The Genetic Background of Three Introduced Leaf Miner Moth Species -Parectopa robiniella Clemens 1863, Phyllonorycter robiniella Clemens 1859 and Cameraria ohridella Deschka et Dimic 1986

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The Genetic Background of Three Introduced Leaf Miner Moth Species - *Parectopa robiniella* Clemens 1863, *Phyllonorycter robiniella* Clemens 1859 and *Cameraria ohridella* Deschka et Dimic 1986

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Abstract – North American and European populations of three invasive leaf miner moth species *Parectopa robiniella* Clemens 1863, *Phyllonorycter robiniella* Clemens 1859 and *Cameraria ohridella* Deschka et Dimic 1986 were investigated using mtDNA sequences and PCR-RAPD. Significant variation (0.7%) in the mtDNA was detected among *Parectopa robiniella* population, allowing differentiation of European from North-American populations In the other two species, *Phyllonorycter robiniella* and *C. ohridella*, no substitutions could be detected among the populations. The complementary PCR-RAPD analysis in *C. ohridella* revealed genetic similarities among populations that reflected historical patterns of spread. However, additional samples from more populations and additional markers would be desirable in the future in order to obtain a more stable dataset.

I. Introduction

The introductions of insect species can have dramatic effects on the particular ecosystems. Accordingly, the field of invasion biology is well researched and new publications appear regularly. The alien arrival processes, the steps of the colonisation and the impacts of alien species have been analysed in detail [1]. But investigations on how the introduction process affects the genetic structure of the invasive species are seldom investigated [2]. It is possible to obtain precise data if the details of the introduction (single or multiple introduction), and how the settlement took place (e.g. founder effect) are known. Among forest-dwelling insects, data can be found on the introduction process of the pine shoot beetle (*Tomicus piniperda* L.) [3] and gypsy moth (*Lymantria dispar* L.) [4] in North-America.

The goal of this investigation was to analyse the genetic structure of three invasive leaf miner moths in order to obtain knowledge on their introduction processes. The three species studied here are: A) *Parectopa robiniella* Clemens 1863, B) *Phyllonorycter robiniella* Clemens 1859 and C) *Cameraria ohridella* Deschka et Dimic 1986

Taxonomic background
All three, invasive, leaf-mining species are members of the Gracillariidae (superfamily Gracillarioidea), a large, cosmopolitan family of over 2000 recognised species, with probably an even greater number of species awaiting discovery. The genus *Parectopa* is a member of the largest, but far most diverse subfamily, Gracillariinae. The genera *Phyllonorycter* and *Cameraria* are the largest groups within the Lithocolletinae, a subfamily currently including approximately 10 genera.

A. *Parectopa robiniella*

The genus *Parectopa* has a holarctic distribution. Eleven species of the genus are known in North America (some not congeneric with *P. robiniella*) [5], but only *P. ononidis* (Zeller, 1839) and the introduced *P. robiniella* currently occur in Europe. Four species have been reported to have been present in Hungary as early as 1956 [6], but according to the latest report only the above two species are currently known to exist there [7].
Parectopa robiniella originated from North America. In Europe it was first found in 1970 in Italy. In Hungary the first specimen was found in 1983 [8]. Its host plant is the black locust (*Robinia pseudoacacia* L.) and it occurs also on variants of black locust. We found mines on the compound leaves (approximately 15 cm long) of the unifolia variant (*R. p.* f. *monophylla*) in the botanical garden of the West-Hungarian University during 2003.

B. *Phyllonorycter robiniella*

The genus *Phyllonorycter* has a more global distribution, and is the most speciose of the three genera represented with nearly 400 species reported [9]. In North America 82 species are known [5, 10]. Nearly 150 species have been reported in Europe [9] and of those, 55 occur in the United Kingdom [11]. There are also a large and growing number of species in Hungary [6, 12].
Phyllonorycter robiniella also originates from North America where its host-plant is also black locust (*Robinia pseudoacacia* L.). In Europe it was first found in 1983 in Switzerland. In Hungary it was first discovered in 1996 [8] and after several years it had spread over the entire country. Mines have been found only on the black locust in Europe.

C. *Cameraria ohridella*

The distribution of the genus *Cameraria* is also holarctic. Until the discovery of *C. ohridella*, no member of the genus was known to occur in Europe. In North America 52 species are known as well as several undescribed species [5]. It is also a significant group in Asia, but it is necessary to note, that we have inadequate information for many areas (e.g. China). We know of seven described Malaysian and three Indian species [13]. In addition, there are 3 described and at least 4 undescribed species in Japan [Kumata pers. comm.].
The species *Cameraria ohridella* was first described in 1986 [14] from the Ohrid lake region in Macedonia. It dispersed by human transmission to Austria and then throughout Europe, except for the Iberian-peninsula and Scandinavia. In Hungary it was first found in 1993 [15] but in only a few years the moth dispersed over the entire country. The host-plant of the *Cameraria ohridella* is the horse chestnut (*Aesculus hippocastanum* L.), but it also mines the leaves of *Acer* species.

II. Material and Methods

A. Materials

a. Species of known origin (*P. robiniella* and *Ph. robiniella*)
In the case of the two leaf-miner species on black locust (*P. robiniella* and *Ph. robiniella*) we investigated populations from the country of origin (USA) and from Hungary. The sampling sites are shown in Table 1.

TABLE 1
Collection sites of the two black locust leaf miners

Insect species	Country	Location
Parectopa robiniella	USA	Louisville, KY (LO)
		Nashville, TN (NA)
	Hungary	Szentes (SZ)
		Kerekegyháza (KE)
Phyllonorycter robiniella	USA	Slade, KY (SL)
	Hungary	Győrszemere (GY)

The larvae were taken from the leaves and stored in absolute ethanol until the DNA was extracted. We present data here from only one individual from each population.

b. Species of unknown origin (*C. ohridella*)
Several locations from different European countries were collected and also stored in absolute ethanol until DNA extraction (Table 2.).

TABLE 2
Collection sites of *Cameraria ohridella* for the sequence analysis and the RAPD-PCR

Insect species	Country	Location	No. of samples	
			mtDNA	RAPD
C. ohridella	Macedonia	Ohrid	1	14
	Bosnia-Herzegovina	Sarajevo		12
	Austria	Vienna		13
	Hungary	Gyermely	1	
	Italy	Verona		13
	Poland	Krakow	1	7
	Germany	Erfurt	1	6
	Netherland	Wageningen		6

B. Methods

a. DNA Extraction - DNA was extracted by homogenising single larvae or pupae in 400µl of homogenisation buffer (100mM Tris HCl pH 8; 100mM EDTA pH8; 1% SDS) with a sterile pestle. The homogenate was incubated with 4µl of proteinase K (10 mg/ml) at 56°C for 90 min. After adding 250µl of 4.5M NaCl, the DNA was extracted with chloroform-isoamylethanol and precipitated with ethanol, according to standard protocols [16].
b. PCR of the mtDNA - a fragment of mitochondrial cytochrome oxidase I (COI) was amplified by using primers listed in [17] or [18]. Different primers were used with different success.
c. Sequence analysis - the PCR fragments were sequenced directly after purification with the QIAquick PCR purification kit (Qiagen). 20ng of the purified PCR product was used for the cycle sequencing reaction with Big Dye (Applied Biosystems). Sequence products were loaded on an automatic sequencer ABI 310 (Applied Life Sciences).
Sequences were aligned using Clustal W [19].
d. RAPD PCR - 37 different primers were used. 12 of them were interpreted (R1, R2, R7, R8, R9, R13, R14, OPA4, OPAB1, OPAB8, OPAC11, OPAC13, sequences can be sent on request).
The software programme Popgene 32 was taken for analysis.

III. Results and Discussion

A. *Parectopa robiniella*

A 552bp stretch of the mitochondrial COI gene was analysed and four haplotypes were detected (sequences can be sent on demand) (Table 3).

TABLE 3
Haplotypes of *P. robiniella* found in the Hungarian (HU) and the American (US) populations. Numbers indicate substitution site on the COI gene.

	32	161	341	506
HU_SZ	A	A	C	A
HU_KE	A	G	C	A
US_KY	A	A	T	A
US_TN	T	A	T	G

We found distinct differences between the populations from Hungary and North America. American and Hungarian haplotypes showed four mutations and substitution site 341 could be a marker for defining the two populations. Each of the two countries revealed differences although locations were geographically close. This indicates that populations are quite polymorphic and the introduction was certainly by more than one haplotype.
The genetic similarities (Kimura) among the populations are shown in the Table 4. Within Hungary 0.18% variation was found whereas within the US variation was 0.36%, indicating that more haplotypes might exist in America. Between the two countries a maximum of 0.72% variation was analysed.

TABLE 4
Similarity matrix (based on Kimura) of *P. robiniella*

	HU_SZ	HU_KE	US_KY	US_TN
HU_SZ	-	0.0018	0.0018	0.0054
HU_KE		-	0.0036	0.0072
US_KY			-	0.0036
US_TN				-

A Neighbour Joining tree was calculated taking Kimura 2-parameter Model (Figure 1.). The tree indicates that the Hungarian haplotypes are derived from the American populations. To determine the exact location of origin, further analysis of populations from North America is necessary.

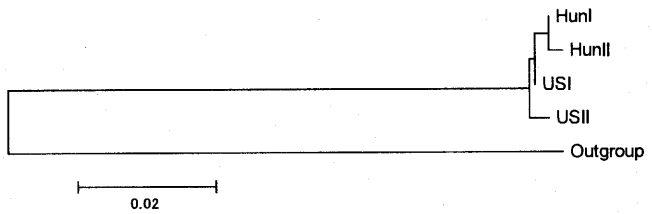

Figure 1. The Neighbour Joining tree based on Kimura 2-distance of the mtCOI gene of *P. robiniella* populations. As outgroup *Phyllonorycter robiniella* was taken. (HunI=HU_SZ, HunII=HU_KE, USI=US_KE and USII=US_TN)

The species has significant genetic diversity both in its country of origin and in Hungary. This provides a good opportunity to conduct successful phylogeographical analysis (geographical pattern of genetic differences).
What are the causes for such large genetic diversity? In North America it may be explained by the greater geographical distances and by the native characteristics of the species. But, what of the situation in Hungary? Were there multiple introductions? Because of the small distance between the two populations sampled (<100km), this is unlikely. It is possible that within *Parectopa* there may exist sibling species that are difficult to distinguish morphologically.

B. *Phyllonorycter robiniella*

A 536bp stretch of the mitochondrial COI gene was analysed and only one haplotype was detected (sequences can be sent on demand). The populations from Hungary and North-America can be considered genetically similar; however other more polymorphic markers like microsattelites should be developed to clarify historical invasion processes.
Because of the low number of populations and individuals surveyed, further research needs to be conducted, taking into account recent studies on the phylogeny of the genus *Phyllonorycter* [10].

C. *Cameraria ohridella*

a. mitochondrial COI gene
A 173bp stretch of the mitochondrial COI gene was analysed (sequences can be sent on demand) and also here only one haplotype was detected. This supports the hypothesis, that either the original population is genetically depauperated or that only one haplotype was introduced into the Ohrid lake district.
Although the number of populations examined was greater (4) than in the case of *Ph. robiniella*, the sequenced DNA section was much shorter.

b. RAPD PCR
The R14 primer had 4 clear bands and results of the statistical evaluation are shown in Table 5.

TABLE 5
Results of RAPD-PCR of *C. ohridella* analysing two different similarity values

Population	Nei's D	Shannon's I	No. of polymorphic bands
Ohrid	0.3436	0.4880	3
Sarajevo	0.3506	0.4948	3
Vienna	0.3083	0.4497	3
Verona	0.2093	0.3034	2
Krakow	0.1244	0.1727	1
Erfurt	0.2071	0.3024	2
Wageningen	0.2428	0.3393	2

The results supports the hypothesis that Ohrid-lake was the original source for the population introduced to Austria from which it subsequently spread through out Europe. The Ohrid population, the population in nearby Sarajevo and the Austrian population revealed the highest values. Values decreased in areas which became infested at later dates indicating a bottleneck effect. The genetic similarity among the locations was plotted using the UPGMA (Unweighted Pair - Group Method using an Arithmetic Average) method (Figure 2.).

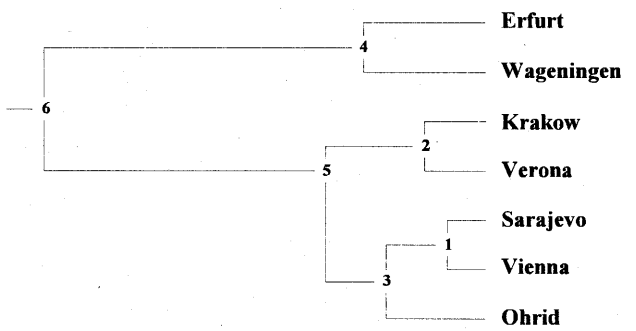

Figure 2. The UPGMA dendrogram of the RAPD-PCR of *C. ohridella* populations

The origin of *C. ohridella* is still not known. Thus it might be reasonable to investigate either the host tree or the origin of closely related species living on the same tree (Table 6.). Both in North America and in Asia (Japan) *Cameraria* species can be found on different *Aesculus* species. Furthermore, possible host switches by *C. ohridella* feeding on *Acer* (e.g. *A. rubrum* L.) (e.g., *Cameraria aceriella* Clem.) should be considered in future analysis [8].

TABLE 6
Cameraria species and its host

Insect species	Host tree	Location
C. ohridella D. & D.	*Aesculus hyppocastanum*	Europe
C. aesculisella Cham.	*Aesculus octandra*	N-America
	Aesculus glabra	N-America
	Aesculus pavia	N-America
C. sp. new Kumata	*Aesculus turbinata*	Japan

The morphotaxonomial analysis of the genus *Aesculus* shows that the sister species of *Ae. hippocastanum* is *Ae. turbinata* [20] which is native to Japan. Our future goal is to conduct comparative genetic analysis of *Cameraria* species from different continents.

IV. Summary and Conclusions

We analysed three invasive leaf-mining moth species (*Parectopa robiniella* Clemens 1863, *Phyllonorycter robiniella* Clemens 1859 and *Cameraria ohridella* Deschka et Dimic 1986) - using mtDNA sequences and genomic RAPD patterns. Populations examined were from North America and from several European localities.

The genetic analysis of the three invasive leaf-mining moth species led to quite different results. Despite the existence of significant variation (0.7%) in the mtDNA sequence of the European and North-American *P. robiniella* populations, the base-sequence of the other two species (*Ph. robiniella* and *C. ohridella*) was the same. The complementary RAPD PCR analysis, which was carried out on the European populations of *C. ohridella* also resulted in a geographic pattern, which showed a significant similarity to the European dispersal route of the species. We are planning further investigations to determine the origin of the species.

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