Identification of PCR-RFLP Haplotypes For Assessing Genetic Variation in the Green Oak Leaf Roller *Tortrix viridana* L. (Lepidoptera, Tortricidae)

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Abstract

PCR-RFLPs were performed to assess intraspecific variation in the green oak leaf roller, *Tortrix viridana*. The cytochrome oxidase I and II genes were amplified with universal and self designed primers, respectively, resulting in three PCR-fragments of 802 bp, 729 bp and 680 bp. 29 restrictions endonucleases were tested for variation in these PCR-patterns. Seven of these enzymes were chosen for further research. We found 13 haplotypes in four populations across a total of 436 individuals. In addition all haplotypes were sequenced. More single nucleotide substitutions were detected in the sequences, particularly in the middle of the cytochrome oxidase I gene, missed by the used restriction enzymes. For these markers intraspecific variation in *T. viridana* is high compared to other insect species. Furthermore we found differences in frequency of haplotypes among the investigated populations which induce that the markers developed so far are suitable for population genetic studies in *T. viridana*.

Key words: Tortrix viridana, Quercus robur, PCR-RFLP, cytochrome oxidase, genetic variation, sequencing

Introduction

The green oak leaf roller, *Tortrix viridana* L., is a major pest on oaks in the west palearctic region. It is an oligophagous herbivore with a host range limited to the genus *Quercus* (HUNTER, 1990; DU MERLE et al., 1999). During outbreak it often leads to defoliation of oaks in spring. Because of its relevance to forest management many experimental studies have been conducted on the coincidence between hatching of *T. viridana* and phenology of oaks (THALENHORST, 1951; HUNTER, 1990, 1992; DU MERLE, 1999; IVASHOV et al., 2002), and its population dynamics (SCHUETTE, 1957; SCHWERDTFEGER, 1971; HORSTMANN, 1984; HUNTER et al., 1997; HUNTER, 1998. But until now relatively little attention has been paid to the level of genetic variation within the species *Tortrix viridana* (SIMCHUK et al., 1999).

Rapid evolution, lack of recombination and maternal inheritance make mitochondrial DNA suitable for investigations at different taxonomic levels (AVISE, 1994). Depending on the chosen mtDNA region studies at the family or genera level (e.g. SPERLING and HICKEY, 1995; KRUSE and SPERLING, 2002; SCHROEDER et al., 2003; WAHLBERG et al., 2003), and intraspecific investigations

(e.g. BOGDANOWICZ et al., 1993; COGNATO et al., 1999) are possible. For population research the cytochrome oxidase subunits I and II are often used (e.g. SPERLING et al., 1999; KRUSE and SPERLING, 2001; RONDAN et al., 2002).

In this study PCR-RFLPs were exerted to establish molecular markers for population genetic studies in *T. viridana* and to get a first insight into which level i.e. within or among populations, variation occurs. First results give indication for differentiation among populations of this major forest pest. This information will be relevant for forest management for predicting future pest outbreaks and expansion.

Material and Methods

Samples

In May 2003 living caterpillars and pupae of *T. viridana* were collected in four pedunculate oak stands (*Quercus robur*) in North Rhine-Westphalia, near the city of Muenster in an area of about 85 x 40 km (*Fig. 1*). The immature stages were reared in the laboratory and then identified as adults (STRESEMANN, 2000). The adult moths were frozen at –20°C until DNA extraction. A total of 436 green oak leaf rollers were used for this research.
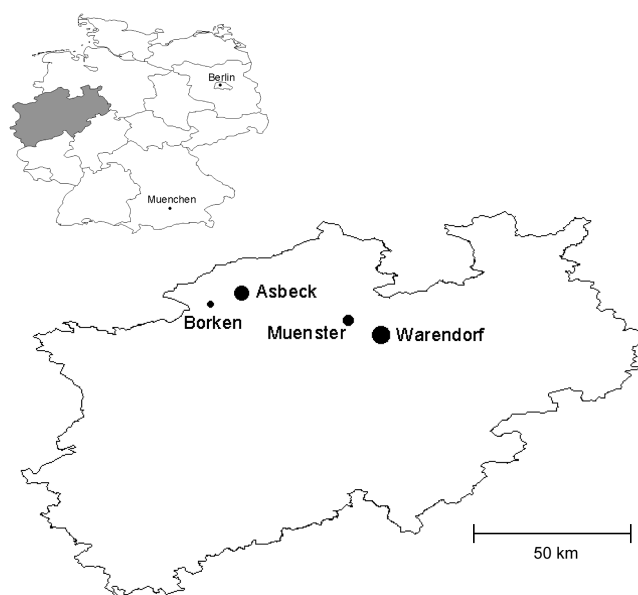

Figure 1. – Collecting areas in North Rhine-Westphalia. The size of the circles is correlated with the sample size.

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DNA extraction

Individual frozen moths were ground to powder in liquid nitrogen. Total genomic DNA was extracted following a CTAB protocol by DUMOLIN et al. (1995) with a slight modification: Proteinase K, in a final concentration of 0,4 µg/µl, was added to the lysis-buffer.

PCR conditions

The PCR reactions contained 50 ng template DNA, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM MgCl$_2$, 200 µM dNTPs, 1 unit Taq polymerase and 0.8 µM of each primer in a total volume of 25 µl. Sequences of the primers used are as follows:

EW2: 5´ GAGACCATTACTTGCTTTCAGTCATCT 3´, EW4: 5´ AGAGCCTCTCCTTTAATAGAACA 3´, EW10: 5' TTGGATGTTTAGGAATAATTTAT 3',

EW14: 5' TAAAATAGGTAATTCATTATA 3', EW15: 5' ATAAATTATTCCTAAACATCC 3',

EW16: 5' TTCAACAAATCATAAAGATATTG 3', whereas primers EW10 and EW15 are complementary to each other.

PCR was carried out in a Biometra Personal thermocycler with a pre-denaturation-step at 94 °C for 4 min, followed by 35 cycles at 94 °C for 1 min, 49 °C (primers EW10 + EW14 and EW16 + EW15) or 60 °C (for the primer combination EW4 + EW2) for 1 min, 72 °C for 1.5 min, final elongation at 72 °C for 10 min.

Restriction digestion conditions

10 to 15 µl of the amplicon were digested with two units of the respective enzyme in a total volume of 20 µl (restriction enzyme buffer) according to the recommendations of the supplier of the restriction enzymes (MBI Fermentas). Separation was carried out with 20 µl of the digested DNA, either on 1.2 % agarose gels, or on 8 % polyacrylamide gels. Bands were visualized using ethidium bromide staining. A 50 bp ladder from GIBCO BRL (Life Technologies) was used as size marker.

Sequencing

For sequencing PCR-products of cytochrome oxidase I and II were first cloned using Topo TA Cloning-Kit (Invitrogen). Afterwards cycle sequencing was performed on both strands using ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Furthermore the first PCR-product of cytochrome oxidase I (802 bp) of 10 individuals, the second PCR-product of cytochrome oxidase I (729 bp) of seven and the PCR-product of cytochrome oxidase II (680 bp) of five individuals were sequenced as described above after purification with High Pure PCR Product Purification Kit from Roche Diagnostics. Purification, cloning and sequencing were performed according to the manufacturer's recommendations.

Results

Primer design

Some universal primers for the cytochrome oxidase subunits I and II were tested not produced an amplifica-
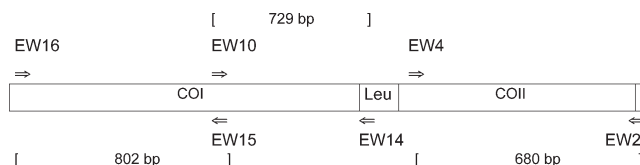
Figure 2. – Used primer combinations for amplification of cytochrome oxidase subunits I and II (COI, COII).

tion product: C1-J-2183, C1-N-2659, C1-J-1751, TL2-N-3014, C2-N-3389 (SIMON et al., 1994). Two further universal primers for COII generated a PCR product: A3772 (BOGDANOWICZ et al., 1993, synonym in the present paper: EW2) and C2-J-3138 (SIMON et al., 1994, synonym in the present paper: EW4, *Fig. 2*). For amplification of the cytochrome oxidase subunit I new primers were designed using known sequences of cytochrome oxidase subunits I of related species (LANDRY et al., 1999; SPERLING et al., 1995).

Subunit I was amplified in two separate reactions and primer combinations, i.e. EW10 + EW14 and EW16 + EW15 (*Fig. 2*). Amplification with the primer pairs EW16 + EW14 and EW16 + EW2 gave no result in spite of using an increased elongation time (2 or 3 min., respectively). The PCR product of the primer pair EW16 + EW15 was a 802 base pair fragment. Amplification with the primer combination EW10 + EW14 resulted in a 729 bp fragment and primers EW4 + EW2 generated a 680 bp fragment.

Table 1. – List of restriction enzymes tested on three fragments (EW4 + EW2 = 680 bp, EW10 + EW14 = 729 bp, EW16 + EW15 = 802 bp). Restriction enzymes written in bold were checked for variation. Restriction enzymes with restriction sites were checked for variation only.

Restriction enzyme	Restriction site			Variation		
	680 bp	729 bp	802 bp	680 bp	729 bp	802 bp
Alu I	Yes	Yes	Yes	No	No	No
Apa I	No	No	No			
BamH I	No	No	No			
Bcl I	No	No	Yes	No	No	No
Bgl I	No	No	No			
BseG I	No	No	No			
Bsp120 I	No	No	No			
Bsu15 I	Yes	No	No	No		
Dde I	Yes	No	Yes	No		*Yes*
Dra I	No	Yes	Yes		No	No
EcoR I	No	No	No			
EcoR V	No	Yes	No		No	
Eco47 I	No	No	No			
Hae III	No	No	Yes			No
Hin6 I	No	No	Yes			*Yes*
Hind III	No	No	No			
Hinf I	Yes	Yes	No	No	*Yes*	
Mbo I	No	Yes	Yes		No	*Yes*
Msp I	No	No	No			
Mva I	Yes	No	Yes	No		No
Pdm I	No	No	No			
Rsa I	No	No	Yes		*Yes*	No
Sac I	No	Yes	Yes		No	No
Sal I	No	No	No			
Sma I	No	No	No			
Ssp I	Yes	Yes	Yes	No	No	*Yes*
Tai I	No	No	No	No	No	No
Taq I	Yes	Yes	Yes	*Yes*	No	No
Xho I	No	Yes	No		No	

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Haplotype identification

29 restriction endonucleases where tested on the three amplified fragments on two to four individuals of the four different populations each for identification of restriction sites (*Tab. 1*). The 17 restriction enzymes showing restriction sites were tested for 10 to 20 individuals of different populations each (*Tab. 1*). Variation was identified at seven restriction sites (*Tab. 1*).

Cutting of the 680 bp fragment with *Taq* I resulted in four to six fragments (*Fig. 3*). The 729 bp fragment produced two patterns with *Rsa* I, a single band (uncut) and two bands, and produced three patterns with *Hinf* I, uncut, two bands, and three bands (*Fig. 4*). The 802 bp fragment was digested with four restriction enzymes. Digestion with *Ssp* I lead to three fragment patterns, cutting with *Dde* I, *Mbo* I and *Hin6* I each produced two patterns (*Fig. 3, Fig. 4*).

Restriction site maps used to survey variation are shown in *Table 2*. Fragment sizes are defined by the sequences of the respective haplotype (*Fig. 5*), which included primers at each end of the original PCR-fragment (Haplotype 1: GenBank accession numbers AY883092 & AY883093). All variation found were point mutations (*Fig. 5*), no length polymorphism was detected.

Figure 4. – Restriction sites of the enzymes *Ssp*I, *Dde*I, *Hin*6I, *Rsa*I. Separated on 1.2% Agarose gel, stained with ethidium bromide. Abbreviations are as explained in *Table 2*.

Table 2. – Restriction site locations for the seven applied restriction enzymes used for haplotype definition. Abbreviations for the variants belong to the respective restriction enzyme.

Ssp I (cutting 802 bp fragment)

variants	Fragment length		
Sp.1	234	148	420
Sp.2	382		420
Sp.3	234	568	

Dde I (cutting 802 bp fragment)

variants	Fragment length		
Dd.1	400	402	
Dd.2	400	132	270

Mbo I (cutting 802 bp fragment)

variants	Fragment length					
Mb.1	110	17	384	99	45	147
Mb.2	110	17	384	99	27	18	147

Hin6 I (cutting 802 bp fragment)

variants	Fragment length	
Hi.1	802	
Hi.2	456	346

Rsa I (cutting 729 bp fragment)

variants	Fragment length	
Rs.1	729	
Rs.2	377	352

Hinf I (cutting 729 bp fragment)

variants	Fragment length		
Hf.1	616		113
Hf.2	729		
Hf.3	47	569	113

Taq I (cutting 680 bp fragment)

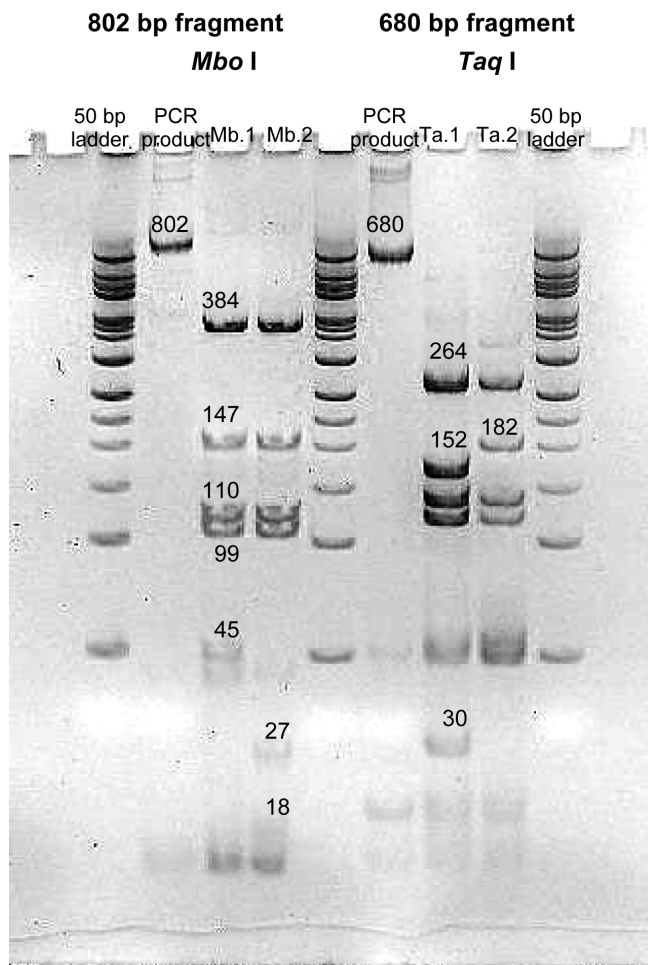| variants	Fragment length				
Ta.1	123	264	111	30	152
Ta.2	123	264	111	182	
Ta.3	387		111	30	152
Ta.4	123	243	21	111	30	152

Figure 3. – Some examples of restriction sites of the enzymes *Taq*I and *Mbo*I. Separated on 8% polyacrylamide gel, stained with ethidium bromide. Abbreviations are as explained in *Table 2*.
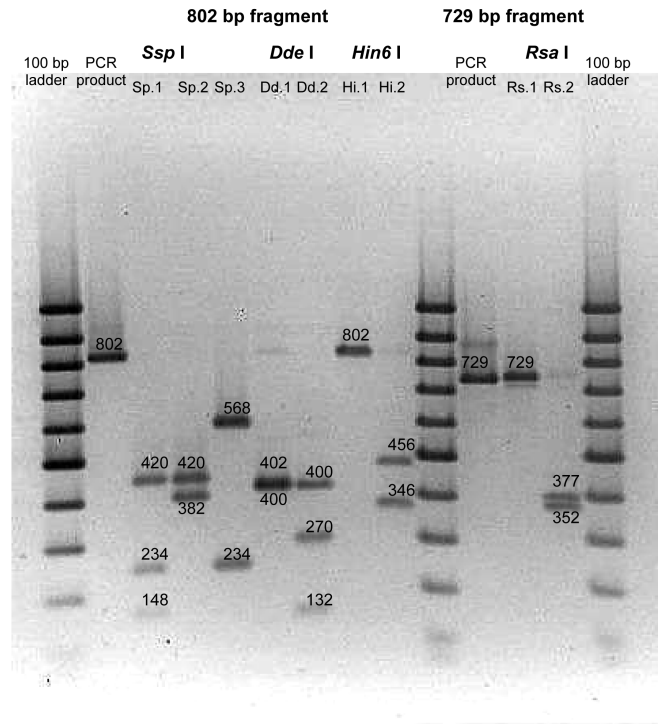
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         1        10        20        30        40        50        60        70        80        90        100
         |         |         |         |         |         |         |         |         |         |         |
Ht1                      TTCAACAAATCATAAAGATATTGGAACATTATATTTTATTTTTGGAATTTGAGCAGGTATAATTGGAACTTCTTT
Ht2-3                    ...............................................................................
Ht4                      ........................................................A....A.................
Ht5-13                   ...............................................................................


        101               150                                              200
         |         |         |         |         |         |         |         |         |         |         |
Ht1   AAGTCTTTTAATTCGAGCAGAATTAGGAAATCCAGGATCATTAATTGGAGATGATCAAATTTATAATACTATTGTAACAGCCCATGCATTTATTATAATT
Ht2-3 ....................................................................................................
Ht4   ..........................................................................C.........................
Ht5-13 ...................................................................................................


        201               250                                              300
         |         |         |         |         |         |         |         |         |         |         |
Ht1   TTTTTTATAGTTATACCTATTATAATTGGAGGATTTGGTAATTGATTAGTACCTTTAATATTAGGAGCTCCTGATATAGCTTTTCCACGAATAAATAATA
Ht2   ............................................................C.......................................
Ht3-13 ...................................................................................................


        301               350                                              400
         |         |         |         |         |         |         |         |         |         |         |
Ht1   TAAGTTTCTGACTTCTCCCCCCCTCTATTATACTTTTAATTTCAAGTAGAATTGTAGAAAACGGAGCAGGAACAGGTTGAACAGTTTATCCCCCCCTTTC
Ht2-13 ...................................................................................................


        401               450                                              500
         |         |         |         |         |         |         |         |         |         |         |
Ht1   TTCTAATATTGCTCATAGYGGAAGCTCAGTAGATTTAGCAATTTTTTCTTTACATTTAGCTGGAATTTCCTCAATTTTAGGTGCAGTAAATTTTATTACA
Ht2   ...................C................................................................................
Ht3   ...................T.......................................................GCGC.....................
Ht4   ...................T................................................................................
Ht5   ...................T.......................................................GCGC.....................
Ht6   .......G...........T................................................................................
Ht7   ...................T................................................................................
Ht8   ...................T.......................................................GCGC.....................
Ht9-13 ...................T...............................................................................


        501               550                                              600
         |         |         |         |         |         |         |         |         |         |         |
Ht1   ACAATTATTAATATACGRCCTAATAATATATCATTAGATCAAATACCCTTATTTGTTTGAGCTGTAGGAATTACAGCTTTATTATTACTTTATCTTTAC
Ht2   .................A..................................................................................
Ht3   .................A...................................CTGAG...............................C..........
Ht4-8 .................A.................................................................................
Ht9   .................A...................................CTGAG..........................................
Ht10-13 ...............A.................................................................................


        601               650                                              700
         |         |         |         |         |         |         |         |         |         |         |
Ht1   CAGTTTTAGCAGGTGCTATTACTATAYTATTAACAGATCGAAATTTAAATACCTCATTTTTTGACCCAGCTGGGGGAGGAGATCCAATTTTTATAYCAACA
Ht2   .........................C...............................................................T.....
Ht3   .........................C...............................GATC............................C.....
Ht4-6 .........................C.............................................................T.....
Ht7   .........................C...............................GATC............................T.....
Ht8   .........................C.............................................................T.....
Ht9   .........................C...............................GATC............................T.....
Ht10-13 .......................C.............................................................T.....


        701               750                                              800
         |         |         |         |         |         |         |         |         |         |         |
Ht1   TTTATTTTGATTTTTTGGCCACCCTGAAGTTTATATTYTAATTTTACCAGGATTTGGTATAATTTCTCATATTATTTCCCAAGAAAGAGGTAAAAAAGAA
Ht2   .............................................C......................................................
Ht3   .............................................T......................................................
Ht4   .............................................C......................................................
Ht5-13 ...........................................T......................................................


        801               850                                              900
         |         |         |         |         |         |         |         |         |         |         |
Ht1   ACATTTGGATGTTTAGGAATAATTTATGCAATAATAGCAATTGGGCTTCTAGGATTTGTAGTTTGAGCTCATCATATATTTACAGTAGGAATAGATATTG
Ht2-12 ...................................................................................................
Ht13  ..................................................GATTC.............................................


        901               950                                              1000
         |         |         |         |         |         |         |         |         |         |         |
Ht1   ATACTCGAGCTTATTTTACTTCAGCAACTATAATTATTGCTGTTCCAACAGGAATTAAAATTTTTAGTTGACTAGCTACTCTTCATGGAACACAAATCAA
Ht2-13 ...................................................................................................


        1001              1050                                             1100
         |         |         |         |         |         |         |         |         |         |         |
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Continued *Figure 5.*

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   1101                           1150                                        1200
   |        |        |        |        |        |        |        |        |        |
Ht1 CTTCATGATACTTATTATGTAGTTGCCCATTTTCAYTATGTTCTTTCTATAGGAGCTGTATTTGCTATTATAGGAGGATTTGTTCATTGATACCCATTAT
Ht2 .................................C..................................................................
Ht3 ...............................T...................................................................
Ht4 .................................C..........................................GTAC....................
Ht5-13...........................T.....................................................................

   1201                           1250                                        1300
   |        |        |        |        |        |        |        |        |        |
Ht1 TTACAGGCTTATCAATAAATCCTTATCTTTTAAAAATCCAATTTTTTATTATATTTATTGGAGTAAATTTAACCTTTTTTCCTCAACACTTTTTAGGRTT
Ht2 ..............................................................................................G..
Ht3 ..............................................................................................G..
Ht4 ..............................................................................................A..
Ht5-13..........................................................................................G..

   1301                           1350                                        1400
   |        |        |        |        |        |        |        |        |        |
Ht1 AGCTGGGATACCTCGACGATATTCAGATTATCCTGATACTTATACATCATGAAATATTATTTCTTCTTTAGGATCTTATATTTCTCTTATTGCAACTATA
Ht2-13..............................................................................................

   1401                           1450                                        1500
   |        |        |        |        |        |        |        |        |        |
Ht1 TTAATATTAATTATTATTTGAGAATCTTTAATTAATAAACGAATTAKTTTATTTCCATTAAATATAAATTCTTCTATTGAATGATATCAAAATCTTCCCC
Ht2-11.....................................................T........................................
Ht12....................AAATC............................T....................................A....
Ht13....................GAATC............................T........................................

   1501                           1550                                        1600
   |        |        |        |        |        |        |        |        |        |
Ht1 CTGCAGAACATTCATATAATGAATTACCTATTTTA
Ht2-13.................................

   1601                           1650                                        1700
   |        |        |        |        |        |        |        |        |        |
Ht1                         AGAGCCTCTCCTTTAATAGACCAAATTATTTTTTTTCACGACCATACTTTAGTTATTT
Ht2-13                      ........................................................

   1701                           1750                                        1800
   |        |        |        |        |        |        |        |        |        |
Ht1 TAATTATAATTACTATTTTAGTAGGTTATTTAATAATTAGTTTATTTTTTAATACTTATATTAATCGATTTTTATTGGAAGGACAAATAATTGAGTTAAT
Ht2-9....................................................................................................
Ht10......................................CCGA....................................................
Ht11-13..............................................................................................

   1801                           1850                                        1900
   |        |        |        |        |        |        |        |        |        |
Ht1 TTGAACTATTCTTCCAGCAATTACTTTAATTTTTATTGCATTACCTTCTTTACGATTACTTTATTTATTAGATGAATTAAATAACCCATTAATTACCTTA
Ht2-13..............................................................................................

   1901                           1950                                        2000
   |        |        |        |        |        |        |        |        |        |
Ht1 AAATCTATTGGACATCAATGATATTGAAGTTATGAATATTCAGATTTTAATAATATTCAATTTGATTCTTATATAATTCCTATAAATGAAATAAAATCTA
Ht2-13..............................................................................................

   2001                           2050                                        2100
   |        |        |        |        |        |        |        |        |        |
Ht1 ATTCTTTCCGACTATTAGATGTTGATAATCGAATTATTTTTACCTATAAATAATCAAATTCGTATTTTAGTTACAGCTACAGATGTTATTCATTCATGAAC
Ht2-10.................................................................................................
Ht11......TCGA...............................................................................
Ht12-13..............................................................................................

   2101                           2150                                        2200
   |        |        |        |        |        |        |        |        |        |
Ht1 TATCCCATCTTTAGGGGTTAAAGTAGATGCTAACCCAGGTCGATTAAATCAAACTAATTTTTTTTATAAATCGACCTGGAATTTTTTACGGTCAATGCTCA
Ht2-4.................................................................................................
Ht5 .........................................................C........................................
Ht6-13..............................................................................................

   2201                           2250                                        2300
   |        |        |        |        |        |        |        |        |        |
Ht1 GAAATTTGTGGGGCAAATCATAGTTTTATACCTATTGTAATTGAAAGAATCTCAATTAAAAACTTCATTAATTGAATTAATAATTATTCTTCATTAGATG
Ht2-13..............................................................................................

   2301
   |        |        |
Ht1 ACTGAAAGCAAGTAATGGTCTC
Ht2-13....................
```

Figure 5. – Sequence of the 13 defined haplotypes of *Tortrix viridana*. Polymorphic restriction sites are underlined, nucleotide substitutions are bold. Only nucleotide substitutions are shown, remaining sequences are as in haplotype 1.

Table 3. – The 13 different haplotypes (Ht) defined (abbreviations are as in *Table 2*).

Haplotype	Sp.	Dd.	Mb.	Rs.	Ta.	Hi.	Hf.
1	1	1	1	1	1	1	1
2	2	1	1	1	1	1	1
3	1	2	2	1	1	2	1
4	1	1	1	2	1	1	1
5	1	1	1	1	2	2	1
6	3	1	1	1	1	1	1
7	1	1	2	1	1	1	1
8	1	1	1	1	1	2	1
9	1	2	2	1	1	1	1
10	1	1	1	1	3	1	1
11	1	1	1	1	4	1	1
12	1	1	1	1	1	1	2
13	1	1	1	1	1	1	3

Table 4. – Frequencies of the haplotypes in the four studied oak stands. N = Total number of each haplotype, [%] = percent within populations.

Haplo-type	N	Warend.		Asbeck		Muenster		Borken	
		N	[%]	N	[%]	N	[%]	N	[%]
1	360	196	85.6	87	79.8	53	77.9	24	80
2	15	6	2.6	5	4.6	4	5.9		
3	35	14	6.1	10	9.2	7	10.3	4	13.3
4	4	2	0.9	2	1.81				
5	1			1	0.9				
6	1	1	0.4						
7	1							1	3.3
8	4	2	0.9	1	0.9	1	1.5		
9	9	6	2.6	2	1.8	0		1	3.4
10	1	1	0.45						
11	1					1	1.5		
12	1			1	0.9				
13	3	1	0.45			2	2.9		
N total	436	229		109		68		30	

Figure 6. – Minimum spanning tree of the 13 haplotypes designed by means of haplotype definition as described in *Table 2*.

Haplotypes were identified by a combination of the fragment patterns (*Tab. 3*).

Haplotype distribution and relationship

Frequencies of the haplotypes vary among the investigated populations (*Tab. 4*). Haplotypes 1 and 3 were found in all populations. Haplotype 2, 8 and 9 in three and haplotype 4 in two of the four populations (*Tab. 4*). A test of homogeneity including Chi-square-test was performed resulting in no significant difference in frequency between populations.

Relationships among the haplotypes were performed by a minimum spanning tree (*Fig. 6*). Nine of the haplotypes are directly connected to haplotype 1 by single site changes. Only two branches have more than one site change from Ht1 (7-9-3 and 8-5). The branch 7-9-3 based on the variants Dd.2 and Mb.2, which are linked, except for one individual, resulted in haplotype 7.

Discussion

Variability in the cytochromoxidase I and II gene is very different throughout insect orders, families and even within generic groups or species. For example pop-
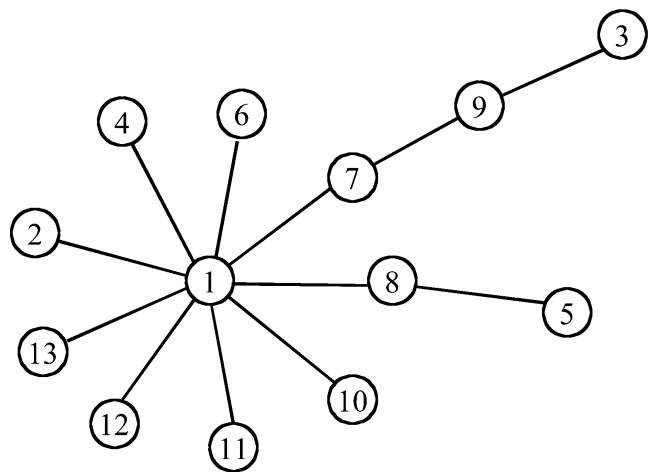
ulations of one species from the milkweed beetle genus, *Chrysochus*, from the East of the USA, all possess the same haplotype as detected by PCR-RFLPs', whereas in populations of another species from the West-USA five different haplotypes exist (DOBLER and FARRELL, 1999). Intraspecific investigations using RFLPs of COI and COII in *Lymantria dispar* allow separation of populations from China, Japan and America (BOGDANOWICZ et al., 1993). In *Aedes aegypti* only two haplotypes were found across several areas in Argentina (RONDAN et al., 2002). These examples are based on studies over wide areas of some thousand kilometers, whereas in our study nine haplotypes were found in *T. viridana* in a small area of only 90 x 40 km.

In addition to point mutations detected using restriction enzyme screening, additional point mutations were detected following sequencing (*Fig. 5*). These nucleotide substitutions are located in parts of the cytochrome oxidase I: The first part of COI (bp 1 to 399) is highly conserved, whereas variability is very high within bp 400 to 800 and also high within bp 1050 to 1500, which agree with the results of LUNT et al. (1996) for several other insect species. The level of single nucleotide polymorphisms (SNPs) is very different across insect taxa, even when allowing for differences in sample size. KRUSE and SPERLING (2001) sequenced the mtDNA COI area from bp 719 to 1193 of 88 individuals of *Archips argyrospila* (Lepidoptera, Tortricidae) and found 20 haplotypes. In our study, within the same area sequencing of only five individuals of *T. viridana*, five nucleotide substitutions produced three (sequence) haplotypes. Between bp 719 of COI and the tRNA leu gene, a screen of 48 individuals of the planthopper, *Nilaparvata lugens* (Hemiptera, Delphacidae), only identified two variable bases (MUN et al., 1999), whereas for *T. viridana*, nine variable bases were deserved in seven individuals in this region. In addition, *Drosophila buzzatii* (Diptera, Drosophilidae) showed a higher level of variability in the sequence from the start of COI to bp 708 (DE BRITO et al., 2002), where 25 haplotypes were identified in a total of 60 individuals. For *T. viridana* this region yielded 10 nucleotide substitutions and seven potential (sequence) haplotypes.

Our data indicate that variation within the cytochrome oxidase gene of *T. viridana* is high as detected by the PCR-RFLP analysis and still higher using sequence analysis. These substitutions could not be integrated in the haplotype definition presented above, since sequencing all the 436 investigated individuals was not practicable. Differences in the sequence of several individuals sharing haplotype 1, defined from PCR-RFLP fragment profile are shown in *Fig. 6* as letters R, Y or K. Further testing of variability with restriction enzymes with sequences that flank the SNPs will be done for refining haplotype identification.

The markers developed so far are suitable for population genetic studies in *T. viridana*. The sampling strategy in this study was governed by the fact that the green oak leaf roller occurred only scarcely in 2003. Thus per population we gathered as many individuals as possible. Even with this limited sample one can see differences for the frequency of haplotypes among the investigated populations. These results are encouraging for future studies of *T. viridana* using increased population size and optimised spatial sampling. Questions to be studied will be the gene flow between populations, origin of the investigated populations and spatial-temporal distribution of haplotypes as verified for the host plants *Quercus robur* and *Q. petraea* (PETIT et al., 1997).

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Assessment of AFLP-Based Genetic Variation in the Populations of *Picea asperata*

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Summary

Picea asperata Mast., which occurs in a restricted habitat in western China, has a wide ecological amplitude. In the present study, ten natural populations of *P. asperata* were studied using AFLP markers to investigate the population genetic structure and the level of genetic diversity. Of the 210 loci identified with two EcoRI/MseI primer combinations, 142 loci were found to be polymorphic. Yet, the level of genetic diversity observed within populations was quite low. The averages of NEI's gene diversities (h) and Shannon's indices of diversity (I) calculated across populations equaled 0.156 and 0.227, respectively. The coefficient of gene differentiation among populations, based on the estimate G_{ST} and the unbiased estimate Φ_{st}, equaled 0.340 and 0.231, respectively. The mean genetic distance (D) between population pairs was 0.119 (range 0.050-0.156). Such high values indicate that there is significant differentiation among populations of *P. asperata*. Several factors could have contributed to the strong population differentiation, including relatively limited gene flow between populations (N_m = 0.968). Variation in environmental conditions and consequent selection pressures may be other factors attributing to the high level of genetic differentiation among populations. In addition, it was discovered that the geographic distances are not correlated with the genetic distances between the populations of *P. asperata*.

Key words: AFLP markers, genetic structure, genetic diversity, *Picea asperata* Mast.

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Introduction

Picea asperata Mast. is an endemic spruce species, which mainly occurs in the northwestern Sichuan province and the southern Gansu province, approximately within the geographical range of 100.1–106.8 °E and 30.2–34.6 °N (LIU et al., 2002). *P. asperata* is one of the most important trees for the production of wood pulp and lumber, as well as one of the keystone species of