

**MORPHOLOGICAL DIFFERENCE BETWEEN UPSIDE AND
UNDERSIDE LEAF-MINING LARVAE OF *PHYLLOCNISTIS*
UNIPUNCTELLA (STEPHENS, 1834) (LEP.: GRACILLARIIDAE)
AND ITS CHANGING PHENOLOGY**

¹ MARTIN P. JORDAN, ² JOHN R. LANGMAID & ³ CAMIEL DOORENWEERD

¹ 52 Poverest Road, Orpington, Kent BR5 2DQ (mpj1@live.co.uk)

² Wilverley, 1 Dorrita Close, Southsea, Hampshire PO4 0NY (johnrendle.langmaid@gmail.com)

³ Naturalis Biodiversity Center, Leiden, The Netherlands (camiel.doorenweerd@naturalis.nl)

Abstract

Larvae of *Phyllocnistis unipunctella* (Stephens) mining the upperside and underside of leaves of *Populus* spp. were compared in terms of gross morphology and the COI DNA barcoding section of mitochondrial DNA. It was discovered that larvae feeding on the underside did not show the dark pigmentation of the prothoracic plate as described in the literature. Larvae were found feeding two months earlier than normal and at least one extra generation was observed. More work is required to determine the possibility of speciation.
Keywords: *Lepidoptera*, prothoracic plate, DNA barcoding, phenology.

Introduction

The literature records *Phyllocnistis unipunctella* (Stephens, 1834) as a species with a Palaearctic distribution and larvae that create leaf-mines on *Populus nigra* Linnaeus, *P. balsamifera* L. and their respective hybrids (Lüders, 1900; Emmet, 1985, Bengtsson and Johansson, 2011, Kobayashi *et al.*, 2013, Sinev, 2008). Where known, the literature describes two generations per year, with the first generation larvae mining primarily on the upperside of the leaves in June and the second generation mining in August. The species overwinters in the adult stage, hibernating from September to April in thatch (Emmet, 1985). In the first three instars, *P. unipunctella* larvae feed just below the epidermal layer by severing the cell wall with specialised horizontal slicing mandibles and imbibing only the sap. The resulting mines are inconspicuous and leaves must be viewed at an angle in order to see the long, sinuous, transparent galleries that have an appearance similar to snail trails (Emmet, *loc. cit.*). The larvae are hyaline with a blackish prothoracic plate that is present until the final instar (Emmet, *loc. cit.*). During the third ecdysis, larvae undergo hypermetamorphosis to produce a non-feeding fourth instar in which the head capsule is modified to generate silk via spinnerets for the construction of a cocoon. In this final stage the prothoracic plate is lost.

The cocoon is formed in a small fold at the edge of the leaf on the side on which the larva fed. Unlike some species of *Phyllocnistis*, for example the willow feeding *P. saligna* (Zeller, 1839), *P. unipunctella* larvae cannot change their feeding orientation from the upper to the lower surface of the leaves or

vice versa (Kobayashi *et al.*, 2011, Lüders, *loc. cit.*). We report our observations on the phenology that do not correspond to the literature records and a morphological difference between upperside and underside mining larvae.

Methodology

Tenanted *P. unipunctella* mines were recorded and collected from *Populus* species by searching upper and lower surfaces of the leaves. A 10x hand lens was used in the field to record the presence or absence of a pigmented prothoracic plate in third instar larvae. First and second instar larvae are less than 6mm in length and if found were not recorded, as identification of the prothoracic plate is difficult in the field due to its diminutive size. Final instar larvae are only found within a prepared chamber at the leaf edge prior to cocoon construction and pupation. Larvae found at this stage were not recorded as the prothoracic plate is absent. Adults were bred through from tenanted mines stored in closed glass vials placed in a shaded position outdoors. Genitalia preparations were made following the standard methods for small Lepidoptera.

DNA barcode analyses were performed to examine potential genetic differences between the two types of larva (Hebert, 2003). DNA was extracted non-destructively from two larvae of each type and sequenced for the COI DNA barcode region. The DNA sequences are made available through the online database BOLD (www.boldsystems.org, Ratnashingham & Hebert, 2007), sample IDs RMNH.INS.30306 and RMNH.INS.30308 are upperside mining larvae, RMNH.INS.30310 and RMNH.INS.30311 are underside mining larvae. The sequences were compared to reference sequences in BOLD and a Maximum Likelihood phylogenetic analysis was performed using PhyML 3.0 (Guindon and Gascuel, 2003) to visualise the relationships between the specimens in a tree. The chitinous remains from the larvae following non-destructive DNA extraction were stained with phenosafranin and fixed in euparal on a microscopic slide.

Results and Discussion

Changing phenology

The first underside mining larva was found on 25 April 2014 at Orpington Kent (N 51.3844 E 00.1077) on *Populus nigra*. Searching at several sites in north-west Kent, MPJ then recorded numerous first generation *P. unipunctella* larvae feeding from late April into May 2014. These larvae primarily mined the underside of the leaves of local poplar species including *Populus nigra* L, *Populus nigra* var. *italica* L. and *Populus x canadensis* Moench with few larvae mining the upperside. This is contrary to what is described in the literature, where upperside mines are always more numerous, particularly in the first generation. Whether these observations represent a change in the preference of the leaf-mining orientation or that previous observers have simply focussed more on the upperside of the leaves remains unclear.



Plate 1 (left). Typical form third instar larva of *Phyllocnistis unipunctella* (Stephens) feeding on the upperside of the leaf, showing typical dark prothoracic plate. N 51.3844 E 00.1077.

Plate 2 (right). Atypical form third instar larva of *Phyllocnistis unipunctella* (Stephens) feeding on the underside of the leaf showing absence of visible dark prothoracic plate.



Plate 3 (left). Typical form third instar larva of *Phyllocnistis unipunctella* (Stephens) from upperside mine showing typical dark prothoracic plate.

Plate 4 (right). Atypical third instar larva of *Phyllocnistis unipunctella* (Stephens) from underside mine showing absence of pigmentation of prothoracic plate.

P. unipunctella larvae were not recorded on adjacent *Populus balsamifera*. These findings were shared with JRL, who also recorded the same unexpected observations in Portsmouth, Hampshire where he and Ian Thirlwell found several underside mines but no upperside mines on one *Populus x canadensis* on 1 May but many both upperside and underside mines on one *P. nigra* var. *italica* on 5 May. CD was then consulted and confirmed similar observations in the field in The Netherlands. The second generation was found in June 2014

and these larvae mined primarily the upperside. Finding first brood *P. unipunctella* larvae feeding from April rather than June, as previously published (Emmet, 1985) suggests extra time was available for the production of more than two generations. The shift also resulted in the overlap of subsequent generations which made determination of the phenology difficult, but certainly three cycles and possibly four were observed in 2014. The mean UK winter 2013/2014 temperature was 5.2 °C which is 1.5°C above the 1981-2010 average and the mean UK temperature for summer 2014 was 14.8°C which is 0.5°C above the 1981-2010 average (Metoffice, 2016). It is unknown at this time if this degree of warming would result in the recorded changes to the number of generations. Studies on the overwinter survival of a related species, *P. populiella* Chambers 1875, have shown that microhabitat conditions are more important than average temperature fluctuations and that changes to winter stress factors can affect insect fecundity (Wagner *et al.* 2012; Williams *et al.* 2015)

Presence of the prothoracic plate correlates with leaf-mine orientation

In the third instar, the larvae that mined the upperside showed the dark prothoracic plate (Plate 1) but the underside feeders did not show this (Plate 2). This difference was consistent for all recorded larvae. The same difference was consistently encountered by CD in the coastal dune areas near Katwijk, The Netherlands. In 2015 larvae were found in north-west Kent from 10th May onwards and these also showed the same correlation of the mining position on the leaf with the presence or absence of pigmentation of the prothoracic plate in the third instar as was observed in 2014. Several larvae were collected for breeding through to examine both larval types for other potential differences in other life stage morphologies. Moths emerged after 12 to 15 days following pupation. Pupae and adults from both upperside and underside mines were typical of the description of *P. unipunctella* and there were no discernible differences by genitalia dissection. Because the morphological characters of *Phyllocnistis* species can be difficult to interpret (e.g. Langmaid and Corley, 2007), we decided to include DNA analysis. Specifically, the COI DNA barcoding region was analysed for four specimens, two of each type, and compared to reference sequences in the global DNA barcoding database BOLD. The genetic relationships are displayed in the form of a phylogenetic tree (Fig. 1) where the length of the branches indicates the genetic distance between specimens. One way of interpreting these results is by examining the pairwise genetic distances between DNA sequences, i.e. the percentage of nucleotides that have mutated. Although this gives an indication of the frequency of, or time since, exchange of genetic material between different populations, the percentage of genetic distance should only be interpreted in relation to the distances between other related species and always in the context of geographic

distance (Nieukerken *et al.*, 2012). Populations of species with a wide distribution may have been isolated from each other for long periods of time, translating into a genetic distance, but can usually still be considered a single species under the biological species concept (De Quieroz, 2007).

There are distinct genetic clusters of *P. unipunctella* DNA barcodes in BOLD from specimens spanning the entire Palearctic region with pairwise genetic differences up to 5.5% between a specimen from Japan compared to European specimens. The Russian specimen has been collected in eastern Russia (Republic of Buryatiya), differs 3.5% from the Japanese specimen and is 4.7% different from the European specimens. Within Europe, the largest distance is 1.1%, between specimens collected south of the Alps, compared to specimens from North-West Europe. As a reference, *P. labyrinthella* is included in the analysis, with a minimum genetic distance to *P. unipunctella* of 7%. A second way to examine DNA barcodes is by looking at the clustering of the phylogenetic tree. A prerequisite for a valid species hypothesis is that it is a monophyletic entity, i.e. all members must have descended from a common ancestor. In a phylogenetic tree this means that all specimens of a presumed species must have a single branch leading to that cluster, and no other presumed species can be present in that same cluster. The four specimens that we analysed are all placed in a monophyletic *P. unipunctella* cluster, more specifically, in the cluster with the North-West European specimens, with three sequences 100% identical, and a fourth sequence being 0.15% different. There is no diagnostic difference in the DNA barcodes of the two types that would indicate genetic isolation or speciation. Although DNA barcodes are in most cases reliable for distinguishing species, it is difficult to completely rule out that recent speciation or natural genetic processes have obscured a detectable speciation trace in the COI gene (van Nieukerken *et al.*, 2012).

Prothoracic plate production

Photomicrographs were taken of the stained chitinous larval remains from which DNA was extracted to compare the head and thorax of the two larval prothoracic plate forms (Plates 3 and 4). These images revealed that the structure of the plate was present in the lower surface feeding larvae but it did not show dark pigmentation. Ventral and dorsal plates in leaf-mining larvae occur in different insect families and orders, including Tischeriidae, Gracillariidae, Nepticulidae (Lepidoptera) and Tenthredinidae (Hymenoptera). It is hypothesized that they offer protection against parasitoid oviposition but to our knowledge there is no scientific evidence that either supports or refutes this idea. Even less is known of the genetic basis or environmental factors that are involved in the production of such plates, we suggest that perhaps exposure to UV radiation could be an important factor.

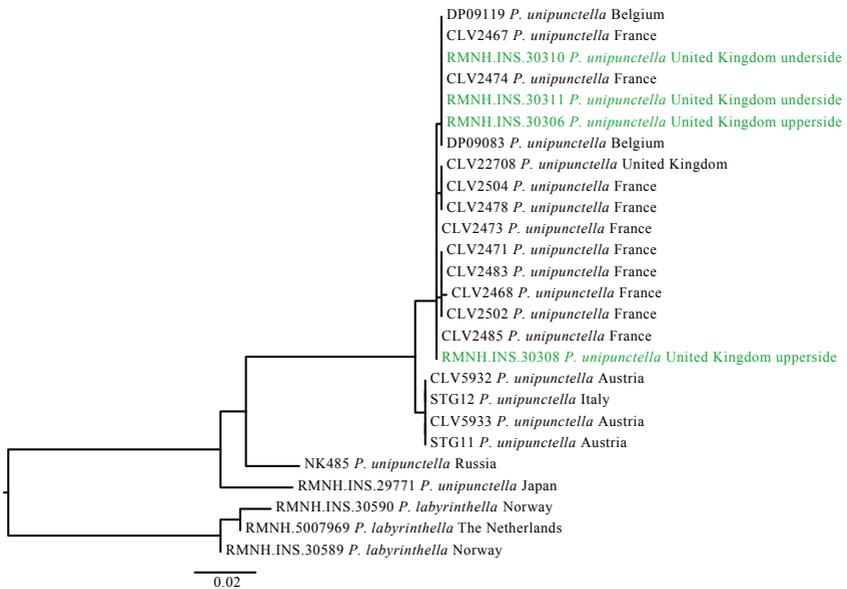


Fig 1. Phylogenetic tree resulting from a Maximum Likelihood analysis displaying the relations between the DNA barcode sequences of the two types of larvae, in relation to other *P. unipunctella* DNA barcodes. The numbers before the specimen names indicate the respective sample-ID in BOLD. The scale bar indicates 0.02 substitutions per nucleotide position.

Conclusion

We have found at least three and possibly four generations of presumed *Phyllocnistis unipunctella* in 2014 and 2015 where the species has previously been recorded to be bivoltine. There is also a shift to a much earlier initial generation, mining in April compared to June as stated in the literature. Larvae of *P. unipunctella* from upperside mines exhibit a dark pigmented prothoracic plate whereas those from underside mines do not show this morphology. The first generation of larvae were primarily underside miners with unpigmented prothoracic plates. Generations 2 and 3 were primarily upper surface miners and typical of the description of *P. unipunctella*. These observations were repeated at locations in the UK and The Netherlands.

DNA analysis using the COI-gene DNA barcode region did not reveal diagnostic characters to distinguish the two types but higher resolution genetic data may reveal recent genetic differentiation. At this time it is uncertain if the repeatedly observed link between larval feeding position and the presence or absence of the dark pigmentation of the prothoracic plate in the third instar is indicative of speciation or is perhaps the result of an environmental factor.

Acknowledgements

We are very grateful to Ian Thirlwell for proof reading and correcting grammatical errors, and we are thankful to Colin Plant for helping to properly format the paper. We would also like to thank Carlos Lopez-Vaamonde and Natalia Kirichenko for allowing us to use the *Phyllocnistis unipunctella* DNA barcodes from their BOLD projects, and the other collectors who contributed specimens that have been barcoded: Paul van Wielink, Wouter Moerland, Kees van den Berg, Alain Cama, Christian Wieser, Jurate De Prins, Willy De Prins, Ian Sims and Gerfried Deschka. Natalia Kirichenko is additionally thanked for providing information on the distribution of *P. unipunctella* in Russia.

References

- Bengtsson, B. Å. & Johansson, R., 2011. *Fjärilar: Bronsmalar–rullvingemalar. Lepidoptera: Roeslerstammidae–Lyonetiidae*. ArtDatabanken, SLU, Uppsala, 512 pp.
- De Queiroz, K., 2007. Species Concepts and Species Delimitation. *Systematic Biology* **56**: 879-886.
- Emmet, A. M., 1985. *Phyllocnistidae*. In: Heath J., Emmet A.M. (Eds) *The Moths and Butterflies of Great Britain and Ireland, Volume 2*. Harley Books, Colchester.
- Guindon, S. & Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**: 696-704.
- Kobayashi, S., Sakamoto, Y., Jinbo, U., Nakamura, A. & Hirowatari, T., 2011. A new willow leaf blotch miner of the genus *Phyllocnistis* (Lepidoptera: Gracillariidae: Phyllocnistinae) from Japan, with pupal morphology and genetic comparison of Salicaceae mining species using DNA barcodes. *Lepidoptera Science* **62**: 75-93.
- Kobayashi, S., Hirowatari, T., 2013. *Gracillariidae, Phyllocnistinae*. In: Nasu, Y., Hirowatari, T. & Kishida, Y., (Eds) *Nihon-san garui hyojun zukan The standard of moths in Japan*. Gakken Kyoiku Shuppan, Tokyo, 154-155, pl 154-108.
- Langmaid, J. R., Corley M. F., 2007. *Phyllocnistis ramulicola* sp. nov. (Lepidoptera: Gracillariidae) in England and Portugal. *Entomologist's Gazette* **58**: 227-237.
- Lüders, L., 1900. Beitrag zur Kenntnis der Lepidopterengattung Phyllocnistis Z. *Beilage zum Bericht über das Schuljahr 1899-1900, Realschule St Pauli: 1-33*.
- Metoffice, 2016 <http://www.metoffice.gov.uk/climate/uk/summaries/2014/summer>. [Accessed 07 February 2016].
- Nieukerken, E. J. van, Doorenweerd, C., Stokvis, F.R. & Groenenberg, D. S. J., 2012. DNA barcoding of the leaf-mining moth subgenus *Ectoedemia* s. str. (Lepidoptera: Nepticulidae) with COI and EF1- α : two are better than one in recognising cryptic species. *Contributions to zoology* **81**: 1-24.
- Ratnasingham, S. & Hebert, P. D. N., 2007. BOLD: The Barcode of Life Data System (www.barcodinglife.org). *Molecular Ecology Notes* **7**: 355-364.
- Sinev, S., (Ed) 2008. *Catalogue of the Lepidoptera of Russia*. KMK Press, Saint Petersburg–Moscow, 425 pp. [in Russian].
- Wagner, D., Doak, P., Sformo, T., Steiner, P. M. & Carlson, B., 2012. Overwintering physiology and microhabitat use of *Phyllocnistis populiella* (Lepidoptera: Gracillariidae) in interior Alaska. *Environmental Entomology* **41**: 180-187.
- Williams, C.M., Henry, H.A. & Sinclair, B.J., 2015. Cold truths: how winter drives responses of terrestrial organisms to climate change. *Biol. Rev. Camb. Philos. Soc.* **90**: 214-235.