSPs), designed on the basis of homologous sequences from several insects. PCR products were extracted from the gel, cloned and subjected to sequencing analysis. Multiple sequencing reactions were performed to test the accuracy and reproducibility of the obtained results. Four cDNA fragments composing I and II domains of VSSC coding sequences were amplified (using primers designed for homologue sequences). Pollen beetle cDNA VSSC sequences were analysed and compared with published VSSC sequences showing point mutations that may confer pyrethroid resistance.
Also metabolic resistance factors, Cytochrome P450, were studied on the same populations of pollen beetles using RT-PCR technique. Point mutations were found in all CYP450 genes (CYP4S and CYP4G8) from the CYP4 family amplified and sequenced.

Results and discussion

Establishment of the AFLP technique for genotyping of pollen beetles (paper I)

The proposed established technique is an example of improvement of an AFLP protocol developed for the analysis of population genetic variability in one of the most important pests of *Brassicaceae*—the Pollen beetle. The pollen beetle (*M. aeneus*) is a pest of a great economic importance, destroying flowering oil seed *Brassicas* throughout Europe. The problem of resistance by pollen beetles to pyrethroid insecticides has lately increased both in frequency and geographical spread, especially in Western Europe. Thus it is very important to describe the genetic variation between different populations of pollen beetles and to understand the basis of insecticide resistance, in order to overcome it.

No genome information is available for pollen beetles; therefore, the AFLP technique was chosen, since it does not depend on any prior sequence information for the samples, and is also a sensitive and robust technique. Difficulties were encountered in the use of standard AFLP conditions on pollen beetles. The unmodified technique was of no use, hence a new AFLP protocol, suitable for analysis of genetic variability of pollen beetles, was established. This analysis may help to understand the basis of insecticide resistance and ways of overcoming it. Such information will support the long-term goal of decreasing insect pest problems on Brassicas in the whole of Europe.

Thus, several modifications were needed to adapt the method for analysis of pollen beetles. Basic modifications included *(i)* alterations of DNA purification, *(ii)* use of two six-cutter restriction enzymes, and *(iii)* modified PCR conditions.

Three methods of DNA isolation were tested (Reineke *et al*., 1998): *(i)* a method modified from Blanchetot (1991), *(ii)* a method modified from Marchant (1988) and *(iii)* a modified CTAB-method from Moeller *et al.* (1992). The Blanchetot method resulted in a genomic DNA preparation of very low concentration, owing to low DNA recovery, while DNA resulting from the use of the Marchant method was not sufficiently pure, even after additional purification steps (Ranamukhaarachchi *et al*., 2000). By contrast, DNA extracted according to the CTAB-method was highly concentrated and quite pure after subsequent spermine precipitation (to remove potential inhibitors of restriction endonucleases), together with additional RNase A treatment. Moreover, the protocols of Blanchetot and Marchant use phenol and phenol/chloroform extractions, that may result in partial digestion of DNA if not efficiently removed, whereas a crucial prerequisite for AFLP assay is the completeness of the digestion of DNA by restriction endonucleases. On the basis of these data, the modified CTAB-method was selected for further experiments (Fig. 8).

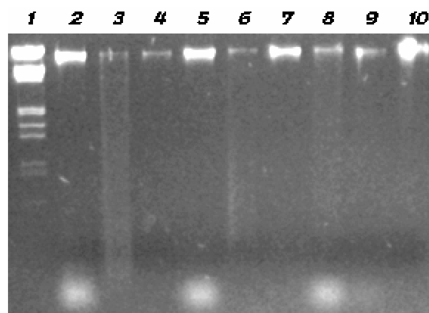


Fig. 8. Lanes 2, 5, 8 correspond to unpurified DNA, lanes 3, 6, 9– to DNA precipitated with spermine, lanes 4, 7, 10 – to DNA precipitated with spermine and RNase A treated, lane 1 – to marker λ/HindIII+EcoRI. Lanes 2-4 – Pollen beetle DNA of Hällberga; lanes 5-7 - of Klockricke; lanes 8-10 - of Kölback. DNA was extracted by the CTAB-method. Samples were analysed on 1.5% TAE agarose gel, 150 A, 90 V, for 40 min.

Initially, two restriction enzymes customary in AFLP analysis were used, the frequent-cutter (four-base cutter) *Mse*I and the rare-cutter (six-base cutter) *Eco*RI. Digestion of pollen beetle DNA with these two enzymes created fragments that were very small and numerous, *i.e.* not appropriate for further analysis. To improve the situation, several alternatives were tested: the use of *Mse*I primer with three selective nucleotides instead of one, to reduce the number of fragments; the use of another four-base cutter (*Taq*I) and the use of a second six-base cutter (*Pst*I) to reduce the number of fragments and improve their size range.

Only the use of two rare-cutters, the six-base cutters *Eco*RI and *Pst*I (18 primer combinations used) gave an acceptable size distribution of fragments up to 700 bp. Based on the observed size distribution and number of fragments, four primer combinations were selected for further studies, E-AT/P-AAT, E-AT/P-ATC, E-GT/ P-AAT, and E-GT/P-ATC. An increase in the concentration of ATP (2 mM instead of 1 mM) and BSA (100 ng/µl instead of 50 ng/µl) at the restriction-

ligation step, improved DNA amplification. In addition, an increase in the amount of DNA (from 11 to 20 ng) and in the concentration of *Eco*RI selective primer (from 2 pM to 5 pM), improved the intensity of bands, and thus facilitated subsequent fragment analysis.

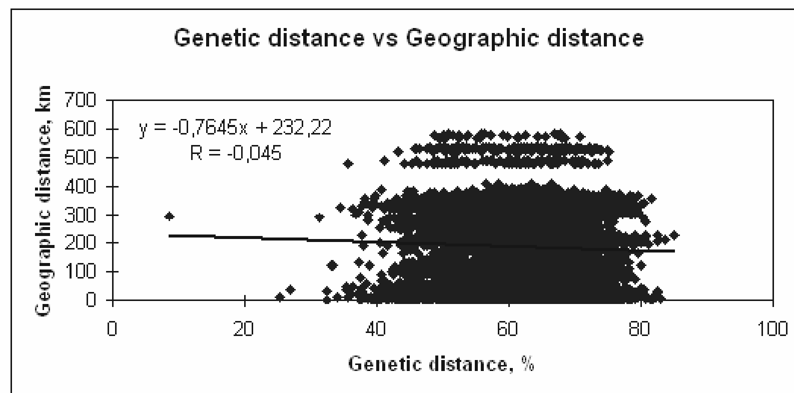
To analyse the potential variability of pooled insect DNA samples, AFLP genotyping of pollen beetle populations, of single insects, of body parts from a single insect and of eggs, was performed. Obtained DNA fragment patterns within a small population were apparently identical with the primer combinations tested. It should be noted that a pollen beetle lay eggs on many plants in the same and adjacent fields, and sampling of insects in a limited area means that offspring can be expected to have a more limited genetic variation, compared to geographically distinct samples. Indeed, insect samples collected in different parts of Sweden were easily distinguished from each other, owing to reasonably high polymorphism levels. A dendrogram was constructed on the basis of an analysis whereby several pollen beetles from each location were pooled before DNA extraction. A high degree of variability was observed between the locations. To study the degree of polymorphism of three pollen beetle populations, peaks resulting after scanning of AFLP fragments in polyacrylamide sequencing gel were compared. For all sources of pollen beetles tested ("Hal", "Klo", "Kol"), a stable repetition of results was observed for *(i)* different DNA extractions; *(ii)* different restriction-ligation steps; *(iii)* different PCR amplifications. The scoring difference was no more than 2% in all cases.

The modified protocol resulted in a favourable number of fragments of an appropriate size range for standard gel analysis by a DNA sequencer, applicable to a single insect and even body parts, enabling different assays to be conducted on a single specimen. Pollen beetles from different areas of Sweden were analysed to verify the reproducibility and efficacy of the protocol, as well as for phenetic analysis. The high reproducibility of the modified AFLP protocol allows it to be used as a reliable tool for genotype analysis of pollen beetles.

Genetic diversity in pollen beetles (*Meligethes aeneus*) in Sweden: role of spatial, temporal and insecticide resistance factors (paper II)

AFLP analysis, using the modified technique (Kazachkova *et al*., 2004) with the restriction endonuclease combination *Eco*RI and *Pst*I, was performed on 133 samples of pollen beetles, both susceptible and resistant to pyrethroid insecticides, collected during the years 2001 to 2004 from five different provinces of Sweden. Using one primer combination, more than 450 polymorphic DNA fragments were obtained, and in total, four primer combinations were used for analysis of pooled samples of three beetles. A sub-sample of 59 single beetles was analysed using one primer combination. AFLP profiles were analysed by similarity measures, using the Nei and Li coefficient, and dendrograms were generated using TREECON software. The dendrogram built using 133 samples showed four distinct groups. Two groups contained beetles from the first generation sampled and the second and third generation each fell into separate groups. Statistical

analysis of single beetle samples by means of AMOVA, showed no evidence of significant genetic difference between resistant and susceptible beetles. Instead, a clear difference between populations, depending on time and generation, was observed. Expected regional population structure, although statistically significant, explained little of the variation. The correlation between genetic and geographic distances was tested with a Mantel test (999 permutations) both for analysis from three beetles and from a single beetle, the results appeared to be identical (Fig.9). It showed that there is no clear dependence of genetic distance on geographic distance, e.g. populations isolated from each other over 5.3 km have almost the same genetic distance (64.7 % and 64.3 % respectively) as populations isolated over 532 km (Högby vs. Flistad and Ransta vs. Hennes respectively). The coefficient R is negative (-0.045 for three beetles analysis and -0.063 for a single beetle analysis) and running to zero shows that there is almost no linear relationship between the variables.



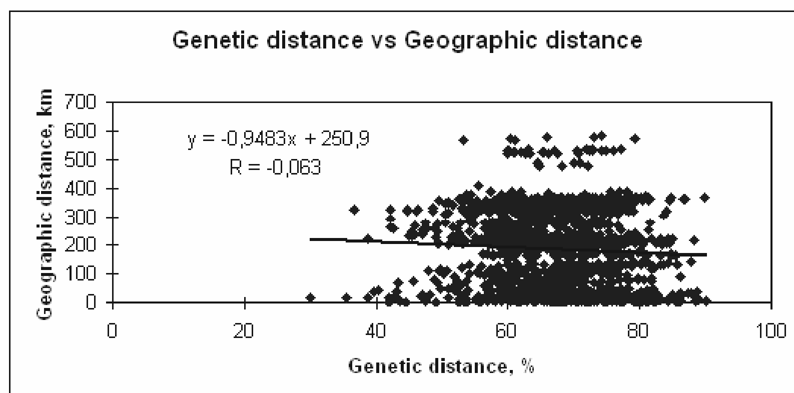


Fig. 9. Mantel test, the correlation between genetic and geographic distances of Swedish pollen beetle populations.

A: Three beetles analysis. B: A single beetle analysis. Test based on 999 permutations.

The levels of genetic variation within populations were very high. There appears to be a high rate of gene flow between pollen beetle populations.

The proportion of variability explained by different generations was the highest among the explored factors. This observation supports the conclusion that there is considerable gene flow among populations, already indicated by migration estimates. There was no significant difference between the percentage of resistant beetles at a location before and after hibernation. This suggests that most of the long-distance dispersal of beetles takes place during the spring and summer. Population differentiation from one generation to the next may be influenced by environmental factors. For instance a cold, wet summer may be more advantageous for some individuals, causing increased survival. If the weather in the following summer is different (which is often the case), then other individuals will have increased fitness, and the genetic makeup of the two generations will be somewhat different.

Our analysis suggests that there is a large gene flow within and among pollen beetle populations. Widespread oilseed Brassica cultivation in Sweden has provided an enormous resource for the feeding and reproduction of pollen beetles, and has resulted in high numbers of individuals. Because this is recent in evolutionary terms, and because genetic drift has not yet had time to sort out variants, one would expect a lack of population structure, especially if pollen beetles migrate extensively. The selection pressure imposed by insecticide use, and information about genetic structure in pollen beetle populations, may provide more information about the extent to which the exchange of individuals between populations—enhancing gene flow—may counteract gene frequency changes, and thereby limit the development of insecticide resistance (Lenormand & Raymond 1998). We have demonstrated that the AFLP technique is a useful tool for studying genetic polymorphism of an important insect pest, the pollen beetle and thereby allow analysis of genetic variability, population structure and gene flow. Such information can be useful for design and evaluation of novel plant protection strategies since pollen beetle resistant Brassica germplasm is not available. For example the long-term effects on pollen beetle population dynamics of a recently developed oilseed rape line containing pea lectin in the pollen (Melander *et al.*, 2003) could be followed.

Genetic diversity in European pollen beetle (*Meligethes aeneus*) populations (paper III)

The modified AFLP technique (Kazachkova *et al.*, 2004) was also applied for study of genetic differentiation between 14 populations of pollen beetles, collected during the year 2004 in six European countries (Denmark, France, Finland, Germany, Sweden and the U.K.).

Evidence of geographic differentiation in the analysed pollen beetle material was relatively strong. The dendrograms constructed from distance matrices revealed well-supported clusters. AMOVA supported the clusters by

comparatively high genetic variation among populations; particularly among populations within countries. Estimates for migration between countries were generally very low suggesting a low level of gene flow between populations. These data were also supported by assignment test, which showed that almost all individuals are assigned to populations of their origin meaning low gene flow. On the other hand the Mantel test revealed no correlation between geographic and genetic distances (Fig. 10). Populations from Finland, Denmark and France showed significant differentiation, but were all found in the same clade in the dendrogram.

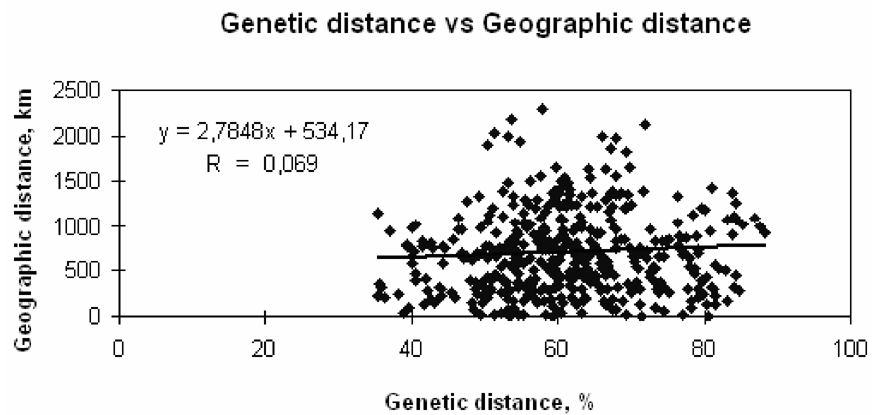


Fig. 10. Mantel test, the correlation between genetic and geographic distances of European pollen beetle populations. Test is based on 999 permutations.

The main result of this study is a clear genetic divergence among European populations of pollen beetle revealed by AFLP markers. The evolutionary forces influencing genetic differentiation among populations are: natural selection, random genetic drift and mutations promote differentiation while phenotypic plasticity and gene flow delay or prevent differentiation. Low gene flow observed between European populations of pollen beetle indicates a high level of genetic diversity. Both intrinsic (i.e., biological; reproductive system, vagility and dispersal behaviours) and extrinsic (i.e., environment; physical barriers and selection gradients) are expected to influence gene flow parameters. Physical barriers (e.g., mountains, rivers etc.) will have a tremendous effect on the genetic connectivity of individuals occurring on either side of such barriers. Thus, gene flow between populations of a species is a complex interaction between the innate vagility/dispersal ability of a species and its physical environment. In our case genetic diversity of pollen beetle populations most likely can be caused by long-term genetic isolation of the separate groups. Baltic Sea forms a barrier to gene flow between Swedish, Finnish populations and the rest of populations. North Sea and English Channel separate UK from the rest of Europe. Danish Flakkebjerg is an island population, German Ruegen population is separated from other German and French populations with the river Elbe, French and German populations are divided by the river Rhine and hilly locality, French population Indre is separated from the rest of populations with the river Loire. All these barriers explain the

division between British and the rest of populations, the separation of Danish Flakkebjerg from Danish Hjörring and Holböh populations, the division between German and French populations and the difference of French Indre population from other French populations. In contrast, we can see the division between two neighbour German populations – Torland and Weendelsgraben. It may be explained by a genetic bottleneck caused by different pest management strategies or by the effect of a small population size, which leads to the increase of genetic drift, as the rate of drift is inversely proportional to the population size, increase of inbreeding due to the reduced pool of possible mates and as a result two neigbour populations become genetically distant. However, we can also see the clustering of Finnish with Danish and French populations, Danish Flakkebjerg with French Indre population and clustering of German Ruegen with German Torland population. Such clustering can be explained by historical processes, when current patterns of gene flow that have only recently been contributing to the genetic structure of a population may be masked by the influence of historical gene flow. During major glaciations many European species were restricted to southern refugia, in which populations were isolated and then expended to the north during the interglacials (Timmermans *et al.,* 2005). Also the transportation of a pollen beetle with *Brassica* crops is possible, when it arrives as a population and then expands after introduction.

Low level of expected heterozygosities observed here also points out the small genetic variability and implies that there is a degree of sib-mating in a small effective population. At positive assortive mating the lack of heterozygotes may be due to Wahlund effect, where individuals treated as one population are actually two or more distinct populations, which are differentiated and have little or no gene flow between them.

Low migration rate observed between European populations is one of the factors that may influence resistance development. Together with the fact that pollen beetles feed preferably on pollen from *Brassicas*, low migration rate becomes a very important biological factor in pest management, especially in conditions, when there is a prolonged exposure to the pyrethroids in these areas and large areas are treated. In these conditions it is very important to minimize selection pressure to keep susceptible insects alive, use mixtures or rotation of insecticides and keep untreated areas of the crop to have refugees.

Our analysis suggests that there is a low gene flow within and among European pollen beetle populations in contrast with a higher rate of gene flow observed in Swedish populations. Low level of expected heterozygosities points out the small genetic variability. This resulted in clear geographic differentiation among European populations of pollen beetles. We have also shown that AFLP technique is a powerful tool for genetic analysis of populations, generating a better understanding of the basic genetic variation among different populations of pollen beetles and may be used for further analysis..

Analysis of common insecticide resistance genes in pollen beetles (Voltage sensitive sodium channel gene and CytochromeP450, paper IV)

To identify possible point mutations associated with pollen beetles resistance to pyrethroids, the primary target site for pyrethroids—voltage-sensitive sodium channels (VSSC, *para* sodium channels in insects)—were studied by means of the RT-PCR technique. Two susceptible (BjökebergS03a, SättunaS03a) and three pyrethroid-resistant populations (BjökebergR03a, SättunaR03a and KölbäckR02s) representing the Swedish province Östergötland were used in this study.

A *para*-specific antisense primer, complementary to the sequence at the 3′ end of the *para* coding sequence, was used in cDNA synthesis of VSSC, and an oligo(dT) primer in cDNA synthesis of CYP450. The first-strand cDNA was used as a template to amplify *para* cDNA fragments using primers designed to homologous sequences from several insects (for VSSC cloning, primer 1, 2, 4; primer 3 was designed from the sequences of a pollen beetle obtained by primers 2 and 4) and using primers specific to CYP4S and CYP4G8 genes (for CYP450 cloning). Primers were designed so that the amplified product would overlap with approximately 30-65 bp of the sequence, in order to confirm unequivocally that the sequence of the product obtained corresponded to the same cDNA. Following reverse transcription, the first-strand cDNA is used directly in amplification of VSSC or CYP fragments.

PCR products were extracted from the gel, cloned using TA-Cloning kit and subjected to sequencing analysis. Multiple sequencing reactions were performed to test the accuracy and reproducibility of the obtained results. Four cDNA fragments composing VSSC domains I and II were amplified and compared by the MacVector and ClustalW software. Pollen beetle VSSC cDNA sequences were analysed and compared with published VSSC sequences, using the Blast search engine, ClustalW and ClustalX programs showing point mutations, which may confer pyrethroid resistance.

The main result of this study was the isolation of the VSSC partial cDNA (almost 3 kb, domain I and II) from susceptible and resistant pollen beetles, not available earlier. Four overlapping fragments were amplified: fragment 1 contains 599 bp, fragment 2 – 677 bp, fragment 3 – 1328 bp and fragment 4 – 474 bp, respectively. The composite VSSC cDNA contains 2943 bp (I and II domains of the VSSC) with an ORF encoding 981 amino acids. The obtained amino acid sequence of *Meligethes aeneus* shares 66 to 80 % overall identities with other insects. The highest similarity of *M. aeneus's* VSSC was to sodium channels of other *Coleoptera* (Fig. 11).

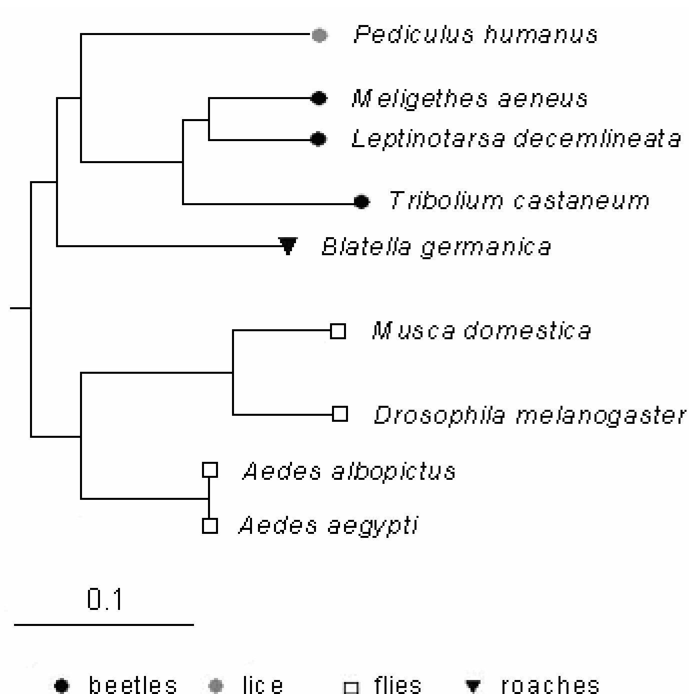


Fig. 11. Dendrogram of insect VSSC.
The tree was produced using ClustalW pairwise alignments.

Compared to susceptible strains resistant strains of cDNA fragment have 11 nucleotide substitutions (seven - in domain I and four – in domain II of VSSC), which do not cause substitutions on amino acid level, i.e. these point mutations are silent. All clones of SättunaR03a and BjökebergR03a have one nucleotide substitution, which causes the substitution of amino acids as well (F124Y), and all clones of KölbäckR02s have two point mutations (G308D and H404R).

Also metabolic resistance sites—Cytochrome P450s, were studied on the same populations of pollen beetles using RT-PCR technique in resistant and susceptible insects. Comparison of CYP450 genes (CYP4S and CYP4G8) sequences of resistant and susceptible strains (BjökebergS/R03a, SättunaS/R03a) revealed five point mutations: three for CYP4S gene (S124N, S142T, A148V) and two (E4K, R142S) – for CYP4G8. Also ten nucleotide substitutions were found, five for each gene. Among insects' CYP4S genes S142 and T142 are quite conserved, N124 is highly conserved while A148 is present only in *Anopheles funestus;* Among CYP4G8 genes, E4 is conserved among many insects and R142 is highly conserved: only *Drosophila pseudoobscura* and *D. melanogaster* have K142.

F124, G308 and H404 are conserved among insect sodium channel proteins. These mutations may be responsible for the high level of *kdr* resistance to pyrethroids in the *Meligethes aeneus*. Pyrethroids slow the kinetics of sodium channel activation and inactivation, resulting in the prolonged opening of individual channels, and leading to paralysis and death of poisoned insects, thus point mutations can directly or indirectly alter pyrethroid binding affinity or they

may modify channel gating kinetics. Thus, a shift of the voltage dependence of activation or inactivation to more depolarizing membrane potentials could counteract (antagonize) the action of pyrethroids (Soderlund & Knippe, 2003).

Northern blot analysis has been initiated to study CYP4S and CYP4G8 transcript levels in pollen beetles. The transcript level varied extensively between different insects showing that these genes are under active transcriptional control. However, no consistent correlation between CYP4 transcript levels and resistance was found. Additional analyses (e.g., real-time PCR) to more exactly determine the level of CYP4S and CYP4G8 overexpression in pyrethroid-resistant strains should be performed. The precise role of CYP4 variants expressed in resistant insects should be assessed via the ability of the expressed protein to metabolize pyrethroids.

Thus, we can conclude that resistance of pollen beetles to pyrethroids can be the result of either mutations in VSSC gene or combination of different type of mutations, like point mutations in VSSC gene and overexpression of specific CYP450 genes.

Conclusions

Summarizing all results presented in this thesis, next conclusions can be drawn:

- The modified AFLP protocol presented here is well suited for a detailed analysis of genetic variability in one of the most important insect pests of Brassicaceae plants - the pollen beetle. All of the modifications applied resulted in well-resolved and evenly distributed DNA fragments within the desired size window. Finally, the established AFLP technique shows a high degree of reproducibility in the species concerned.
- On analysis of Swedish and European populations of pollen beetles the AFLP technique was demonstrated as a useful tool for analysing the genetic polymorphisms of insect pest populations and thereby population structure and gene flow.
- Analysis of Swedish pollen beetle populations suggests that there is a large gene flow within and among populations. Expansion in Brassica cultivation during the last decades in Sweden provided an enormous resource for feeding and reproduction of pollen beetles and allowed a rapid spread. If this occurred recently from a mixed origin and genetic drift has not yet had time to sort out variants one would expect a lack of population structure, especially if pollen beetles migrate extensively. The selection pressure imposed by insecticide use and information about genetic structure in pollen beetle populations may provide more information about the interaction between these two processes e.g. to what extent exchange of individuals between populations enhancing gene flow may counteract gene frequency changes and thereby limit development of insecticide resistance.
- Analysis of European pollen beetle populations reveales a low gene flow within and among populations. This resulted in clear geographic differentiation among populations. Geographic differentiation of European pollen beetle populations can also be explained by such factors of natural selection and adaptation as weather conditions (warm or cold, wet or dry summer), the use of the particular insecticides and their different dosage etc. as well as mutations, genetic drift, which leads to fixation of alleles or genotypes in populations.
- Three point mutations potentially associated with pollen beetle resistance to pyrethroids, were identified in the primary target site for pyrethroids - voltage-sensitive sodium channel and five point mutations were found in CYP450 genes (CYP4S and CYP4G8) by RT-PCR technique and cloning/ sequencing analyses. A pollen beetle VSSC partial cDNA was obtained.

Future perspectives

- Analysis of further European populations of pollen beetles in context of their susceptibility to insecticides.

- Study of pollen beetle populations dynamics, prey-predator relationship and distribution (e.g. using different models such as Lotka-Volterra model).

- Continuation of VSSC gene analysis (III and IV domains) for identification of possible mutations in this gene and to obtain a VSSC full-length sequence.

- Characterization of pharmacological effects of VSSC point mutations using an in vivo expression system (such as Xenopus oocytes) probed with pyrethroids.

- Test of different novel sources for protection of *B. napus (Bacillus thuringiensis* crystal proteins etc) to pollen beetles.

- Analysis of other metabolic “resistance genes”, such as CYP450, GST for identification of possible mutations and analysis of overexpression levels associated with pyrethroid resistance.

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Acknowledgements

This work has been carried out at the Department of Plant Biology and Forest Genetics at the Swedsih University of Agricultural Sciences, Sweden and has been financially supported by grants from Stiftelsen Lantbruksforskning, the project "Molecular Ecology" financed by the Faculty of Natural Resources and Agricultural Sciences at SLU, Helge Ax:son-Johnsons Stiftelse and Nilsson-Ehle Stiftelsen.

First of all I would like to thank my main supervisor Johan Meijer for all your support, help and encouragement during these unforgettable years. Thank you for our group meetings and activity, for all interesting discussions, not only scientific, for the possibility to learn a lot from you. Also I would like to express my sincere gratitude to my assistant supervisors, Barbara Ekbom and Janne Fahleson. Thank you for sharing your experience with me, for your help and moral support.

My special thanks for Anna-Karin Kuusk for a collection of pollen beetles from Swedish provinces, without it my work would be impossible. Thank you very much and all the best in your study and research.

Thanks to Inger Åhman for our interesting and effective collaboration that helped me to obtain extra knowledge and ended with the publication. Thanks to the entomology group for our journal club discussions, to the members of the biotic stress journal club for scientific discussions, to our Department for organizing interesting seminars.

My very special thanks to Dr. Jeremy Flower-Ellis, who taught me how to write and publish scientific papers, for all your help with my first paper and this thesis, for your wisdom, energy and optimism shared with me, for our discussions and the possibility to listen to your perfect English.

Also I would like to thank my first scientific supervisor Svetlana G. Makarova for the introducing me to a wonderful world of genetics. Thanks to my first teacher of English Svetlana Haritonovna and my University teachers of English Elena Aleksandrovna and Nataliya Georgievna for all your patience, nice discussions and all I have learned from you.

Special thanks to Academician of NASU professor Vitaly A. Kordium, Head of the Dept. of Cell Regulation Mechanisms of the Institute of Molecular Biology and Genetics in Kyiv for the pleasure to work at your Department and possibility to learn from you. Thanks to Svetlana E. Rymar for introducing me to the basic genetic and molecular biology methods and for the first steps of my career under your guidance. My thanks to Irina

Isakova, Nina Aleksandrovna D. and Lyubov Petrovna Z. for your help and moral support, to Nataliya A. Kozyrosvka for support and interesting discussions. Special thanks to Boris Troyanovsky for your help, optimism and support. Thank Oksana and Inna for your friendship, our chatting and all the fun. Thanks to all members of the Department for sharing your experience, for all scientific discussions and different kinds of activities.

I would like to express my sincere gratitude to Dr. Anatoliy I.Potopalsky for the pleasure to collaborate with you, for all you have done and will do for Ukrainian science, for a wonderful preparation izatizon invented by you that helped me and my family so much during these years. Я щиро дякую Вам за все, бажаю творчої наснаги та нових досягнень!

Thanks to our Prefect Christer Jansson, our rector Ann-Christin Bylund and the whole Department for your unforgettable support during February-April of the last year. It will always be in my heart.

Also I would like to thank my group, for all work and fun together, thanks to Derek for our discussions and some practical advises, for being my friend during these five years. Thanks to Jeppe for your friendship and all special moments, for the possibility to share everything with you, either sad or happy, for all the jokes, for always being patient and nice with me. Thanks to Ritu and Romit for your energy, friendship and our trips, for good food and lots of fun. Thanks to Sarosh for your support, optimism, good mood and help. Thanks to all present and past members of our group and GC – Wang Jing, Anne-Marie, Ulrika, Oleg, Ilona, Sasha, Harald, Lena Holmberg, Ingrid E. and many others for your help and friendship. Also I thank Mattias P. for your friendship, support and our chattings. Thanks to Birgitta, Lotta and Mona for your help and support, to Ingrid S. and Yvonne for the help with AFLP gels, Björn and Lena for the help with computers. Special thanks to my friend Lena Marklund for our friendship, discussions of different aspects of life, for all your help and support! My special thanks to Elke for the friendship, our great activities, chattings and sushi.

Also special thanks to our Russian and Ukrainian-speaking community for all the helpful advices, keeping our traditions, for all the fun, interesting trips together: to Anja, Oksana, Marina, Olga, Lena K., Vlad, Masha, Sasha, Peter, Lada, Eugen, Nastya, Ilona, Jurga, Ira Iv., Tanya Nussli, Yana T., Andrey, Tatiana Zhoukova and your big family, Julia and Maksim. Thanks to Göran Hjälm for your friendship, hospitality and very tasty apple-pies. Дорогие друзья, сердечно благодарю вас всех за вашу дружбу, поддержку, за все радостные моменты нашей здешней жизни!

Окремо хочу від щирого серця подякувати п. Стефанії та п. Стефану за те, що дарували мені часточку рідної України, за вашу гостинність, за чудові українські страви, за тепло ваших сердець, за вашу любов до

України, яка надихала мене, за той український дух, якого тут мені так не вистачало!

My very special thanks to all my friends and dear people in Kyiv and whole Ukraine. Огромное спасибо всем тем, кто поддерживал меня в самые трудные минуты моей жизни, кто делил со мной горе и радость, кто помогал во всем. Спасибо жителям родного поселка Димитрово в Кировоградской обл. – О.А.Маломуж, Игорю, М.Д.Магдюк, Н.А. Дибровой, т. Дайне, а также Л.П. Горбанёвой, Л.П. Давыдовской, всей моей семье и др., кто поддержал меня в трудную минуту. Спасибо Натали, моему настоящему и преданному другу, приходящему на помощь в любое время суток, спасибо тебе за всё, что ты для меня сделала, за поддержку, веру в мои способности, за то, что всегда находишь слова, чтобы преободрить и вернуть веру в свои силы. Дуже дякую Антонині Андріївні за родинну гостинність та підтримку. Спасибо Марине и Тамаре Григорьевне, Александру Алексеевичу, Наталье И.К., семье Макаровых и Руслану в частности, Юле, Наташе Ф. и всем остальным моим друзьям за моральную поддержку и дружбу. Спасибо Володе за помощь с компьютером и дельные советы.

Finally, I would like to express my sincere gratitude to my family. Thank you, my parents Ivan and Olga, for putting so much love, trust and support in me. This work is also your work. Thank you, father, for bringing up the love to Science in my heart that I always wanted to be a Professor, like my father, for guiding me in scientific world. This work would not be possible without you. Спасибо за всё, чему ты меня научил! Спасибо, дорогая мама, за твою любовь и заботу, без которых бы мне никогда не удалось достичь того, чего я достигла. Низкий тебе поклон. Спасибо моей сестре Алёне и дорогой племяннице Маргарите за любовь, поддержку и радость, которую вы мне дарите.

Огромное спасибо моим дорогим бабушкам Марии и Анне, дедушкам Николаю и Василию и всем родным, кого уже нет с нами, за их любовь, заботу и доброту. Вы всегда будете в моем сердце.

Щиро ваша, Надія

Искренне ваша, Надя

Sincerely yours, Nadiya

Obstacles cannot crush me. Every obstacle yields to stern resolve. He who is fixed to a star does not change his mind.

Leonardo Da Vinci