

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

By H. SCHROEDER¹⁾ and F. SCHOLZ

Federal Research Centre for Forestry and Forest Products (BFH),
Institute for forest genetics and forest tree breeding

(Received 8th February 2005)

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.

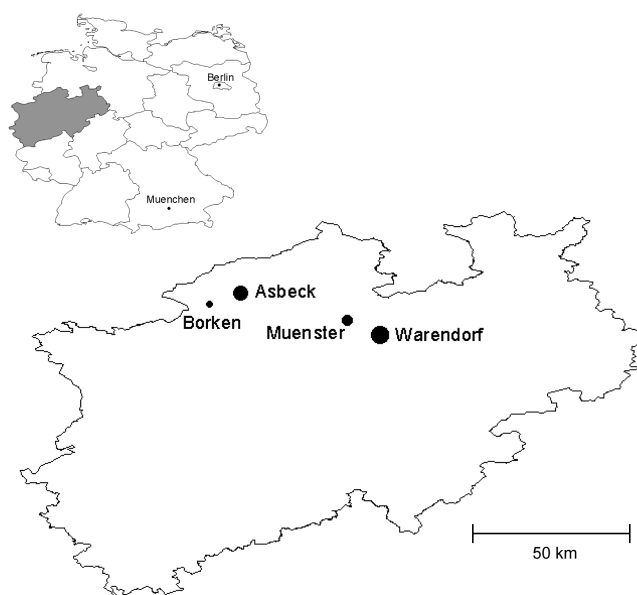


Figure 1. – Collecting areas in North Rhine-Westphalia. The size of the circles is correlated with the sample size.

¹⁾ Author's address: Sieker Landstrasse 2, 22927 Großhansdorf, Tel: 0049-4102-696148, Fax: 0049-4102-696200, e-mail: h.schroeder@holz.uni-hamburg.de

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 MgCl₂, 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' GAGACCATTACTTGCTTTTCAGTCATCT 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-

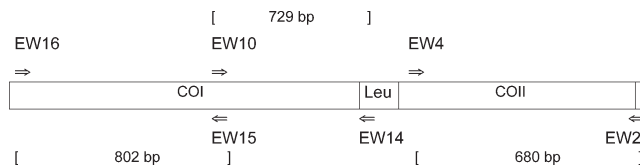


Figure 2. – Used primer combinations for amplification of cytochrome oxidase subunits I and II (COI, COII).

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
<i>Alu I</i>	Yes	Yes	Yes	No	No	No
<i>Apa I</i>	No	No	No			
<i>BamH I</i>	No	No	No			
<i>Bcl I</i>	No	No	Yes	No	No	No
<i>Bgl I</i>	No	No	No			
<i>BseG I</i>	No	No	No			
<i>Bsp120 I</i>	No	No	No			
<i>Bsu15 I</i>	Yes	No	No	No		
<i>Dde I</i>	Yes	No	Yes	No		Yes
<i>Dra I</i>	No	Yes	Yes		No	No
<i>EcoR I</i>	No	No	No			
<i>EcoR V</i>	No	Yes	No		No	
<i>Eco47 I</i>	No	No	No			
<i>Hae III</i>	No	No	Yes			No
<i>Hin6 I</i>	No	No	Yes			Yes
<i>Hind III</i>	No	No	No			
<i>Hinf I</i>	Yes	Yes	No	No	Yes	
<i>Mbo I</i>	No	Yes	Yes		No	Yes
<i>Msp I</i>	No	No	No			
<i>Mva I</i>	Yes	No	Yes	No		No
<i>Pdm I</i>	No	No	No			
<i>Rsa I</i>	No	No	Yes		Yes	No
<i>Sac I</i>	No	Yes	Yes		No	No
<i>Sal I</i>	No	No	No			
<i>Sma I</i>	No	No	No			
<i>Ssp I</i>	Yes	Yes	Yes	No	No	Yes
<i>Tai I</i>	No	No	No	No	No	No
<i>Taq I</i>	Yes	Yes	Yes	Yes	No	No
<i>Xho I</i>	No	Yes	No		No	

Haplotype identification

29 restriction endonucleases were 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.

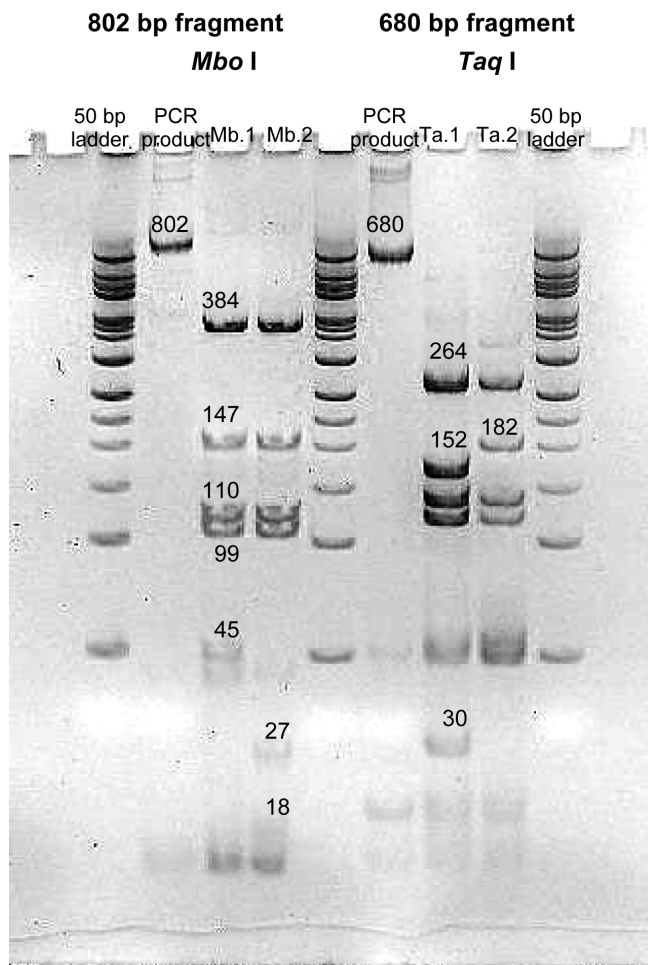


Figure 3. – Some examples of restriction sites of the enzymes *Taq*I and *Mbo*I. Separated on 8% polyacrylamide gel, stained with ethidium bromide. Abbreviations are as explained in Table 2.

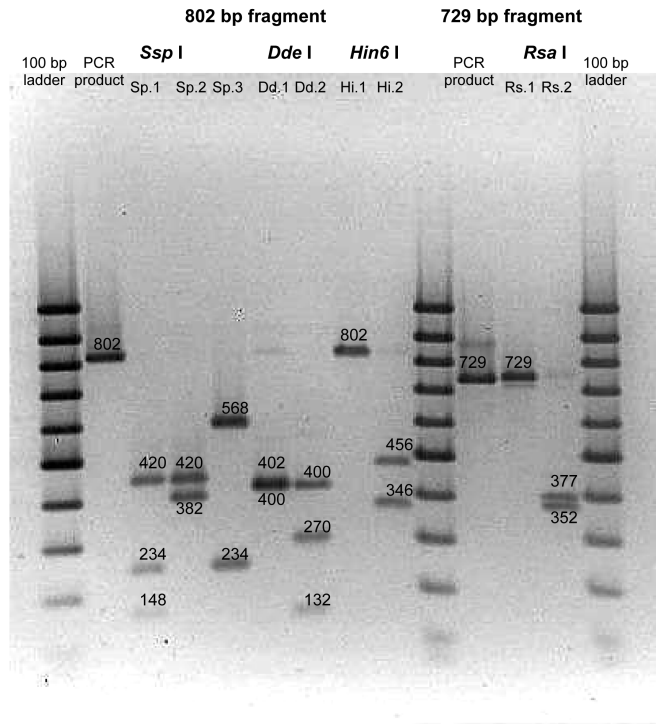


Figure 4. – Restriction sites of the enzymes *Ssp*I, *Dde*I, *Hin6*I, *Rsa*I. Separated on 1.2% Agarose gel, stained with ethidium bromide. Abbreviations are as explained in Table 2.

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

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
Ta.2	123		264		111	182
Ta.3			387		111	30
Ta.4	123	243	21	111	30	152

```

1      10      20      30      40      50      60      70      80      90      100
|      |      |      |      |      |      |      |      |      |
Ht1    |      |      |      |      |      |      |      |      |      |
Ht2-3  |      |      |      |      |      |      |      |      |      |
Ht4    |      |      |      |      |      |      |      |      |      |
Ht5-13 |      |      |      |      |      |      |      |      |      |
      TTCAACAAATCATAAAGATATTGGAACATTATATTTTATTTTGGAAATTTGAGCAGGTATAATTGGAACTTCTTT

101    |      |      |      |      |      |      |      |      |      |      |
Ht1    |      |      |      |      |      |      |      |      |      |      |
Ht2-3  |      |      |      |      |      |      |      |      |      |      |
Ht4    |      |      |      |      |      |      |      |      |      |      |
Ht5-13 |      |      |      |      |      |      |      |      |      |      |
      AAGTCITTTAATTCGAGCAGAATTAGGAAATCCAGGATCATTAATTGGAGATGATCAAAATTTATAATACTATGTACAGCCCATGCATTTATATAAAT

201    |      |      |      |      |      |      |      |      |      |      |
Ht1    |      |      |      |      |      |      |      |      |      |      |
Ht2    |      |      |      |      |      |      |      |      |      |      |
Ht3-13 |      |      |      |      |      |      |      |      |      |      |
      TTTTTTATAGTTATACCTATTATAATTGGAGATTGGTAATTGATTAGTACCTTTAATATTAGGAGCTCCTGATATAGCTTTCCACGAATAAATAATA

301    |      |      |      |      |      |      |      |      |      |      |
Ht1    |      |      |      |      |      |      |      |      |      |      |
Ht2-13 |      |      |      |      |      |      |      |      |      |      |
      TAAGTTTCTGACTTCTCCCCCTCTATTATACTTTTAATTTCAAGTAGAATTGTAGAAAACGGAGCAGGAACAGGTTGAACAGTTATCCCCCTTTC

401    |      |      |      |      |      |      |      |      |      |      |
Ht1    |      |      |      |      |      |      |      |      |      |      |
Ht2    |      |      |      |      |      |      |      |      |      |      |
Ht3    |      |      |      |      |      |      |      |      |      |      |
Ht4    |      |      |      |      |      |      |      |      |      |      |
Ht5    |      |      |      |      |      |      |      |      |      |      |
Ht6    |      |      |      |      |      |      |      |      |      |      |
Ht7    |      |      |      |      |      |      |      |      |      |      |
Ht8    |      |      |      |      |      |      |      |      |      |      |
Ht9-13 |      |      |      |      |      |      |      |      |      |      |
      TTCTAATATTGCTCATAGYGGAAAGCTCAGTAGATTAGCAATTTTTCTTTACATTTAGCTGGAATTTCTCAATTTAGGTGCAGTAAATTTTATTACA

501    |      |      |      |      |      |      |      |      |      |      |
Ht1    |      |      |      |      |      |      |      |      |      |      |
Ht2    |      |      |      |      |      |      |      |      |      |      |
Ht3    |      |      |      |      |      |      |      |      |      |      |
Ht4-8  |      |      |      |      |      |      |      |      |      |      |
Ht9    |      |      |      |      |      |      |      |      |      |      |
Ht10-13 |      |      |      |      |      |      |      |      |      |      |
      ACAATTATTAATATACGRCCATAATAATATATCATTAGATCAAATACCCTTATTTGTTGAGCTGTAGGAATTACAGCTTTATTATTACTTTATCTTTAC

601    |      |      |      |      |      |      |      |      |      |      |
Ht1    |      |      |      |      |      |      |      |      |      |      |
Ht2    |      |      |      |      |      |      |      |      |      |      |
Ht3    |      |      |      |      |      |      |      |      |      |      |
Ht4-6  |      |      |      |      |      |      |      |      |      |      |
Ht7    |      |      |      |      |      |      |      |      |      |      |
Ht8    |      |      |      |      |      |      |      |      |      |      |
Ht9    |      |      |      |      |      |      |      |      |      |      |
Ht10-13 |      |      |      |      |      |      |      |      |      |      |
      CAGTTTTAGCAGGTGCTATTACTATAYTATTAACAGATCGAAATTTAAATACCTCATTTTTGACCCAGCTGGGGGAGGAGATCCAATTTTATAYCAACA

701    |      |      |      |      |      |      |      |      |      |      |
Ht1    |      |      |      |      |      |      |      |      |      |      |
Ht2    |      |      |      |      |      |      |      |      |      |      |
Ht3    |      |      |      |      |      |      |      |      |      |      |
Ht4    |      |      |      |      |      |      |      |      |      |      |
Ht5-13 |      |      |      |      |      |      |      |      |      |      |
      TTTATTTTGATTTTGGCCACCCTGAAGTTTATATTTAATTTTACCAGGATTGGTATAAATTTCTCATATTATTCCCAAGAAAGAGGTAAGAAAGAA

801    |      |      |      |      |      |      |      |      |      |      |
Ht1    |      |      |      |      |      |      |      |      |      |      |
Ht2-12 |      |      |      |      |      |      |      |      |      |      |
Ht13  |      |      |      |      |      |      |      |      |      |      |
      ACATTTGGATGTTAGGAATAATTTATGCAATAATAGCAATTTGGGCTTCTAGGATTTGTAGTTTGGAGCTCATCATATATTACAGTAGGAATAGATATTG

901    |      |      |      |      |      |      |      |      |      |      |
Ht1    |      |      |      |      |      |      |      |      |      |      |
Ht2-13 |      |      |      |      |      |      |      |      |      |      |
      AFACTCGAGCTTATTTACTTCAGCAACTATAATTATTGCTGTCCACAGGAATTAATAATTTTAGTTGACTAGCTACTCTTCATGGACACAAATCAA

1001   |      |      |      |      |      |      |      |      |      |      |
      1050   |      |      |      |      |      |      |      |      |      |      |
      1100   |      |      |      |      |      |      |      |      |      |      |

```

Continued *Figure 5.*

```

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

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

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

1401 |                                     | 1450 |                                     | 1500 |
Ht1 | TTAATATTAATTATTATTTGAGAATCTTAAATTAATAAACGAATTAKTTTATTTCCATTAAATATAAATCTTCTATTGAATGATATCAAATCTTCCCC |
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 | TAATATAAATTACTATTTTAGTAGGTTATTTAATAATTAGTTTATTTTTTAATACTTATATTAATCGATTTTATTGGAAAGGACAAATAATTGAGTTAAT |
Ht2-9 | ..... |
Ht10 | .....CCGA..... |
Ht11-13 | ..... |

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

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

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

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

2201 |                                     | 2250 |                                     | 2300 |
Ht1 | GAAATTTGTGGGGCAAATCATAGTTTATACCTATTGTAATGAAAGAATCTCAATTA AAAACTTCATTAATGAATTAATAATTATCTTCATTAGATG |
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.

Haplotype	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	

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-

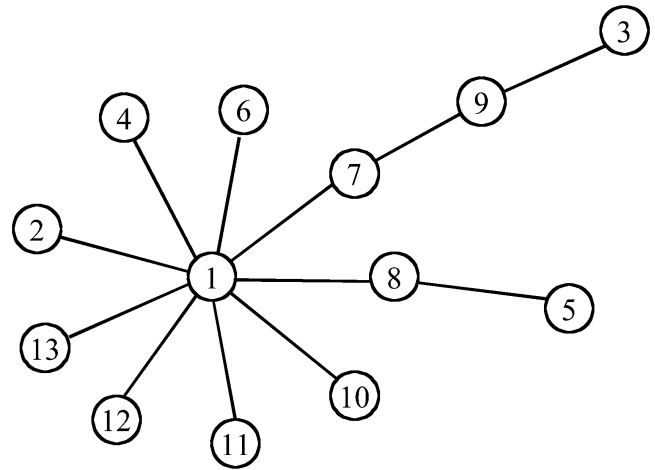


Figure 6. – Minimum spanning tree of the 13 haplotypes designed by means of haplotype definition as described in Table 2.

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 observed 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).

Acknowledgement

This project was supported by the “Deutsche Forschungsgemeinschaft” (DFG). We thank CHRISTOPH ZIEGLER and HEINZ-PETER SCHMITT from the “Landesanstalt für Ökologie, Bodenordnung und Forsten” (LÖBF) for very helpful hints to the investigated stands and our technical assistants SUSANNE JELKMANN, VIVIA KUHLENKAMP and INGE SCHULZE for the laboratory work.

Literature

AVISE, J. C. (1994): Molecular markers, natural history and evolution. Chapman & Hall, New York.

BOGDANOWICZ, S. M., W. E. WALLNER, J. BELL, T. M. ODELL and R. G. HARRISON (1993): Asian gypsy moths (Lepidoptera: Lymantriidae) in North America: Evidence from molecular data. *Ann. Entomol. Soc. Am.* **86** (6): 710–715.

COGNATO, A. I., S. J. SEYBOLD and F. A. H. SPERLING (1999): Incomplete barriers to mitochondrial gene flow between pheromone races of the North American pine engraver, *Ips pini* (Say) (Coleoptera: Scolytidae). *Proc. R. Soc. Lond. B* **266**: 1843–1850.

DE BRITO, R. A., M. H. MANFRIN and F. M. SENE (2002): Mitochondrial DNA phylogeography of Brazilian populations of *Drosophila buzzatii*. *Genetics Mol. Biol.* **25** (2): 161–171.

DOBLER, S. and B. D. FARRELL (1999): Host use evolution in *Chrysochus* milkweed beetles: evidence from behaviour, population genetics and phylogeny. *Mol. Ecol.* **8**: 1297–1307.

DU MERLE, P. (1999): Egg development and diapause: eco-physiological and genetic basis of phenological polymorphism and adaptation to varied hosts in the green oak tortrix, *Tortrix viridana* L. (Lepidoptera: Tortricidae). *J. Insect Physiol.* **45**: 599–611.

DUMOLIN, S., B. DEMESURE and R. J. PETIT (1995): Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method. *Theor Appl Genet* **91**: 1253–1256.

HORSTMANN, K. (1984): Untersuchungen zum Massenwechsel des Eichenwicklers, *Tortrix viridana* L. (Lepidoptera: Tortricidae), in Unterfranken. *Z. ang. Entomol.* **98** (1): 73–95.

HUNTER, M. D. (1990): Differential susceptibility to variable plant phenology and its role in competition between two insect herbivores on oak. *Ecol. Entomol.* **15**: 401–408.

HUNTER, M. D. (1992): A variable insect-plant interaction: the relationship between tree budburst phenology and population levels of insect herbivores among trees. *Ecol. Entomol.* **16**: 91–95.

HUNTER, M. D. (1998): Interactions between *Operophtera brumata* and *Tortrix viridana* on oak: new evidence from timeseries analysis. *Ecol. Entomol.* **23**: 168–173.

HUNTER, M. D., G. C. VARLEY and G. R. GRADWELL (1997): Estimating the relative roles of top-down and bottom-up forces on insect herbivore populations: A classic study revisited. *Proc. Natl. Acad. Sci. USA* **94**: 9176–9181.

IVASHOV, A. V., G. E. BOYKO and A. P. SIMCHUK (2002): The role of host plant phenology in the development of the oak leaf roller moth, *Tortrix viridana* L. (Lepidoptera: Tortricidae). *Forest Ecol. Manag.* **157**: 7–14.

KRUSE, J. J. and F. A. H. SPERLING (2001): Molecular phylogeny within and between species of the *Archips argyrospila* complex (Lepidoptera: Tortricidae). *Ann. Entomol. Soc. Am.* **94** (2): 166–173.

KRUSE, J. J. and F. A. H. SPERLING (2002): Phylogeny of nearctic species of the Xylosteana group of *Archips* Hübner (Lepidoptera: Tortricidae) based on combined analysis of morphological and mitochondrial DNA data sets. *Ann. Entomol. Soc. Am.* **95** (3): 288–301.

LANDRY, B., J. A. POWELL and F. A. H. SPERLING (1999): Systematics of the *Argyrotaenia franciscana* (Lepidoptera: Tortricidae) species group: evidence from mitochondrial DNA. *Ann. Entomol. Soc. Am.* **92**: 40–46.

LUNT, D. H., D. X. ZHANG, J. M. SZYMURA and G. M. HEWITT (1996): The insect cytochrome oxidase I gene: evolutionary patterns and conserved primers for phylogenetic studies. *Insect Mol. Biol.* **5** (3): 153–165.

MUN, J. H., Y. H., SONG, K. L. HEONG and G. K. RODERIC (1999): Genetic variation among Asian populations of rice planthoppers, *Nilaparvata lugens* and *Sogatella furcifera* (Hemiptera: Delphacidae): mitochondrial DNA sequences. *Bull. Entomol. Res.* **89**: 245–253.

PETIT, R. J., E., PINEAU, B. DEMESURE, R. BACILIERI, A. DUCOUSO and A. KREMER (1997): Chloroplast DNA footprints of postglacial recolonization by oaks. *Proc. Natl. Acad. Sci. USA* **94**: 9996–10001.

RONDAN DUENAS, J. C., G. M. PANZETTA-DUTARI, A. BLANCO and C. N. GARDENAL (2002): Restriction Fragment-Length Polymorphism of the mtDNA A+T rich region as a genetic marker in *Aedes aegypti* (Diptera: Culicidae). *Ann. Entomol. Soc. Am.* **95** (3): 352–358.

SCHROEDER, H., H. KLOTZBACH, S. ELIAS, C. AUGUSTIN and K. PUESCHEL (2003): Use of PCR-RFLP for differentiation of *calliphorid larvae* (Diptera, Calliphoridae) on human corpses. *Forensic Sci. Int.* **132** (1): 76–81.

SCHÜTTE, F. (1957): Untersuchungen über die Populationsdynamik des Eichenwicklers (*Tortrix viridana* L.). *Z. ang. Entomol.* **40** (3): 1–36 und 285–331.

- SCHWERDTFEGER, F. (1971): Vergleichende Untersuchungen an der Kronenfauna der Eichen in Latenz- und Gradationsgebieten des Eichenwicklers (*Tortrix viridana* L.). 3. Die Bedeutung der Parasiten für den lokalen Fluktuationstyp des Eichenwicklers. *Z. ang. Entomol.* **67**: 296–304.
- SIMCHUK, A. P., A. V. IVASHOV and V. A. COMPANYYTSEV (1999): Genetic patterns as possible factors causing population cycles in oak leaf roller moth, *Tortrix viridana* L. *Forest Ecol. Manag.* **113**: 35–49.
- SIMON, C., F. FRATI, A. BECKENBACH, B. CRESPI, H. LIU and P. FLOOK (1994): Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Ann. Entomol. Soc. Am.* **87** (6): 651–701.
- SPERLING, F. A. H. and D. A. HICKEY (1995): Amplified mitochondrial DNA as a diagnostic marker for species of conifer-feeding *Choristoneura* (Lepidoptera: Tortricidae). *Can. Entomol.* **127**: 277–288.
- SPERLING, F. A. H., J. LANDRY and D. A. HICKEY (1995): DNA-based identification of introduced ermine moth species in North America (Lepidoptera: Yponomeutidae). *Ann. Entomol. Soc. Am.* **88** (2): 155–162.
- SPERLING, F. A. H., A. G. RASKE and I. S. OTVO (1999): Mitochondrial DNA sequence variation among populations and host races of *Lambdina fiscellaria* (Gn.) (Lepidoptera, Geometridae). *Insect Mol. Biol.* **8** (1): 97–106.
- STRESEMANN, E., H. J. HANNEMANN, B. KLAUSNITZER and K. SENGLAUB (2000): Exkursionsfauna von Deutschland. Wirbellose: Insekten. Band 2. 9. Auflage, Spektrum Akademischer Verlag Heidelberg, Berlin, 959 pp.
- THALENHORST, W. (1951): Die Koinzidenz als gradologisches Problem. Eine synökologische Studie. *Z. ang. Entomol.* **32** (1): 1–48.
- WAHLBERG, N., OLIVEIRA, R. and J. A. SCOTT (2003): Phylogenetic relationships of *Phyciodes* butterfly species (Lepidoptera: Nymphalidae): complex mtDNA variation and species delimitations. *Syst. Entomol.* **28**: 257–273.

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

By X. XUE^{1,2}, Y. WANG^{1,2}, H. KORPELAINEN³ and C. LI^{1,*}

(Received 21st February 2005)

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.

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

¹) Chengdu Institute of Biology, Chinese Academy of Sciences, P. O. Box 416, Chengdu 610041, P. R. China.

²) Graduate School of the Chinese Academy of Sciences, Beijing 100039, P. R. China.

³) Department of Applied Biology, P. O. Box 27, FIN-00014 University of Helsinki, Finland.

^{*}) Correspondence address: CHUNYANG LI, Chengdu Institute of Biology, Chinese Academy of Sciences, P. O. Box 416, Chengdu 610041, P. R. China, Tel: +86-28-85221347, Fax: +86-28-85222753, Email: licy@cib.ac.cn, chunyang.li@helsinki.fi