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# SHAPE OF CLINES IN MELANISM IN LADYBIRDS AND THE POSSIBLE ROLE OF MIGRATION

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**SUMMARY**

*Adalia bipunctata*, the two spot ladybird beetle, has different colour morphs.

A few decades ago, there were large differences in frequencies of these colour morphs in The Netherlands: in the West, the non-melanic morph was predominant, but in the South-East, the melanic one was more common. More recently, the local differences in frequency of colour morphs have become less pronounced.

Migration might be important to explain the percentage of melanic and non-melanic ladybirds at different localities, because migration can influence the steepness of the clines. A cline is a gradual change in character or allele frequency along a geographic transect, that can be gradual if migration exists and more stepped if it doesn't exist.

With this thesis I want to investigate whether migration may play a role in shaping the clinal variation in *A. bipunctata* using microsatellites. Microsatellites, also called SSR (Simple Sequence Repeats), are a class of genetic polymorphism commonly used for population studies.

The other aim is to investigate whether the selection-pressures of the putative force of thermal melanism continue to change in *A. bipunctata*, and if this selection also influences colour polymorphism in *Harmonia axyridis* (Coleoptera: Coccinellidae).

# INTRODUCTION

## 1. Climate change and colour morphs

*Adalia bipunctata*, the two spot ladybird beetle, has different colour morphs. They can be black with usually 4 or 6 red spots, called melanic morph, or red with two black spots, called non-melanic (de Jong and Brakefield 1998).

A few decades ago, there were large differences in frequencies of these colour morphs in The Netherlands: in the West, the non-melanic morph was predominant, but in the South-East, the melanic one was more common (de Jong and Brakefield 1998).

One hypothesis to explain these differences involves the thermal biology of the beetles. A black surface absorbs radiation (including sunlight) more efficiently, so the melanic ladybirds can warm up faster and reach higher body temperatures. When sunshine is limiting for ladybird activity, the black ones may have a thermal selective advantage (de Jong, Gussekloo and Brakefield 1996).

More recently, the local differences in frequency of colour morphs have become less pronounced. This could be possible because of the climate change (de Jong and Brakefield 1998).

Additionally, migration might be important to explain the percentage of melanic and non-melanic ladybirds at different localities, because migration can influence the steepness of the clines in melanism (see figure 1). A cline is a gradual change in character or allele frequency along a geographic transect, that can be gradual if migration exists and more stepped if it doesn't exist.

So far, we have never studied migration in the two spot ladybird. This thesis describes a study of migration using microsatellites.

In the period of 1980-1995, the clines in melanism in *Adalia* were thoroughly studied by repeated sampling of a number of localities in The Netherlands (Figure 1).

The places where the samples were taken around The Netherlands were: Eindhoven, Waalre, Tilburg, Prinsenbeek, Oudenbosch, Etten L., Zevenbergen, Klundert, Willemstad, Ooltgensplaat, Achthuizen, Oude Tonge, Middelharnis, Dirksland, Stellendam, Goedereede, Ouddorp, Delft, Rotterdam, Rhoon, Barendrecht, Heinenoord, Mijnsheerenland, Klaaswaal, Numansdorp, Helwijk, Dinteloord, Steenbergen, Bergen op Zoom, Antwerpen, Waalwijk, Utrecht C (Figure 1).

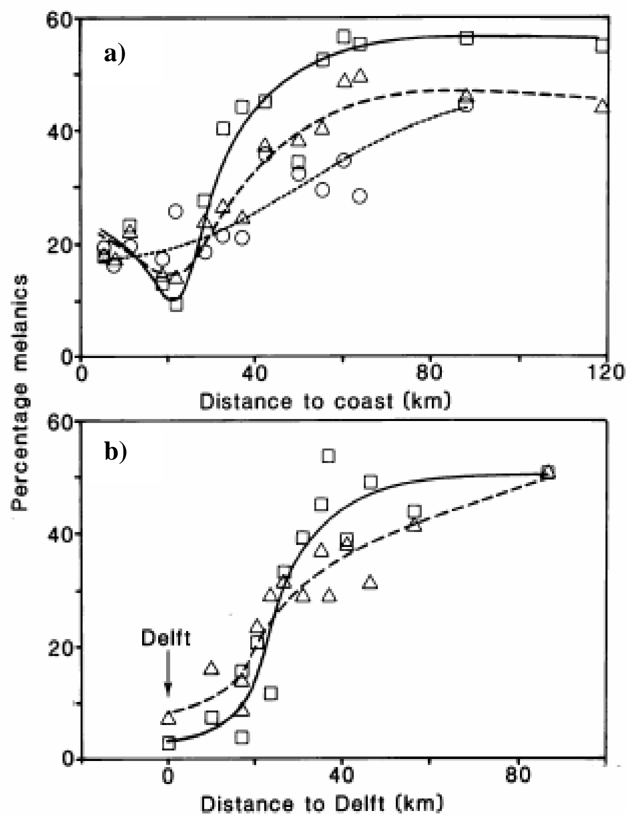



**Figure 1. Frequencies of melanics in different places and years.**

**a)** Clinal variation in the relative frequency of melanic two-spot ladybirds along the east-west transect.

**b)** The north-south transect for successive collections.

**Symbols:**

□ Collections in 1980.

△ Collections in 1991 (east-west cline) and 1992 (north-south cline).

O Collections in 1995.

(de Jong and Brakefield 1998).

*Adalia bipunctata* shows clinal variation in the frequency with which melanic and non-melanic morphs occur. The clines in melanism in The Netherlands have changed over the past two decades, becoming shallower (Figure 1). Most populations in the south-east of The Netherlands showed a significant decrease in relative frequency of melanics, while in the west some populations showed a significant increase. A coinciding increase in local ambient spring temperatures was found for most weather stations close to the samplings sites. This is important data because the change in cline shape is correlated only with an increase in spring temperatures. A general increase in temperature is expected to reduce the contribution of radiation to warming up of the ladybirds, and hence reduce the thermal difference between the morphs. However, the role of migration in producing the precise shape of these clines has remained unknown until now. Therefore, we here present data using microsatellites, and evaluate the possible role of migration.

## 2. Microsatellite analysis

Microsatellites are also called SSR (Simple Sequence Repeats). They are a class of genetic polymorphism commonly used for population studies, conservation biology and other applications like mapping, forensics, diagnosis and identification of human diseases, linkage analysis and to trace inheritance patterns.

Microsatellites are tandemly repeated sequences, where the repeating unit is 1 to 4 nucleotides long (Table 1). The number of times the unit is repeated in a given microsatellite can be highly variable, a characteristic that makes them useful as genetic markers.

**Table 1.** Examples of microsatellites
(www.bakerlab.berkely.edu/blims/documents/uploads/0625040454_microsatellites.ppt).

| | |
|---|---|
| **Mononucleotide SSR (A) 11** | AAAAAAAAAAA |
| **Dinucleotide SSR (GT) 6** | GTGTGTGTGTGT |
| **Trinucleotide SSR (CTG) 4** | CTGCTGCTGCTG |
| **Tetranucleotide SSR (ACTC) 4** | ACTCACTCACTCACTC |

Diploid individuals have two copies of a microsatellite (MS) locus, and the copies need not be equally long. If they are equally long, the individual is said to be homozygous at the MS locus, and when not, heterozygous:

**a) Homozygous**

…CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCT**ATCGGTACTACGTGG…

…CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCT**ATCGGTACTACGTGG…

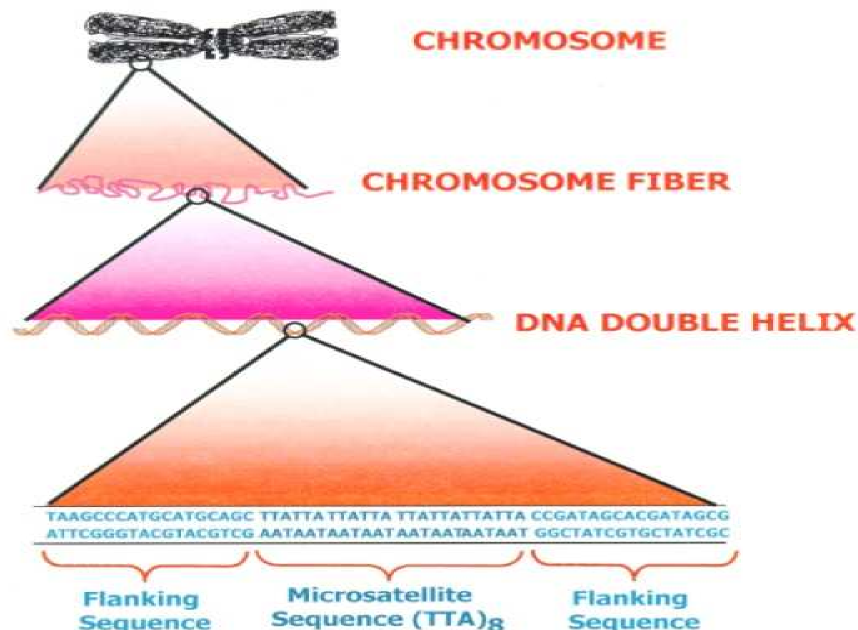
5’ flanking region microsatellite locus 3’ flanking region

**b) Heterozygous**

…CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCT**    ATCGGTACTACGTGG…

…CGTAGCCTTGCATCCTT**CTCTCTCTCTCTCTCTCT**ATCGGTACTACGTGG…

**Figure 2.** Place where the microsatellites are found (www.bakerlab.berkely.edu/blims/documents/uploads/0625040454_microsatellites.ppt).




The majority of MS are in non-coding regions (Figure 2). There may exist many alleles (up to 70 or 80) at a single microsatellite locus.

Microsatellites may exist because they're a necessary source of genetic variation, "junk" DNA or they regulate gene expression and protein function.

Their advantages for molecular studies are the next ones:

- Relative ease of use.
- High levels of accuracy.

Microsatellites can be studied by means of PCR. The region containing the microsatellite is amplified by PCR using primers that bind to the flanking regions of the microsatellite.

The size of the DNA amplified is determined by the number of repeats present in the MS at that locus. Diploid individuals typically have two alleles for all microsatellites. If the number of repeats of one allele is different from the other, then two separate bands can be detected.

We applied microsatellites to study population structure in *Adalia bipunctata*. In doing so, we assume that microsatellites are neutral to selection. In other words, allele frequencies are only influenced by migration and genetic drift. This enables us to draw conclusions about the extent of migration by comparing microsatellite allele frequencies in different samples of *Adalia*.

**3. *Harmonia axyridis***

In this project we also studied the % of *Harmonia axyridis* (Coleoptera: Coccinellidae). This lady beetle is native to Asia, arrived to The Netherlands in 2002 and actually has established and is expanding (www.issg.org/database/species/distribution_detail.asp?si=668&di=28870&sts=).

This beetle is also polymorphic for its body colour (Figure 3). To investigate the hypothesis of thermal melanism to explain the maintenance of colour polymorphism, we aimed to compare the morph frequencies of *H. axyridis* with *A. bipunctata*, to see if a common selection pressure is influencing the morph frequencies in both species.

*H. axyridis* is known to colonise a wide range of habitats. They are found in cropping areas, meadows and semi-natural areas (Koch, 2003a).

The invasion pathways to new locations could be explained because of Biological control; *H. axyridis* has been widely used for reducing aphid pest populations in greenhouses, orchards and gardens in North America since 1916 and in Western Europe since 1982 (Koch et al. 2003b; Adriaens et al. 2003). Some examples of the biological control are:

1. In pecan against pecan aphid.
2. In apple orchards against *Aphis spiraecola*.
3. In soybeans against *Aphis glycines*.
4. In sweet corn against *Ostrinia nubilalis* and *Rhopalosiphum maidis*.

*H. axyridis* has also been documented in alfalfa, cotton, tobacco and winter wheat, where it may be contributing to biological control (Koch, 2003a).

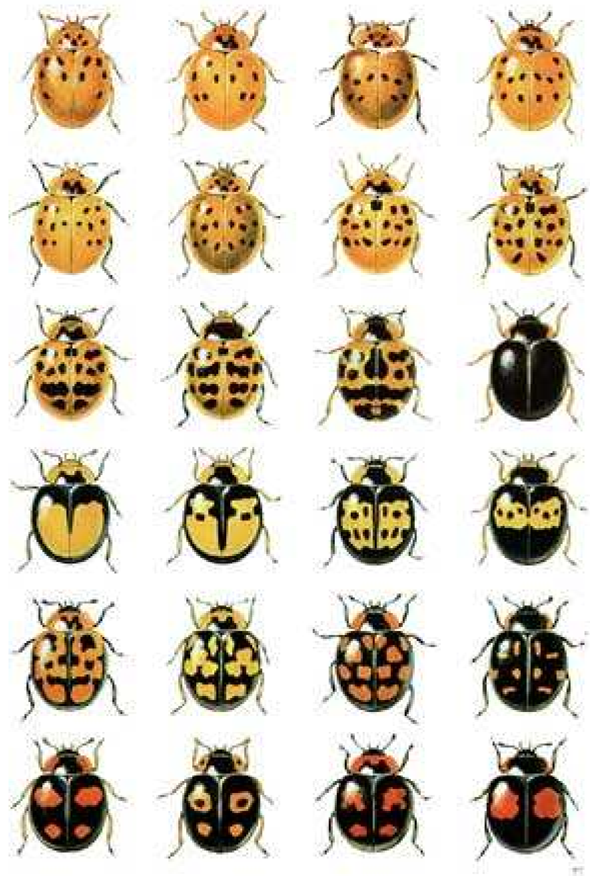
Unfortunately exotic natural enemies may impact organisms other than the targeted pests. Adverse effects of *H. axyridis* on insects, humans, and crops are beginning to be identified (Louda et al, 2003). Some of the negative impact of this coleoptera are (Koch, 2003a):

1. In hemlock trees against *Adelges tsungae*.
2. Predator of *Adalia bipunctata*.
3. Predator of *Coccinella septempunctata*.
4. Predator of *Propilea japonica*.
5. Predator of *Chrysoperla carnea*.
6. Many people become annoyed by the swarms of *H. axyridis* flying toward their homes because often they make their way inside the buildings, where they will overwinter.

7. Some people have developed an allergic rhinoconjuntivitis to *H. axyridis*.
8. *H. axyridis* has been reported to bite humans, and *Adalia bipuntata* is also known to occasionally bite humans.

In The Netherlands, the most common forms of *H. axyridis* can be clearly distinguished into melanic (lower row in fig. 3) and non-melanic (upper two rows) morphs.

**Figure 3.** Color variants (as of about 1975) for *Harmonia axyridis*, the multicolor Asian ladybeetle (www.ent.orst.edu/urban/Harmonia.html).



**OBJECTIVE**

1) To investigate whether the selection-pressures of the putative force of thermal melanism continue to change in *A. bipunctata*, and if this selection may also influence colour polymorphism in *H. axyridis*.

2) To investigate whether (limited) migration may play a role in shaping the clinal variation in *A. bipunctata*.

## MATERIAL AND METHODS

### a) Collection

During several days of May – July 2005 four *Adalia bipunctata* populations in The Netherlands have been sampled (Figure 4).

**Figure 4.** Example of the two transects that were previously sampled in The Netherlands.
Dots indicate collection sites along the transects and ° the places studied in this work.
Delft is taken as a reference site to relate distances along the north-south cline (de Jong and Brakefield, 1998).

We chose a limited number of localities with a relatively large distance between each pair. Therefore, if there is not any differentiation between these, neither there will be between the populations between them. The strategic places to search for the samples were: Wageningen, Tilburg, Leiden and Utrecht.

The samples were collected in different species of trees and plants that we found in the streets:

- Wageningen: *Rosa rugosa*.
- Tilburg: *Rosa sp.* and *Corylus avellana*.
- Leiden: *Rosa sp.*
- Utrecht: *Rosa sp.*, *Hydrangea macrophilla* and *Tilia cordata*.

The material necessary to collect ladybirds was:

- Small bottle with plastic top that has a hole in the center.
- A piece of cotton to cover the bottle's hole.

### b) Identification and sexing

The next step was to bring the samples to the entomology laboratory to identify (Table 6 appendix) and sex the samples. If we had to wait until the next day, we stored the insects in a fridge at 4 °C. Then we differentiated the several species of ladybirds found and between the melanic and non-melanic morphs (of *Adalia bipunctata* and *Harmonia axyridis*) and we put them in different plastic bottles.

Finally we sexed only the two-spot ladybirds. The method followed was the same used by de Jong et al, 1991. To do it we used:

- Stereo-microscope.
- Small Petri plate.
- Tweezers.
- A piece of cotton to cover the Petri plate.
- Phorexpan box with ice.

First we were placing the ladybird in the Petri plate, then we put cotton on top of it and turned the badge so we saw the ventral part of the insect and we placed it under the Stereo-microscope. By observing the differences in caudal segments, the sex could be determined (de Jong et al., 1991).

**c) Isolation of DNA**

The "*Genomic DNA Purification kit*" (Puregene, Gentra) was used to isolate DNA of the samples (Table 2 of appendix). It consists of five different parts: Cell lysis, RNase treatment, protein precipitation, DNA precipitation and DNA hydration.

Finally the DNA was stored at 4 °C.

**d) Loci amplified by polymerase chain reaction (PCR)**

PCR reactions were carried out in a PCR Mastercycler gradient machine (eppendorf).

The cycling conditions used in the PCR reactions were the same used by Haddrill, Majerus and Mayes (2002):

- 94 °C for 15 min.
- 35 cycles of 94 °C for 1 min. Annealing temperature for 1 min.
- 72 °C for 1 min.
- 72 °C for 10 min.

Amplifications were carried out in 25 µL, containing 12,5 µL HSTaq Master Mix (QIAGEN), 8,5 µl RNase-free water (QIAGEN), 1 µL Reverse primer (25 pmol/µL), 1 µL Forward primer (25 pmol/µL) and 2 µL *A. bipunctata* genomic DNA.

**Table 2.** Primers used to amplify microsatellites (Haddrill, Majerus and Mayes 2002).

| Locus | Primer sequence (5'-3') | Repeat motif | Allele size range (bp) |
|---|---|---|---|
| Ab1 | F: CCAAATCCGTTAGTGGAACG<br>R: CATTTTCCCAAGAGAAGTTTGG | $(GA)^{27}$ | 234 - 282 |
| Ab3 | F: TTCTTGACAGAATGATATCCTGAAC<br>R: GGCAACGATTGTTGATCCTTTC | $(GT)^{10}$ | 142 - 176 |
| Ab 7 | F: CACGAGTGTCAACTTATTCAAACTG<br>R: GGACTAACAGGATATCACCTCAAGA | $(TG)^{32}$ | 144 - 220 |
| Ab 9 | F: AGCCACAATTAATCCCCTATCG<br>R: TGTCCATAATTTTGAGCAGAGC | $(TC)^{31}$ | 184 - 286 |
| Ab 11 | F: AATAAATATTTTTGCAGCCCTGAAG<br>R: TGTTGGTTAACATAAATTTCGATTC | $(GA)^{32}$ | 286 - 356 |
| Ab 19 | F: GTGTCATTTCCGGGGTCATTT<br>R: AATCGGACTTATGATGGTTTATTTTC | $(GT)^{11}$ | 210 - 224 |
| Ab 31 | F: AAAGGATAACTGGAGTAGCGGTAG<br>R: TAAATCTGTCAATGCAACATTCATC | $(TA)^{3}(CT)^{2}$ | 304 - 348 |
| Ab 32 | F: TATCCATATCATTCCTGCTCATTCC<br>R: AAGATCAGACCTTGCTTCTCTTAG | $(AC)^{12}$ | 170 - 240 |
| Ab 35 | F: GAAATTGTTAGGTTAGGATCGGAAG<br>R: ATTCACTGTGCGGAGTATTACGTTC | $(GA)^{14}$ | 218 - 260 |
| Ab 38 | F: GGTAATGCTATCCGTAGATATAGACC<br>R: GAAAATATTCACCTTCCGAAGAACC | $(CGG)^{5}(TGG)^{2}$ | 182 – 188 |

These primer-sets were used (Table 2) to identify the loci that showed polymorphism in the Dutch samples. Four polymorphic loci were used to score differences within and between populations.

To determine the allele size of the samples we performed PCR with modified forward primers which contained fluorescent dye (Fam: For Ab1, Hex: For Ab3, Hex: For Ab 11 and Ned: For Ab 19) See figures 1- 4 of appendix.

**e) Electrophoresis**

The PCR products were run on a 1% agarose gel for 90 minutes at 80 Volts, and 400 mA. The Gel electrophoresis used was the Power-Pac 300 (BIO-RAD).

First, with the ten primers the samples were tested on a normal agarose gel. Next heterozygosity was visualized on a high resolution agarose gel (MetaPhor Agarose) to

show the bands more clearly. But finally, with the Forward primers with dyes we again used a normal agarose gel because the resolution was acceptable.

All gels were photographed with a digital camera and Kodak Digital Science software.

**f) Analysis of allele sizes**

Allele sizes were determined on an ABI sequencer (3700 DNA analyzer; Applied Biosystems).

PCR products of each DNA sample were pooled in a 96-well plate (see table 7 appendix for concentration). Purification of these PCR products was achieved by use of Sephadex G-50 columns (Millipore).

The final mix (for size determination) contains per well 5µL of a sizer, formamide mix and 1 µL of the purified PCR products.

Genotyper 3,5 HT software (Applied Biosystems) was used to analyze the data.

**g) Statistical analysis**

For the statistical analysis, the software package Genetic Data Analysis (GDA) was used to analyze the differences between populations in allele frequency (Table 8 appendix).

## RESULTS

The first PCR result found was the number of alleles (Table 3) of 10 microsatellite loci. They were found against 11 individuals, one from Wageningen and 10 from Leiden (these samples were not studied later).

**Table 3.** Primers used with their annealing temperature and the number of alleles found.

| Loci | T (°C) | No. alleles |
|---|---|---|
| Ab1 | 55 | 5 |
| Ab3 | 54 | 4 |
| Ab7 | 55 | 1 |
| Ab9 | 65 | 3 |
| Ab11 | 60 | 3 |
| Ab19 | 60 | 5 |
| Ab31 | 60 | 3 |
| Ab32 | 65 | 3 |
| Ab35 | 65 | 4 |
| Ab38 | 60 | 1 |

With these gels we could see that eight out of ten loci were polymorphic within this limited sample. Then we selected just four of these polymorphic ones for the rest of the study.

With the bootstrap analysis using GDA (table 8 appendix) we analyzed the differences between populations in allele frequency and the result was (table 8 appendix) Theta-P (F-st) = 0.02. This value means that there is high migration; the value can potentially vary from 0 (no limitation to migration) to 1 (complete isolation between samples)

The analysis was done with 1000 replicates and a confidence interval of 95 % and the bootstrap interval found of Theta-P was 0.057 to –0.009, including the value 0, hence, we can conclude that there are no overall significant differences between the samples from different localities. The analysis was repeated by comparing the samples pairwise, and this led to the same conclusion.

In the next table (number 5) we can observe the percentage of melanic in the samples of *A. bipunctata* that is relatively low in all four samples.

**Table 5.** Collection places, dates and the percentage of melanic morphs of *A. bipunctata* found.

| Site | Collection date | N ° total samples | % melanic |
|---|---|---|---|
| Wageningen | 18 May | 175 | 16.6 % |
| Tilburg | 23 May | 51 | 23.5 % |
| Leiden | 26 May | 41 | 7.3 % |
| Utrecht | 7 July | 16 | 12.5 % |

With the data of *Adalia bipunctata* shown in the table 1 of the appendix we can see if there is any difference in the percentage of melanic morphs with the statistical method Chi Square (Table 6).

**Table 6.** Chi Square between the proportion of melanic beetles in the samples of *Adalia bipunctata* in 2005.

| Site | Wageningen | Tilburg | Leiden | Utrecht |
|---|---|---|---|---|
| Wageningen | - | 1.28 | 2.25 | 0.18 |
| Tilburg | 1.28 | - | 4.38 * | 0.90 |
| Leiden | 2.25 | 4.38 * | - | 0.39 |
| Utrecht | 0.18 | 0.90 | 0.39 | - |

* The difference in % melanic is significant.

Table 7 shows the percentage of melanic in the samples of *H. axyridis*.

**Table 7.** Collection localities, dates and the percentage of melanic morphs of *H. axyridis*.

| Site | Collection date | N ° total samples | % melanic |
|---|---|---|---|
| Wageningen | 18 May | 2 | 0 % |
| Tilburg | 23 May | 71 | 28.2 % |
| Leiden | 26 May | 6 | 33.3 % |
| Utrecht | 7 July | 85 | 14.1 % |

The comparison between the percentage of *H. axyridis* in the different samples are shown in Table 8.

**Table 8.** Chi Square between the samples of *Harmonia axyridis* in 2005.

| Site | Wageningen | Tilburg | Leiden | Utrecht |
|---|---|---|---|---|
| Wageningen | - | 0.76 | 0.89 | 0.33 |
| Tilburg | 0.76 | - | 0.07 | 4.68 * |
| Leiden | 0.89 | 0.07 | - | 1.59 |
| Utrecht | 0.33 | 4.68 * | 1.59 | - |

* The difference between places is significant.

With the numbers of the collections of other years (realized by de Jong and Brakefield 1998) we can make a comparison with the ones found this year. Chi Square between the samples of *Adalia bipunctata* in 1998 (or 1999) and 2005:

- Tilburg 1998 – 2005: 2.41, not significant.
- Leiden 1998 – 2005: 0.50, not significant.
- Utrecht 1999 – 2005: 0.70, not significant.

Remarks:

- Wageningen is not calculated because we don't have data of 1998.
- The values from Leiden were collected exactly in Oegstgeest.

These results show inexistence of significant differences between the values of both years, but they show the general trend that the melanic ones are decreasing (Table 5 appendix). Perhaps if we had more samples the results would have been significant.

It is also very important to calculate the frequency of *Harmonia axyridis* (*H. a.*) to compare with the values of the two-spot ladybird found (Table 9), and then we can see if the frequency of one of the species is higher than the other way round.

**Table 9.** Frequency of *Harmonia axyridis*: % total = [n° *H. a*/ (n° *H. a* + n° *A. bipunctata*)]*100

| Site | % total *H.a.* |
|---|---|
| Wageningen | 1.13 % |
| Tilburg | 58.2 % |
| Leiden | 12.8 % |
| Utrecht | 84.2 % |

Comparing the different cities, we can observe that there are still places where the native species is more common, like in Wageningen and Leiden. But the high frequency of *H. axyridis* like in Tilburg and above all Utrecht is remarkable.

## DISCUSSION AND CONCLUSIONS

This is the first time that someone studies the migration of *Adalia bipunctata* and we can conclude that this is a first indication that there isn't any difference between the samples of *Adalia bipunctata* from the four localities studied, and that there is a high migration in The Netherlands.

The stepped shape of the cline that existed in 1998 was probably not because of limited migration, but was because of strong selection in the west in favour of non melanics.

The percentage of melanics in each sample (Table 5) of *Adalia bipunctata* is considerably lower than the non-melanic one, with values ranging between 7.3 and 23.5 %. In conclusion, the frequency of melanic *A. bipunctata* has not significantly chanced since 1998 and now is more equal between localities. Then, that the percentage of melanics is more or less the same everywhere could be because migration is high and/or because selection pressures are approximately equal at different localities.

With regard to the percentage of melanics forms of *Harmonia axyridis* we found values (Table 7), ranging between 0 and 28.2 %.

We also can conclude that in 2005 *H. axyridis* is more common in the South of The Netherlands (Table 3 appendix).

In comparison with *A. bipunctata* there are places in The Netherlands where we can find frequencies of *Harmonia axyridis* really high (Table 9), like Utrecht with 84.2 % and Tilburg with 58.2 %. In conclusion *H. axyridis* in some localities is already more common than *A. bipunctata*, especially in the South of the Netherlands.

By means of the statistical method Chi Square we can conclude that only there are significant differences in the percentage of melanic morphs of the two spot ladybird when we compare the samples from Leiden and Tilburg (Table 6), with a Chi Square of 4.38.

Table 8 shows the same statistical study but with the Harmonia axyridis samples and the result is also that only there are significant differences between Tilburg and Utrecht with a Chi Square of 4.68.

If we compare the data of the collections of this study with the ones from 1998 and 1999 we can conclude that there are no significant differences between them. But they generally show that the melanic forms are decreasing in the last years (Table 5 appendix).

**APPENDIX**

**Table1.** Numbers of *Adalia bipunctata* found.

| Site | N ° Melanics | N ° non melanics |
|---|---|---|
| Wageningen | 29 | 146 |
| Tilburg | 12 | 39 |
| Leiden | 3 | 38 |
| Utrecht | 2 | 14 |

**Table 2.** Numbers of males and females of sexed *Adalia bipunctata* in 2005.

| Site | $N_{Total}$[a] | Melanics | | Non melanics | |
|---|---|---|---|---|---|
| | | N ° males | N ° females | N ° males | N ° females |
| Wageningen | 25 | 0 | 3 | 9 | 13 |
| Tilburg | 25 | 1 | 7 | 0 | 17 |
| Leiden | 25 | 0 | 0 | 5 | 20 |
| Utrecht | 16 | 1 | 1 | 5 | 9 |

[a] Total number of beetles sampled including melanics and non melanics.

**Table 3.** Numbers of *Harmonia axyridis* found in 2005.

| Site | N ° Melanics | N ° non melanics |
|---|---|---|
| Wageningen | 0 | 2 |
| Tilburg | 20 | 51 |
| Leiden | 2 | 4 |
| Utrecht | 12 | 73 |

**Table 4.** Numbers of *Adalia bipunctata* found in 1998 and 1999.

| Site | N ° melanics | N ° non melanics |
|---|---|---|
| Wageningen | - | - |
| Tilburg (1998) | 37 | 66 |
| Leiden (1998) | 16 | 128 |

| Utrecht (1999) | 53 | 196 |
|---|---|---|

**Table 5.** Percentage of melanic morphs of *A. bipunctata* found in 1998 and 2005 (with values from table 1 and 4 of appendix).

| Site | % melanics | |
|---|---|---|
| | 1998 | 2005 |
| Wageningen | - | 16.6 |
| Tilburg | 35.9 | 23.5 |
| Leiden | 11.1 | 7.3 |
| Utrecht | 21.3 | 12.5 |

**Table 6.** Other species found in 2005.

| Site | N ° individuals | Species |
|---|---|---|
| Wageningen | 2 | *Coccinella septempunctata* |
| Tilburg | 6 | *Coccinella quinquepunctata* |
| | 6 | *Coccinella septempunctata* |
| Leiden | 2 | *Exocomus quadripustulatus* |
| Utrecht | 5 | *Adalia decempunctata* |
| | 4 | *Coccinella septempunctata* |
| | 1 | *Calvia quatuordecimguttata* |
| | 3 | *Propillea quatuordecimpunctata* |

**Table 7.** Concentrations (µL) of PCR products in the 96 well plate.

| Site | Primer | | | | Water | Total |
|---|---|---|---|---|---|---|
| | Ab 1 | Ab 3 | Ab 11 | Ab 19 | µL | µL |
| Wageningen | 2.5 | 5 | 2.5 | 5 | - | 15 |
| Tilburg | 4 | 3 | 2.5 | 3 | - | 15 |
| Leiden | 3 | 5 | 3 | 5 | 2 | 15 |
| Utrecht | 2.5 | 4 | 4 | 2.5 | 2.5 | 15 |

**Table 8.** Data introduced in GDA statistical analysis.

begin gdadata; dimensions npops=4 nloci=4; format missing=? separator=/;
locusallelelabels

1 Ab01, 2 Ab03, 3 Ab11, 4 Ab19; matrix

Wag:

| | | | | |
|---|---|---|---|---|
| 1 | G/G | A/H | I/K | D/O |
| 2 | C/E | G/I | J/J | F/? |
| 3 | ?/? | F/F | I/J | F/? |
| 4 | D/D | F/T | D/M | F/H |
| 5 | Q/Q | M/O | A/L | E/? |
| 6 | O/O | C/F | J/? | G/O |
| 7 | J/J | M/M | L/M | E/P |
| 8 | ?/S | ?/? | ?/? | E/Q |
| 9 | C/C | F/R | B/K | D/? |
| 10 | M/M | ?/? | L/? | E/G |
| 11 | C/? | E/? | K/N | I/P |
| 12 | C/S | E/O | K/N | E/H |
| 13 | C/? | A/I | ?/? | B/J |
| 14 | B/H | B/G | C/M | D/N |
| 15 | K/K | E/? | L/? | E/G |
| 16 | C/K | H/L | F/K | E/H |
| 17 | B/J | A/C | ?/? | E/? |
| 18 | M/R | D/F | I/K | G/? |
| 19 | H/H | E/H | H/I | E/? |
| 20 | M/M | I/L | L/? | E/H |
| 21 | C/C | G/J | B/F | E/? |
| 22 | E/J | B/? | E/K | E/G |
| 23 | A/C | C/? | B/J | D/? |
| 24 | B/B | N/N | J/? | D/? |
| 25 | C/C | G/G | L/M | H/J, |

Lei:

| | | | | |
|---|---|---|---|---|
| 1 | ?/? | ?/? | ?/? | ?/? |
| 2 | G/G | G/L | F/J | H/? |
| 3 | K/? | J/N | L/M | E/G |
| 4 | K/K | D/D | J/? | E/? |
| 5 | J/J | E/H | J/Q | F/? |
| 6 | J/J | ?/? | ?/? | D/O |
| 7 | D/K | F/F | C/J | E/K |
| 8 | K/K | F/K | I/O | G/K |
| 9 | F/F | M/M | H/? | F/? |
| 10 | C/K | B/E | F/I | E/? |
| 11 | C/H | ?/? | I/L | M/P |
| 12 | J/J | B/B | I/K | ?/? |
| 13 | B/K | E/K | C/H | H/L |
| 14 | B/J | C/C | D/K | G/K |
| 15 | C/C | D/J | K/M | F/J |
| 16 | K/K | ?/? | K/M | R/? |
| 17 | P/T | F/F | K/? | D/? |
| 18 | G/L | A/? | B/I | ?/? |
| 19 | ?/? | ?/? | G/K | E/K |
| 20 | C/F | ?/? | I/J | G/? |
| 21 | C/K | J/K | ?/? | E/I |

```
22      F/F     K/N     C/K     E/G
23      O/O     ?/?     I/L     D/P
24      C/G     M/M     ?/?     I/P
25      F/J     H/?     A/I     I/O,
Utr:
1       C/K     J/J     I/P     H/?
2       C/G     J/M     ?/?     ?/?
3       B/?     C/?     B/E     E/G
4       B/N     M/S     D/J     G/?
5       B/B     E/E     C/L     D/?
6       C/K     ?/?     A/I     ?/?
7       D/K     H/J     I/K     E/H
8       F/F     C/C     J/M     D/?
9       ?/?     N/N     K/?     D/?
10      C/?     ?/?     E/L     H/P
11      G/G     H/M     B/J     D/?
12      C/K     K/K     I/L     D/F
13      C/K     D/H     ?/?     D/F
14      B/J     C/E     E/F     D/O
15      C/?     M/M     G/J     D/F
16      C/J     J/L     B/L     A/F,
Til:
1       B/J     E/I     I/J     E/K
2       B/G     B/D     L/M     D/H
3       B/K     H/O     B/K     D/J
4       C/G     D/N     I/K     D/F
5       C/?     H/L     B/I     ?/?
6       B/?     B/E     J/L     E/?
7       C/K     C/F     G/M     F/H
8       B/?     ?/?     ?/?     F/P
9       B/G     K/M     K/P     F/H
10      ?/?     ?/?     ?/?     ?/?
11      C/C     I/I     I/M     E/?
12      B/?     F/F     K/K     E/P
13      B/?     J/N     G/M     E/G
14      B/?     D/F     J/J     E/O
15      B/J     ?/?     F/G     E/P
16      C/?     C/M     I/?     E/?
17      B/?     ?/?     C/F     E/G
18      F/H     ?/?     ?/?     F/C
19      B/E     ?/?     I/J     H/?
20      B/K     ?/?     J/K     F/?
21      B/G     ?/?     I/J     E/P
22      C/?     K/N     C/L     E/?
23      B/K     ?/?     I/M     E/G
24      B/?     ?/?     L/N     E/O
25      B/?     C/C     B/K     E/H;
end;.
```

**Table 9.** Results from statistical analysis.

Analysis of variance. There are 4 active loci, two-level analysis and 4 active populations.

| Locus | Allele | Theta-P |
|---|---|---|
| **Ab01** | All | 0.067143 |
| | N | 0.017442 |
| | L | -0.009580 |
| | T | -0.009580 |
| | P | -0.009580 |
| | F | 0.019824 |
| | A | -0.008026 |
| | R | -0.008026 |
| | K | 0.015637 |
| | H | -0.016771 |
| | B | 0.370176 |
| | M | 0.062870 |
| | S | 0.016042 |
| | J | -0.018055 |
| | O | -0.038598 |
| | Q | -0.016085 |
| | D | -0.031974 |
| | E | 0.001606 |
| | C | -0.000611 |
| | G | -0.019728 |

| Locus | Allele | Theta-P |
|---|---|---|
| **Ab03** | All | -0.007225 |
| | S | 0.009447 |
| | K | 0.011503 |
| | N | -0.040800 |
| | J | 0.038620 |
| | D | -0.014828 |
| | L | -0.029783 |
| | B | -0.014402 |
| | E | -0.031719 |
| | R | -0.007671 |
| | C | -0.027144 |
| | O | -0.000935 |
| | M | -0.020341 |
| | T | -0.007671 |
| | F | 0.000653 |
| | I | 0.017363 |
| | G | 0.053913 |
| | H | -0.023833 |
| | A | 0.018155 |

| Locus | Allele | Theta-P |
|---|---|---|
| **Ab11** | All | -0.010065 |
| | P | -0.006786 |
| | G | -0.000852 |
| | O | -0.001701 |
| | Q | -0.001701 |
| | E | 0.062544 |
| | H | 0.005710 |
| | F | -0.027827 |
| | C | -0.017052 |
| | N | 0.006154 |
| | B | -0.009116 |
| | L | -0.006736 |
| | A | -0.015713 |
| | M | -0.012281 |
| | D | -0.015713 |
| | J | -0.029737 |
| | K | -0.006789 |
| | I | -0.009313 |

| Locus | Allele | Theta-P |
|---|---|---|
| **Ab19** | All | 0.028252 |
| | C | -0.011034 |
| | A | 0.036407 |
| | R | -0.001910 |
| | L | -0.001910 |
| | M | -0.001910 |
| | K | 0.070977 |
| | N | -0.005352 |
| | J | -0.020341 |
| | B | -0.005352 |
| | I | 0.037436 |
| | Q | -0.005352 |
| | P | -0.026779 |
| | G | -0.016589 |
| | E | 0.052327 |
| | H | -0.012894 |
| | F | -0.000922 |
| | O | -0.037453 |
| | D | 0.152066 |

| Locus | Theta-P |
|---|---|
| **Overall** | 0.020069 |

Bootstrapping over loci
Number of replicates = 1000
Nominal confidence interval (CI) = 95%

| Bound | Theta-P |
|---|---|
| Upper | 0.056797 |
| Lower | -0.009341 |

**Figure 1.** Gel of 25 samples from Wageningen with primer Hex Ab. 11.

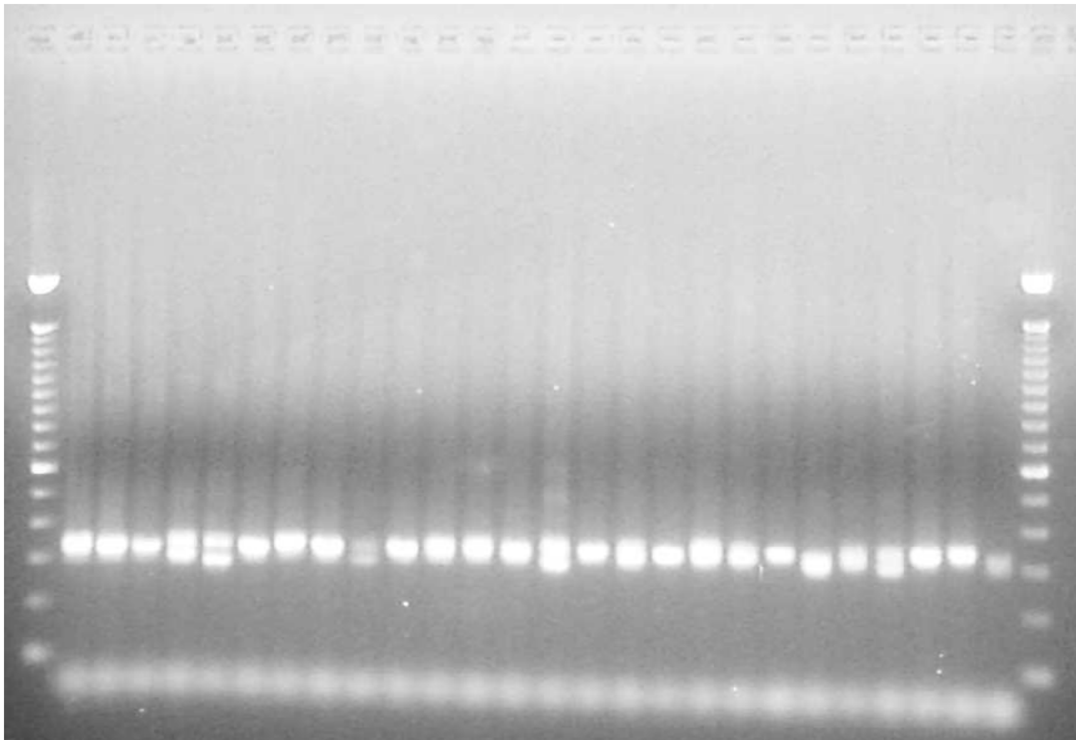


**Figure 2.** Gel of 25 samples from Tilburg with primer Ned Ab 19.

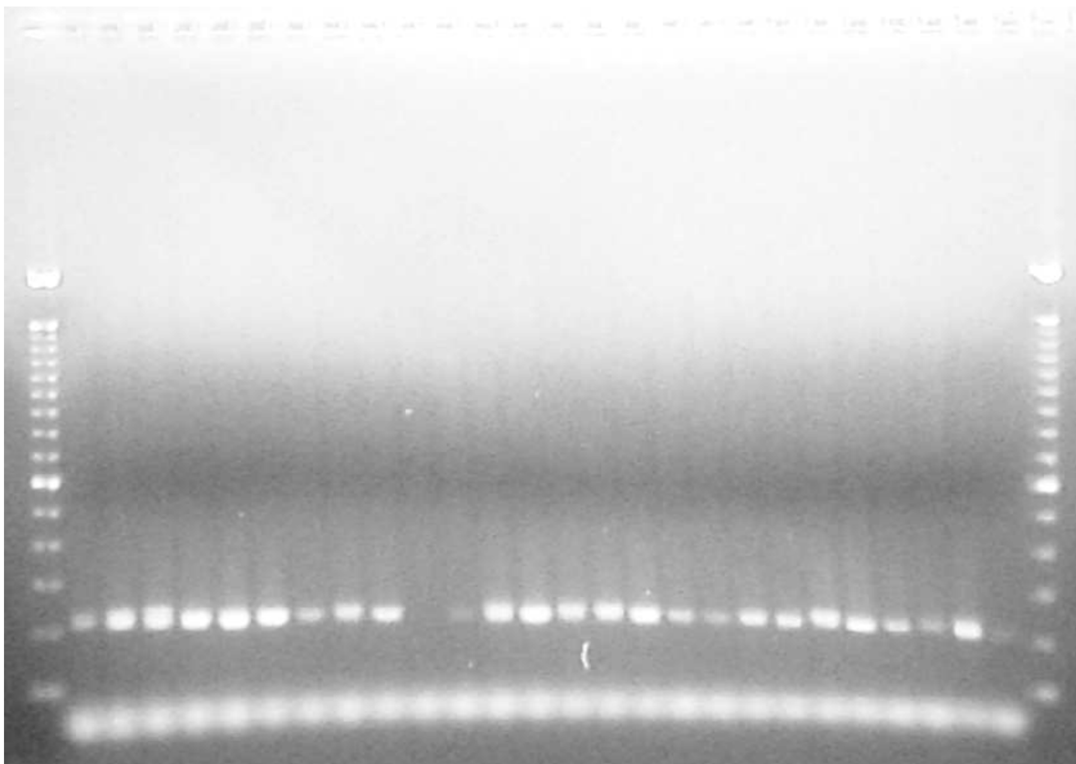


**Figure 3.** Gel of 25 samples from Leiden with primer Fam Ab 1.

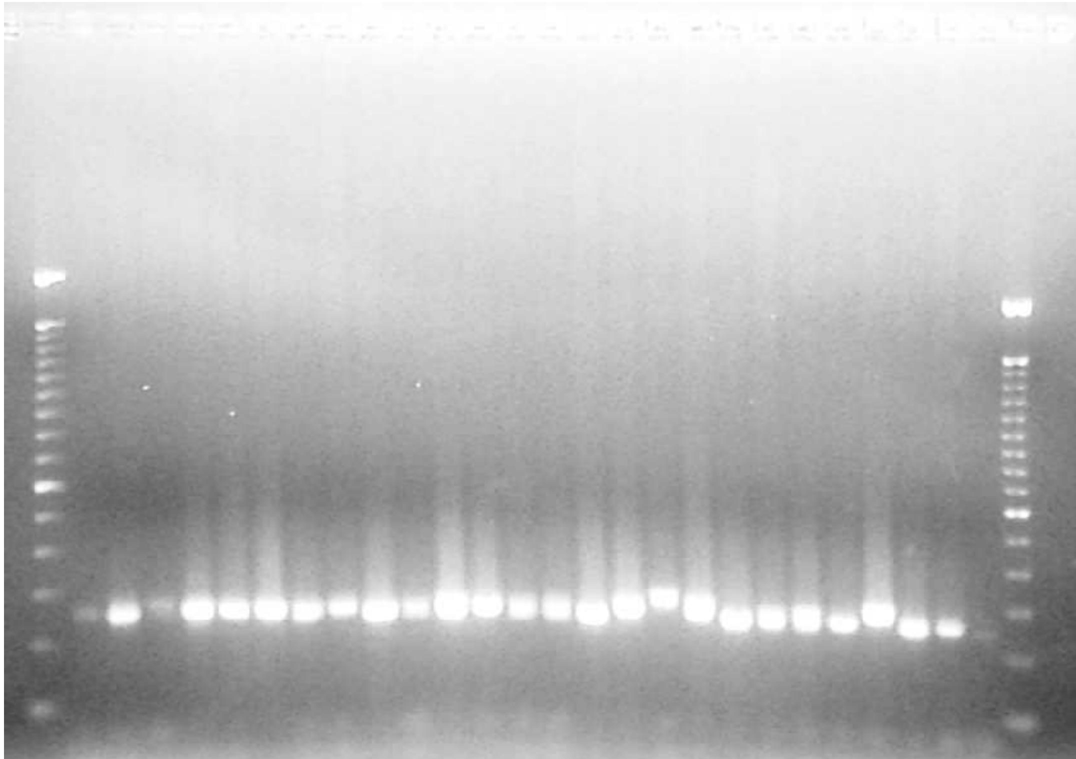
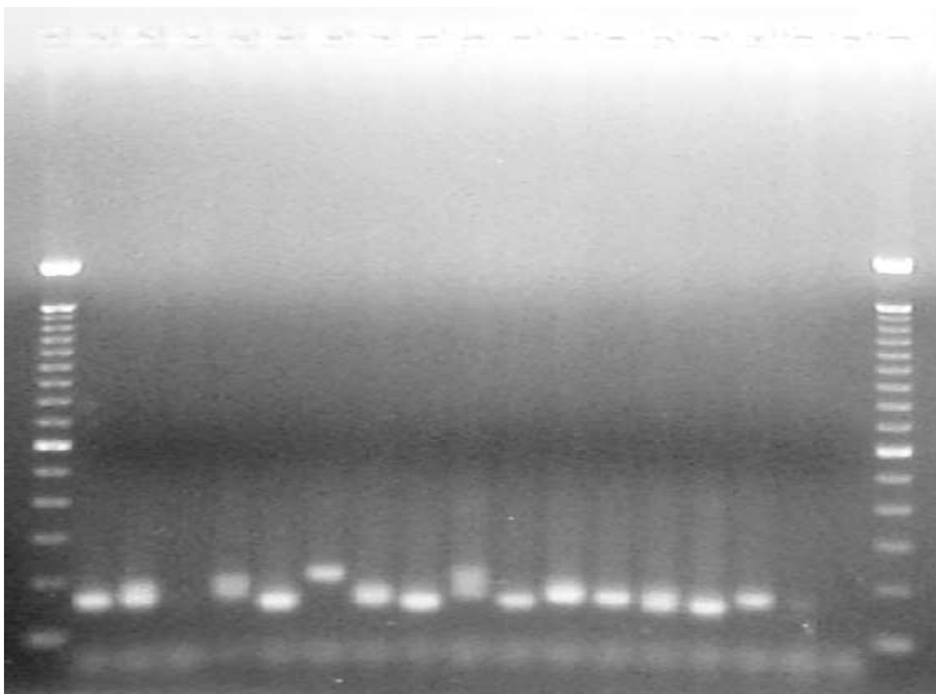


**Figure 4.** Gel of 16 samples from Utrecht with primer Hex Ab 11.



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