University of West Hungary

Doctoral theses

Data on the biology, spreading and control of the horse chestnut leaf miner (*Cameraria ohridella* Deschka et Dimič 1986, Lepidoptera, Gracillariidae)

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1. Importance of the topic

A characteristic tree species of Hungary's parks and alleys, the horse chestnut (*Aesculus hippocastanum)* had no significant pest until the beginning of the 1990ies and it also resisted well the ever increasing environmental pollution in the cities. In Europe in the 1990ies, however, there was a rapid spread of a miner species belonging to the family Gracillariidae which was the horse chestnut leaf miner (*Cameraria ohridella* Deschka et Dimič, 1986, Lep. Gracillariidae). Due to its damage, bigger and bigger yellowish brown mines had appeared on the leaves of the trees since the beginning of June. In parallel with the development of the subsequent horse chestnut leaf miner generations, the trees by mid-August lost the majority of the foliage often followed by a forced second blooming.

To protect and maintain the horse chestnut trees is an important task of the experts working in the field of plant protection. To be able to work out an effective preventive method against the pest unknown until its first occurrence, it is essential to have a thorough all-round knowledge of the species. Consequently, all research work aiming to discover more and more details, and obtain better knowledge on the horse chestnut leaf miner is very important.

2. Scientific background

Because of the fact that the horse chestnut leaf miner species was unknown before 1986, a relatively small number of relevant sources are available.

2.1. Scientific background in Hungary

The appearance of the horse chestnut leaf miner in Hungary was first reported by **Csaba Szabóky** who at the same time described the morphological features of the species too. Detailed information on the lifestyle and habits of the species was provided by **Kornélia Czencz** and **György Bürgés. Klára Balázs, György Bürgés, Kornélia Czencz,** and **Klára Nemestóthy Kerényiné** significantly contributed to the further descriptions of the species, lifestyle analysis and to the development of the first preventive methodologies. In connection with the identification of the parazitoids relying on the Hungarian populations of the horse chestnut leaf miner, we have to mention **Csaba Thúróczy,** and results in this field were also provided by **Klára Balázs. Gábor Szőcs** and **Miklós Tóth** successfully worked on the specification of the horse chestnut leaf miner's sex feromon. **György Bürgés** and **István Szidonya** elaborated on the protection of horse chestnut trees by way of injections.

2.2 International scientific backgrounds

When overviewing the international scientific literature on this subject, we have to highlight two names, **Deschka** and **Dimic** who gave the scientific name and the first morphological description of the new species. Since the first appearance of the horse chestnut leaf miner in Austria, several scientists have investigated the species. Concentrating on its morphology and lifestyle, significant investigations were carried out by **Blümel, Butin, Freise, Führer, Heitland, Hausdorf, Hellrigl, Holzschuh, Krehan, Pschorn-Walcher** and **Puchberger**. Great achievements in the specification of the parazitoids of the horse chestnut leaf miner were provided by **Grabenweger** and **Lethmayer**. Lots of authors contributed to the determination of the moth's spreading route in Europe, for example, **Simova-Tosic** and **Filov**, **Dautbašić** and **Dimić**, **Maceljski** and **Bertic**, **Milevoj** and **Maček**, **Lastuvka**, **Kenis** and **Forster Hellrigl** and **Ambrosi**, **Del Bene** et al. to mention only the most important ones. The Asian species of the genus *Cameraria* were investigated in

detail by **Kumata.** Investigations and taxonomic revisions of the American *Cameraria* species were carried out by **Davis.**

3. Objectives

The habits of the horse chestnut leaf miner, and the question of its spreading and control were addressed by several authors and their examinations. Nevertheless, for lots of questions the answer is still not known or not precisely known because of the short time that has passed since the first occurrence of the species. Thus, the principal objective of the research and hence the dissertation is to obtain a broader and more accurate knowledge on the biology of the horse chestnut leaf miner, and gain new data on its spreading. In accordance, the main goals of the research were as follows.

- To investigate and discuss the overwintering of the populations of the species living in different habitats (alley, stand).

- To investigate and discuss the swarming dynamics of the populations of the species living in different habitats (alley, stand).

- To determine the cold hardiness of the overwintering pupae of the species.

- To compile a gene collection suitable for the genetic investigations of the spreading of the horse chestnut leaf miner.

- To find efficient methods of the primary genetic investigation on the spreading of the species.

- To define the potential spreading route of horse chestnut leaf miner in Europe.

- To develop effective preventive and control method against the horse chestnut leaf miner in the horse chestnut stand in Gyarmatpuszta, Hungary.

4. Materials and methods

4.1. Investigations on overwintering and swarming dynamics

A comparative study was carried out on samples trees in alleys and stands in the fields of Gyermely. 17 trees were selected in stands (13 in unmixed stand, 4 in mixed stand), while 5 trees were selected in alleys. The size of the overwintering population of the horse chestnut leaf miner was determined on the basis of the mould collected from 1 m^2 under the selected trees on 23 April 1997. The samples were placed immediately after the collection in insectariums in standard circumstances (at 20-21 ºC temperature, in covered and aired place). The analysis was carried out on 20 May 1997 based on daily count of the developed adults.

The swarming dynamics of the horse chestnut leaf miner was examined using leaves collected from the sample trees weekly. The mines on the leaves were dissected, and the larvae, pupae and empty mines found were counted and recorded. The change in the number of the items in different development stages correlated to the number of the developed generations in the year in question and also the time necessary for the development of the actual generations. These examinations were carried out between 25 May and 21 September 1997.

4.2. Investigations on cold hardiness

Horse chestnut leaf miner pupae were collected in the winter seasons between February 1998 and March 2000. The pupae were studied in the Department of Forest Protection, Universität für Bodenkultur, Wien. The repeated examinations happened monthly on overwintering pupae from different habitats collected immediately before the examinations. During the experiments, the temperature of the pupae in liquid bath were gradually lowered using „Lauda MGV" equipment by 2 Cº /minute until the so-called „Supercooling point,

(SCP)". The process of cooling and reaching the target temperature value was registered by a thermograph fixed on the body of the pupae.

4.3. Genetic investigations

4.3.1. Sampling

As a first step of the genetic investigations, a genetic sample collection was compiled from the horse chestnut leaf miner populations from the following countries: Austria, Bosnia, Bulgaria, Bohemia, the Netherlands, Croatia, Poland, Macedonia, Hungary, Germany, Italy, Romania, Switzerland, Slovakia and Slovenia. The samples consisted of mainly pupae, but for some populations larvae were also collected. The samples were stored in uniquely labelled glass carriers in ethanol at +4 ºC during the investigations, and at –20 ºC between the investigations.

4.3.2. Extraction, amplification

The deoxyribonucleic acid (DNA) necessary for the Polymerase Chain Reaction (PCR) - which serves as a basis of the genetic investigation - was extracted using a version of Hoy's method, which employs sodium chloride solution and chloroform, optimized for the extraction of horse chestnut leaf miner DNA.

The DNA extracted from the horse chestnut leaf miner pupae were compared by two methods using a HYBAID PCR machine.

Applying the RAPD-PCR (Random Amplified Polymorphic DNA) method, the reactions happened in eppendorfs in amounts of 30 µl. The reaction results after run on 1% agarose gel (1g agarose, 100 ml TBE, 1 µl ethidium bromide) showed up in UV light. While using this method, 37 primers were tested out of which only 12 proved to be suitable for the genetic investigations of the species. 71 individuals from 7 populations (Vienna, Erfurt, Krakow, Naalwijk, Ohrid,

Sarajevo, Verona) were studied. Statistical analysis of the data was done with the PopGen 32 software.

Using the other method, a mitochondrial DNA sequence which is responsible for the coding of citochrome-oxidase I (COI) was extracted from the samples and then sequenced. The first step was to amplify the target DNA part. The COI DNA sequence was selected by a pair of primers, the so-called Pat&Dick forward and reverse primers. The reaction for amplification happened in amounts of 50 μl. The PCR reactions were repeated using nucleotides labelled by Sanger's procedure. After the second PCR, the selected DNA sequence was purified with the help of sodium acetate solution (NaAC). The base sequence of the samples prepared as above was determined by an automatic sequencer. As the sequencing results of the total 9 individuals from 7 populations (Vienna, Erfurt, Krakow, Naalwijk, Ohrid, Sarajevo, Verona) were identical, there was no need for statistical analysis.

5. Summary of scientific results

5.1. Investigations on overwintering

The adults developing from the leaf miner's overwintering pupae swarmed around mid-, and end of April, in parallel with the daily average temperature reaching 10 ºC. (In 1997 the spring swarming started on 29 April.) The overwintering populations under Hungary's climate suffer relatively little during the winter. The results are in line with the relevant published data. At the same time, the results of the studies on alleys and stands show that the number of adults developed from the pupae in the two habitats was different. In samples from alleys, an average of 24 adults fly out in the spring, while in samples from the stands only 9.

5.2. Investigations on swarming dynamics

In 1997 three generations of the pest developed in all the three areas studied. This statement is in agreement with several authors' conclusion so it can be considered as generally acceptable in this geographical region (Carpathian basin). Obviously, under extreme weather conditions or in areas with special microclimate the number of generations can be different.

The time necessary for the development of the generations was an average of 35 days in alleys, 42 days in unmixed and 49 days in mixed stands. Since the trends in the two stands are very similar, it can be stated that in 1997 the development of the leaf miner generations took an average of 35 days in alleys, and 45 days in stands.

In 1997 the swarming peak of the horse chestnut leaf miner's second and third generations was on 13 July and 17 August in the alley, in unmixed stand on 20 July and 31 August, while in the mixed stand on 20 July and 7 September. These facts can explain the differences found during the overwintering studies, because the third generation flying out earlier in the alley can lay its eggs earlier, and consequently, the larvae developing earlier from those eggs have better chance for successful pupae formation.

5.3. Investigations on cold hardiness

The freezing tolerance of the horse chestnut leaf miner's overwintering pupae was found to be between -19,4 and -23,2 ºC, which values are usually characteristic of the insects living in the habitats of cooler latitudes. In connection with the measured cold hardiness of the horse chestnut leaf miner's pupae it can be noted that the temperature values of the area inhabited by horse chestnut trees do not restrict the living and spreading of the damaging horse chestnut leaf miner.

The closer the end of the overwintering period was, the decreased the average freezing tolerance was found, at the same time the bigger the difference was in the tolerance between the individuals of the overwintering population (increased deviation). The first phenomenon is caused by the gradual decrease of the stored nutritive material (glycogen), as a consequence of which the pupae has reduced freezing tolerance towards the end of the overwintering period. The explanation for the bigger deviation values, characteristic at the end of the overwintering period, lies on one hand with the different level of food consumption during the winter, and on the other hand with the different timing of the transformations inside the pupae. However, the values around –20 ºC found in March do not mean any limitation on the overwintering of horse chestnut leaf miner pupae which could restrict or prevent its damage on the areas inhabited by horse chestnut trees.

5.4. Genetic investigations

5.4.1. RAPD-PCR studies

During the RAPD PCR analysis of the horse chestnut leaf miner 37 different primers were used. Out of these primers only the below 12 showed valid results (patterns): R1, R2, R7, R8, R9, R13, R14, OPA4, OPAB1, OPAB8, OPAC11, OPAC13.

The largest diversity can be found in the samples from Sarajevo, Ohrid and Vienna, and these rates decrease when we diverge from the centre of the spread. The explanation of the phenomenon is in connection with the steps of the spreading process. If we diverge from the centre, fewer individuals and lower genetic diversity can be found. A kind of genetic impoverishment is observable. This process dominates especially in species spread by human activities. Based on the available data, the horse-chestnut leaf miner moth can also be considered as such species.

By analysing the similarities between the samples (populations), three sample groups can be outlined. The first and most important one is the group including the samples from Ohrid, Vienna and Sarajevo. The significant similarity between the three samples confirms that the samples from Vienna and Sarajevo primarily originates from Ohrid. In the case of the Sarajevo sample, the reason for this can be the short geographical distance, while in the case of the Vienna sample, it is the introduction of the moth in Linz. The group including samples from Erfurt and Naalwijk can also be clearly separated and most likely shows a spreading route from Linz westbound. The similarities of the samples from Krakkow and Verona can be accounted for by a common origin. (It is to be noted, though, that this level of similarity between these two samples is not in accordance with the published and generally accepted spreading route.)

The members in the first group are populations from Ohrid, Sarajevo and Vienna. Near the Lake Ohrid, the horse chestnut leaf miner was discovered in 1985, in Bosnia and Austria in 1989 so these three populations can be considered the oldest ones in Europe. This is also suggested by the high rate of genetic diversity among the populations. Furthermore, the intensive similarity suppose that the introduction of the horse chestnut leaf miner in Linz was not limited to only a few individuals. In order to have a genetic diversity of the populations in Vienna similar to those around the Lake Ohrid, several dozens of moths must have been introduced in Linz. The characteristics of the second group can also be sufficiently explained by a westbound spreading of the moth from Linz, and also account for the German and Dutch data on the occurrence of the species. The moth appeared in Germany in 1993/94, and in the Netherlands in 1999/2000 most probably via human activities (public and goods transit). In the spreading process, this way, only a few individuals played a significant role which contributed to a relatively small genetic diversity of the population in the new habitat. The data on samples from Krakow and Verona cannot be explained easily or not at all. Although the low diversity values found in the populations perfectly fit into the hypothesis of a Linz-centered spreading, the similarities between the two populations would rather suggest a spreading direction from Verona to Krakow than a radial spreading

from Linz. The species appeared in Italy in 1992 and in Poland in 1995/96 so this option cannot be excluded though seems less likely than the spreading route from Linz to Krakow.

5.4.2. COI studies

During the analysis of the sequencing results, we determined 173 subsequent base pairs of the DNA fragment encoding the citochrome-oxidase I enzyme. These were identical in all the populations studied. The investigations did not succeed in determining the base sequence of the whole DNA encoding the COI.

The results of the studies, though not providing the entire code of the COI, were unambiguous. The 173 bp-long mitochondrial DNA fragment in all samples were identical. Since the COI enzyme has a maternal inheritance, changes in the genes can be caused only by mutations. The inheritable mutations, those that manifest in the offspring too, require quite a long time. It is unlikely that in the last two decades inheritable mitochondrial mutations causing visible changes in the offspring happened. Consequently, if the DNA sequence of the mitochondrial DNA in the European populations would provide different patterns it would have been caused by separate genealogy. But the common mitochondrial DNA fragment in all 9 populations studied strengthen the hypothesis on one origin for all European populations - which is most likely the Lake of Ohrid.

5.5. Plant protection of horse chestnut alleys and stands in Gyarmatpuszta, Hungary

The horse chestnut alleys and stands in Gyarmatpuszta, especially the old trees, have been heavily damaged by the horse chestnut leaf miner in the recent 10 years. The owner of the land found it very important to protect and preserve the stands of significant sylvicultural, hunting and cultural historical value and gave way and support to the development a complex preventive technology

which takes into account the gaming and public function of the area too. The underlying element of the procedure is to attack the damaging horse chestnut leaf miner in as many developmental stages as possible. To achieve this, the land under the loosely-networked chestnut trees were first disked in the spring before the leaf miner could fly out. Disking controls the number of the individuals in the population flying out, and also improves the airing and absorption of precipitation of the otherwise heavily trod land. Disking was followed by planting seeds of mixed grass which helps later on the chemical protection from the ground by providing clear and viable road. The meadow created this way is important source of food for games and also contributes to a higher aesthetic value of the area. The chemical protection is timed according to the swarming peaks of the leaf miner detected by feromon traps, usually twice a year. During the chemical protection, professionals from the local Institute for Plant Health and Soil Protection disseminate Nomolt 15 SC, which inhibits the chitin synthesis, in doses of 0,75 l/ha with the help of an axial ventilator fixed on a Unimog truck. Eight days after, the chemical protection is repeated to enhance the efficiency of the chemical treatment. The second chemicalization takes place only if the swarming is very intensive. When handling the stands, the preservation of dead still upstanding tree trunks is prioritised because by giving home to members of the local fauna they contribute to the biodiversity of the area to an invaluable extent.

The preventive method developed for the horse chestnut stands in Gyarmatpuszta exemplifies well that by applying the relevant scientific results and harmonising the different interests associated with the area, a widely- acceptable treatment of the valuable forest stands can be realised.

6. Suggestions for utilisation of the results

The results of the investigations on horse chestnut leaf miner from utilisation point of view can be divided into two. A significant part of the results contribute to a better understanding and knowledge of the biology of the species, thus enabling further researches on how to control the pest. The other part of the results directly help and can be utilised in the preventive actions.

The spring swarming of the horse chestnut leaf miner takes place in mid-, end of April as soon as the daily average temperature reaches 10 ºC. If the mould fallen during the previous autumn has not been cleared the swarming is more intensive on trees exposed to sunshine and warm climate than on trees standing in cooler and shady locations. This has to be taken into consideration when timing the protection in the spring.

It takes less time for the moth generations to develop on leaves of trees in sun-lit and warm places than in the foliage of trees standing in cooler places. The difference can be very significant and must be taken into account when planning the proper timing of the protection in the summer.

The winter climate does not affect the number of the overwintering pupae thus cannot be taken advantage of during the protection.

The results from genetic investigations on the spreading of the horse chestnut leaf miner provide a stable basis for further researches. By taking more samples and employing other procedures and research methods, the spreading route of the moth within Europe can be drawn more accurately. The knowledge on the spreading process can help to clarify what factors play role in the spreading of species similar to the leaf miner.

By genetic investigations of the *Cameraria* samples from Asia and America the taxonomic place of the species and perhaps even the

place of origin can be defined. Knowledge on the original spread of the species makes it possible to work out more effective methodologies against the horse chestnut leaf miner.

The method developed for the protection of the horse chestnut stands and alleys in Gyarmatpuszta can be directly used in the prevention activities elsewhere. The method described can of course be further elaborated by taking use of the results of new researches.

7. Relevant lectures, posters and list of publications

7.1. Lectures

Kovács Z. (2000): A vadgesztenyelevél-aknázómoly vizsgálata. A VEAB régió doktoranduszainak tudományos fóruma, Sopron, 10 Nov 2000. (in Hungarian) (Investigations on horse chestnut leaf miner, Forum of doctorandi in VEAB region)

Lakatos F. and **Kovács Z.** (1999): Physiological and genetic investigations on *Cameraria ohridella* (Deschka et Dimič 1986, Lep. Lithocolletidae) populations. Diplomanden- und Dissertantenseminar BOKU, Institut für Forstentomologie, Forstpathologie und Forstschutz, Vienna, 9 Nov 1999.

Lakatos, F., **Kovács, Z.**, **Stauffer, Ch.**, **Kenis, M.**, **Tomov, R**. and **Davis, D. M.** (2003): 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). Proceedings of International Symposium of the Kanazawa University 21st Century COE Program (ISSN 1348-3048) sponsored by Kanazawa University. Under press.

7.2. Posters

Kovács Z. and **Lakatos F.** (2000): Physiological examinations on horse chestnut leaf miner (*Cameraria ohridella* Deschka et Dimič 1986, Lep. Gracillariidae). Methodology of Forest Insect and Disease Survey in Central Europe. Third Workshop of the IUFRO WP.7.03.10., Busteni, Romania, 24-28 Sep 2000.

Kovács Z., **Stauffer Ch.** and **Lakatos F.** (2000): Genetic investigations on *Cameraria ohridella* (Deschka et Dimič 1986, Lep. Lithocolletidae) 6th Meeting of PhD Students in Evolutionary Biology, Vaalbeek, Belgium, 15-17 Mar 2000 (6th Meeting of PhD Students in Evolutionary Biology, Vaalbeek, Belgium, 15-17 Mar 2000. Abstract book: p. 29.)

7.3. Publications

Kovács Z. and **Lakatos F.** (1999): Megfigyelések a vadgesztenyelevél-aknázómoly (*Cameraria ohridella* Deschka et Dimič 1986, Lep. Lithocolletidae) áttelelésével és egyedfejlődésével kapcsolatban. Növényvédelem, 35: 57-59. (in Hungarian) (Observations on the overwintering and ontogenesis of the horse chestnut leaf miner (*Cameraria ohridella* Deschka et Dimič 1986, Lep. Lithocolletidae))

Kovács Z. and **Lakatos F.** (2001): Honnan jöttél Cameraria? Növényvédelem, 37: 71-72. (in Hungarian) (Where are you from Cameraria?)

Kovács Z., **Stauffer Ch.** és **Lakatos F.** (2000): A vadgesztenyelevél-aknázómoly (*Cameraria ohridella* Deschka et Dimič 1986, Lep. Lithocolletidae) európai elterjedésének genetikai vizsgálata. Növényvédelem, 36: 288-290. (in Hungarian) (Genetic investigation of the spreading of horse chestnut leaf miner (*Cameraria ohridella* Deschka et Dimič 1986, Lep. Lithocolletidae) in Europe)

Lakatos F., **Traser Gy.** and **Kovács Z.** (2003): A gyarmatpusztai vadgesztenyés. Erdészeti lapok, 138: 143-144. (in Hungarian) (The horse chestnut stand in Gyarmatpuszta)