

UNIVERSITY OF WEST HUNGARY

SUMMARY OF PhD THESIS

THE POPULATION GENETIC STUDY OF THE PLANE LEAF MINER
(*PHYLLONORYCTER PLATANI* STGR. 1870)

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1. Introduction

The increasing rate of invasive species is becoming a big economical and conservation biological problem in these days.

The plane leafminer (*Phyllonorycter platani* STAUDINGER 1870) is one of the most important invasive *Gracillariidae* species (Šefrová 2003; Lopez-Vaamonde *et al.* 2010). Its invasion history is well documented in Europe. Its colonization has started at the second half of the 19th century (Šefrová 2001, 2003). The invasion process includes several jumps from the native origin (SE_Europe) to the northern and north-western part of Europe (Šefrová 2003). The dispersal occurred in anemochoral and atropochoral ways with passive transportation of mined leaves and/or saplings (Šefrová 2001).

The species have been detected in North Africa, Caucasus, Central Asia, Asia Minor, Crimean Peninsula (Vasil'eva & Shkarlet 1989; Kuznetsov 1990; Deschka 1995; Lees 2010) and USA. The North-American occurrence dates back exclusively to California (Heinrich 1920; Burke 1933; Koehler & Campbell 1968; Gates *et al.* 2002).

According to Deschka (1995) the plane miner has holarctic distribution range. However, Šefrová (2001) believes that it is native to South Europe. Lopez-Vaamonde *et al.* (2010) consider the area of origin of *P. platani* as not known.

The plane leafminer is a monophagous species. Host shifts are very rare events in the *Phyllonorycter* genus (Lopez-Vaamonde *et al.* 2006). Leaf mining is a special relationship between insect and its host plants because they are internal feeders. Its host plants are plane trees (*Platanus*): *Platanus orientalis* L., *P. occidentalis* L., *Platanus acerifolia* (AIT.) WILLD., *P. racemosa* NUTT. (Lees 2010).

Plane trees are the most common ornamental and lining trees in the temperate and subtropical cities (Liu & Bao 2003). That is why the aesthetical damage or the early defoliation caused by the mining of *P. platani* is of high significance (Heinrich 1920; Györfi 1941; Halperin 1990).

2. Aims

1. Reveal the genetic structure of a *Phyllonorycter platani*.
2. Exploration of this species' phylogeographic pattern.
3. Determine the native area of *P. platani*. Whether the invasion direction of *P. platani* was North America from Europe or has it come to Europe from North America? Are there any demonstrable refuges?
4. Reveal what factors influence the recent genetic pattern. Can we detect an isolation by distance? Is there any geographical isolation? What kind of spatial level has it materialized?

3. Materials and methods

3.1. Sampling and molecular methods

Samples were collected in 2007-2013. We have obtained larvae and pupae from 13 European, 1 Caucasian, 2 Central Asian countries, Asia Minor and North America. Overall, 26 populations were investigated. The identification of the species was based on damage symptoms (type and locality of the mine) and the host plants. 10-20 individuals per locality were collected. From some of the populations (Bishkek, Brussels, Csongrád, Dávod, Diósvizsló, Hajós, Milopotamos, Rotterdam) we could collect less than ten specimens. All samples were stored in 96% ethanol at 4°C.

DNA was extracted from entire bodies using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) and processed following the manufacturer's protocol. Exceptionally were used E.Z.N.A.® Tissue DNA Kit for four specimens and AquaGenomic Kit for three specimens. Eluted DNA was stored at -20°C.

We amplified a 530bp portion of the 28S rDNA for 35 individuals using a D1F (5'-ACC CGC TGA ATT TAA GCA TAT-3') and D3R (5'-TAG TTC ACC ATC TTT CGG GTC-3') primers (Lopez-Vaamonde *et al.* 2001). We used the polymerase chain reaction as described by Lopez-Vaamonde *et al.* (2001).

A 1243bp region of the COI gene was amplified for 227 individuals by using Pat (5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3'), and Lep2F (5'-ATT CAA CCA ATC ATA AAG ATA TTG G-3') primers (Lunt *et al.* 1996; Dinca *et al.* 2011), as well as two additional primers Dick (5'-CCA ACA GGA ATT AAA ATT TTT AGA TGA-3') (Dinca *et al.* 2011) and Pc6F (5'-GCC CCA GAT ATA GCA TTT CC-3') (Avtzis *et al.* 2006). PCR conditions included an initial denaturation step at 94 °C for 2 minutes, followed by 34 cycles at 94 °C for 30 s, 47 °C for 1 minute and 72 °C for 1 minute 30 s with a final extension step that lasted 10 minutes at 72 °C.

Sequences were generated at the Eurofin's Laboratory.

3.2. Data analysis

a, 28S rDNA

35 individuals were used for nuclear DNA analyses. Sequence data of *P. issikii* were used as out-group and additional sequences were downloaded from GenBank (accession numbers: AF477559 *P. aemula*, AF477569 *P. esperella*, AY521521 *P. issikii*, AF477565 *P. platani*, AY521507 *P. pygmaea*, AF477562 *P. nicellii*).

A neighbor-joining (NJ) tree was generated utilizing the Kimura 2-parameter model (Kimura 1980) by bootstrapping with 5000 replicates. Genetic distances were calculated with MEGA 5.02 (Tamura *et al.* 2011).

b, COI

227 individuals used for mitochondrial DNA analyses. Sequences were visualized using Sequence Scanner and then aligned using ClustalX (Thompson *et al.* 1994). After haplotypes were identified, those represented by only a single individual were verified by additional sequencing of an independent amplicon.

In addition sequences of *P. issikii* (own results), and *Cameraria ohridella* (downloaded from GenBank, accession number: AF477544) were used as outgroups.

Phylogenetic analyses

A neighbor-joining (NJ) tree was generated utilizing the Kimura 2-parameter model (Kimura 1980) by bootstrapping with 5000 replicates.

Both maximum-parsimony (MP) and maximum-likelihood (ML) analyses were performed using MEGA 5.02. The level of support for individual nodes was evaluated by bootstrapping with 5000 replicates.

Maximum likelihood (ML) analysis was performed under GTR+I model. We used jModeltest 2.1.2 (Guindon & Gascuel 2003; Darriba *et al.* 2012) to select the best model of nucleotide substitution with Akaike Information Criterion (AIC) (Akaike 1974).

Bayesian-based inference was performed with TOPALi v.2 (Milne *et al.* 2008) using the nucleotide substitution model GTR+I. The number of generations was 2.000.000, with a sampling frequency of 1000 generations.

Population structure

Patterns of molecular diversity based on the mtDNA sequences between and within populations were assessed by estimating: nucleotide diversity (π) (Nei 1987), transition/transversion ratio, haplotype diversity (h) (Ewens 1972; Zouros 1979), mean number of pairwise differences (k) using the software Arlequin version 3.5.1.2 (Excoffier & Lischer 2010). Shannon diversity indices were calculated with PAST (Hammer *et al.* 2001).

Demographical expansion

For the estimation of Tajima's D statistics (Tajima 1989), Fu's F_s and mismatch distribution (Rogers & Harpending 1992) Arlequin 3.5.1.2 was used with 50 000 permutations (Excoffier & Lischer 2010). Additionally DnaSp 5.10 (Librado & Rozas 2009) was used to estimate R2.

Phylogeographical analysis

Spatial analysis of molecular variance (SAMOVA) was performed using SAMOVA v1.0 (Dupanloup *et al.* 2002). The program was run 1023 iterations. K values were tested, starting from two until the value for which FCT reached a plateau (Chiari *et al.* 2012).

In addition, alternative geographical groups were tested with Analysis of Molecular Variance (AMOVA) (Weir & Cockerham 1984; Excoffier *et al.* 1992; Weir 1996) with Arlequin 3.5.1.2 (Excoffier & Lischer 2010). The statistical significance of variance components in AMOVA was tested with 1000 permutations.

Isolation by distance was evaluated using Mantel-test (Mantel 1967) with MANTEL NON-PARAMETRIC CALCULATOR ver. 2.0 (Liedloff 1999). Natural algorithms of geographical linear distances (km) between localities were correlated with the respective Tamura-Nei genetic distances (Tamura & Nei 1993), calculated with MEGA v.5.02 (Tamura *et al.* 2011), with 1000 random iterations to obtain statistical inferences at $\alpha=1\%$.

Nested Clade Phylogeographic Analysis (NCPA) is the other way of inferring historical phylogeographic processes. Statistical parsimony network (SP) (Templeton *et al.* 1992) was created using a TCS 1.2.1 (Clement *et al.* 2000). The nesting design was constructed on the SP network following the rules in Templeton *et al.* (1995); Templeton (1998). The resulting nested network was statistically evaluated with GeoDis v. 2.6 (Posada *et al.* 2000).

4. Results and thesis

1. The genetical structure of *P. platani* have been revealed.

Twenty haplotypes were detected on the 1243bp long fragment of the mitochondrial DNA (COI gene) among the 227 individuals. The number of variable sites was 38 (3.1%). The sequence was translated into 414 amino acids. The average nucleotide composition for all haplotypes was as follows: T 41.3%, C 13.7%, A 30.9%, G 14.1%. 87.2% of the mutations were silent.

2 alleles were identified on the 530bp long fragment of the 28S rDNA. Variation only at one position was identified among the 35 specimens sequenced. Nucleotide composition for this gene: T 21.3%, C 27.8%, A 20.4%, G 30.5%.

2. All examined populations of P. platani represented one species.

The genetic divergence between European and North American haplotypes were rather low (0.2 %) in compare to other *Phyllonorycter* species (5.2 – 12.4%). They are similar on 28S rDNA.

Pairwise genetic distances between Asian and European or between Asian and North American populations are lower (2.04%) than the general distance among other *Phyllonorycter* species (5.2 – 12.4%) on COI gen.

Genetic divergence between „Asian” and „European” allels was 0.2% on 28S rDNA, in the *Phyllonorycter* genus the average intraspecific divergence is higher than this (0.5%).

3. There are two major haplo groups („Asian” and „European–North American”) present on COI.

Average sequence divergence between „Asian” and „European–North American” haplotype groups (2.04%) was higher than the intrapopulation level (0.21%; 0.06%). Divergence data shows that the population from Asia Minor is closer to „Caucasian–Central Asian” group (0.48%), than the European (1.80%). Both, MP and ML trees support two haplotype groups with 99% probability.

4. The effects of geographical isolation were medium to the genetic divergence between the Asian and European-North American haplotype group. At least two refuges (Balkan Peninsula, Caucasus) were During the Pleistocene.

Frequencies data revealed that at least two glacial refuges (Balkan Peninsula and Caucasus) were existing. The effects of geographical isolation was medium (Mantel test $r=0.4525$), or high (SAMOVA $V_a=95\%$) on the genetic divergence among the two major haplotype group.

5. On European population genetic diversity pattern was sudden demographic expansion the most effective factor. The effect of isolation by distance was weak.

Mantel test indicated weak ($r=0.2049$) correlation between genetic population structure and geographic distances, while on the other hand SAMOVA indicated either a medium ($V_a=64.46\%$) correlation. The effects of small population size and rapid expansion (after bottleneck event) were the most important effects on *P. platani* populations in Europe (unimodal shape of mismatch distribution, neutrality tests $D=-1.744$, $F_s=-9.430$). Similar inference was supported by the star-shape structure of haplotype networks (NCPA).

6. The North-American population suggests sudden demographic expansion, for this reason native area of P. platani in North-America was not supported.

The appearance of temporary haplotype (HT16) out of Asian and North American (European) haplotypes indicates that we can reject the hypothesis of *P. platani* colonised Europe from North America. The outcome of neutrality tests ($R_2=0.188$) and the diversity indices ($h=0.600$, $\pi=0.056\%$) suggest that sudden demographic expansion shaped the current pattern of intraspecific diversity of the North American population.

7. *Sudden expansion was detected from Asia Minor and Central Asia by P. platani.* Based on the high rate of the Caucasian (Telavi) diversity indices ($h = 0.511$, $\pi = 0.054\%$), and the homogeneity of Central Asian populations (Samarkand, Bishkek) may have a recent expansion to Central Asia from the Caucasus. The homogeneity of the population from Asia Minor suggested founder effect.

8. *P. platani expanded through Turkey to the Caucasus and Central Asia.*

The haplotype (HT16) from Asia Minor is closer to the Caucasian haplotypes (HT13, HT14, HT15) than to the European haplotypes (99% probability of MP and ML tree).

5. List of publications related to dissertation

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TÓTH V. – LAKATOS F. (2011): A platánlevél-sátorosmoly (*Phyllonorycter platani* Strg. 1870) tápnövényei. (The host plant range of the plane leaf-miner (*Phyllonorycter platani* Strg. 1870) [In Hungarian with English abstract]) – In: LAKATOS F. –POLGÁR A. – KERÉNYI-NAGY V. (szerk.): *Tudományos Doktorandusz Konferencia, Nyugat-magyarországi Egyetem Erdőmérnöki Kar*, (Konferencia-kötet, 2011.április 13., Sopron), pp.: 163-166

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6. List of publications not closely related to dissertation

Book chapter

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- HORVÁTH B. – SÁFIÁN SZ. – TÓTH V. – LAKATOS F. (2012): Genetic Investigation of Protected Lepidoptera Species in West Hungary (Presentation of methods, modell species and aims). International Scientific Conference on Sustainable Development & Ecological Footprint, The Impact of Urbanization, Industrial and Agricultural Technologies on the Natural Environment, Sopron May 26-27 2012
- LAKATOS F. – TUBA K. – TÓTH V. – MÉSZÁROS B. (2011): Jönnek az idegenek – inváziós rovarok a hazai fás növényeken. (Aliens are coming - Invasive insects on native tree species in Hungary) In: LAKATOS F. – SZABÓ Z. (szerk.): *Nyugat-magyarországi Egyetem Erdőmérnöki Kar, Kari Tudományos Konferencia Kiadvány*, NymE Kiadó Sopron. (Konferencia-kötet, 2011. október 5., Sopron), pp.: 18-22.

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