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A Native Insect on a Non-Native Plant: The Phylogeography of the Leafminer *Phyllonorycter populifoliella* (Lepidoptera: Gracillariidae) Attacking the North American Balsam Poplar in North Asia

Natalia I. Kirichenko [1,2,3,4,*], Maria A. Ryazanova [2], Evgeny N. Akulov [3], Svetlana V. Baryshnikova [5], Anton A. Efremenko [1], Konstantin V. Krutovsky [2,6,7,8], Victor Ya. Kuzevanov [9], Andrei V. Selikhovkin [10], Pathour R. Shashank [11], Sergey Yu. Sinev [5], Paolo Triberti [12], Evgeny V. Zakharov [13] and Dmitrii L. Musolin [14]

1 Federal Research Center «Krasnoyarsk Science Center SB RAS», Siberian Branch of the Russian Academy of Sciences, Sukachev Institute of Forest, Krasnoyarsk 660036, Russia; efremenko2@mail.ru
2 Institute of Ecology nd Geography, Siberian Federal University, Krasnoyarsk 660041, Russia; rznv.m@mail.ru (M.A.R.); konstantin.krutovsky@forst.uni-goettingen.de (K.V.K.)
3 All-Russian Plant Quarantine Center, Krasnoyarsk Branch, Krasnoyarsk 660020, Russia; akulich80@yandex.ru
4 Department of Forest Protection, Slovenian Forestry Institute, 1000 Ljubljana, Slovenia
5 Laboratory of Insect Taxonomy, Zoological Institute RAN, Saint Petersburg 199034, Russia; parornix@zin.ru (S.V.B.); sinev@zin.ru (S.Y.S.)
6 Laboratory of Population Genetics, N. I. Vavilov Institute of General Genetics, Russian Academy of Sciences, Moscow 119333, Russia
7 Department of Forest Genetics and Forest Tree Breeding, Georg-August University of Göttingen, 37077 Göttingen, Germany
8 Scientific and Methodological Center, G.F. Morozov Voronezh State University of Forestry and Technologies, Voronezh 394036, Russia
9 Institute of Management and Finance, Baikal State University, Irkutsk 663003, Russia; victor.kuzevanov@gmail.com
10 Department of Forest Protection, Wood Science and Game Management, Saint Petersburg State Forest, Technical University, Saint Petersburg 194021, Russia; a.selikhovkin@mail.ru
11 Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi 110012, India; spathour@gmail.com
12 Museo Civico di Storia Naturale, 37129 Verona, Italy; caloptilia@alice.it
13 Canadian Center for DNA Barcoding, Centre for Biodiversity Genomics, University of Guelph, Guelph, ON N1G 2W1, Canada; zakharov@uoguelph.ca
14 European and Mediterranean Plant Protection Organization, 75011 Paris, France; musolin@gmail.com
* Correspondence: nkirichenko@yahoo.com

Abstract: The trans-Palearctic moth *Phyllonorycter populifoliella* (Lepidoptera: Gracillariidae) is a major pest of the North American *Populus balsamifera* and its hybrids widely planted as ornamentals in North Asia (i.e., the Asian part of Russia). We DNA barcoded *Ph. populifoliella* from distant geographical populations in Russia and analyzed them together with the data from eight European countries and India to estimate intraspecific variability and the haplotype richness in the Palearctic, and specifically in North Asia. Furthermore, using next-generation sequencing (NGS, Sequel platform, PacBio), we investigated larval and pupal remnants found in an old herbarium from the Nearctic, where *P. balsamifera* occurs naturally, to verify if any events of the moth introduction to this biogeographic zone happened in the past. Relatively high intraspecific variability in the COI gene of mtDNA, reaching 3.73%, was recorded in *Ph. populifoliella*. Overall, 30 COI haplotypes were defined in 83 specimens from the Palearctic, with a noticeable richness in North Asia (21 haplotypes). Using NGS, the remnants of 14 *Phyllonorycter* specimens dissected from up to 174-year-old herbaria from the Palearctic and Nearctic were sequenced, and four moth species were identified. Among them, there were three Palearctic species, *Ph. populifoliella*, *Ph. pastorella* (Zeller), and *Ph. apparella* (Herrich-Schäffer), and one Nearctic,

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Academic Editor: Tiziana Panzavolta

Received: 24 November 2024
Revised: 16 January 2025
Accepted: 17 January 2025
Published: 21 January 2025

Citation: Kirichenko, N.I.; Ryazanova, M.A.; Akulov, E.N.; Baryshnikova, S.V.; Efremenko, A.A.; Krutovsky, K.V.; Kuzevanov, V.Y.; Selikhovkin, A.V.; Shashank, P.R.; Sinev, S.Y.; et al. A Native Insect on a Non-Native Plant: The Phylogeography of the Leafminer *Phyllonorycter populifoliella* (Lepidoptera: Gracillariidae) Attacking the North American Balsam Poplar in North Asia. *Forests* **2025**, *16*, 190. https://doi.org/10.3390/f16020190

Ph. nipigon (Freeman). No evidence of *Ph. populifoliella* introduction to North America was documented based on the examination of the herbarium dated 1850–1974. Three specimens of *Ph. populifoliella* identified from herbaria from Austria and Poland (dated 1879–1931) represented one haplotype (H7) known from the recent time. Overall, our study clarifies the modern range, provides insights into phylogeography, and defines the haplotype richness of the native leafminer outbreaking on the alien host. Furthermore, it underlines the use of old herbaria to explore the historical distribution of endophagous insect species.

Keywords: leafmining moth; alien host plant; DNA barcoding; NGS; intraspecific genetic variability; haplotypes; old herbaria; Asian part of Russia; Palearctic; Nearctic

1. Introduction

The poplar moth, *Ph. populifoliella* (Treitschke, 1833) (Lepidoptera, Gracillariidae), is a trans-Palearctic species distributed in Europe and North, Eastern, and Central Asia [1,2]. In 2017, it was recorded for the first time in North India, which is a part of the Palearctic [3].

Ph. populifoliella is a small moth with a wingspan of up to 6 mm [4]. In most of the Palearctic, it coexists with other poplar moths, in particular, *Ph. pastorella* (Zeller, 1846) and *Ph. comparella* (Duponchel, 1843). They share host plants and produce indistinguishable leaf mines [4,5]. Adult of *Ph. populifoliella* can be distinguished from these two species by forewing patterns and male genitalia [4,5], but at the larval stage, species identification is possible only through DNA barcoding [6–8]. *Ph. populifoliella* typically produces two generations (from May to early October) and overwinters as an adult in cracks on poplar bark, building walls, or, occasionally, within human dwellings [4,5].

This moth is a common pest of poplars (Salicaceae) in urban ecosystems of Russia, particularly in its Asian part [9–11], which is classified as North Asia [12]. Within this region, *Ph. populifoliella* outbreaks on the North American balsam poplar, *P. balsamifera* L. (the taxonomic section Tacamahaca) and its hybrids, especially with black poplar *P. nigra* L. (Aigeiros) [10]. The damage to *Ph. populifoliella* becomes noticeable by the end of June when the leaves are covered by oval upper-side mines (up to 18 mm in length). By early August, affected trees turn brown and start shedding foliage [9,10].

The balsam poplar is native to North America [13]. It was introduced to the former USSR over 150 years ago for ornamental purposes [14,15]. In Russia, this species and its hybrids were intensively used in city and other manmade plantings (along intercity roads, next to agricultural fields as windbreak hedges, and as greenbelts) [16]. As a result, balsam poplar and its hybrids can now be found across a vast territory of the country, from the southern limits of the woodlands and forests to the Arctic Circle [15,17–20]. In North Asia, where woody plant diversity is much lower than in the European part of Russia, the proportion of balsam poplar and its hybrids in ornamental plantings is significant [21,22]. Despite their promising ornamental characteristics (rapid growth, substantial biomass, etc.), balsam poplar and its hybrids turned out to be susceptible to *Ph. populifoliella.*

In Russia, the first outbreak of *Ph. populifoliella* was recorded in the 1910s in the Ural Region [23]. By 1929, noticeable damage caused by this leafminer was documented in Moscow [24,25]. In North Asia, the moth was recorded as a pest of ornamental plantings in the late 1940s, with the first outbreak reported from Irkutsk [9]. The outbreaks in urbanized areas and other planted stands were regularly reported in Russia, especially in its Asian part [10,26–30].

Several papers targeted the biology and ecology of *Ph. populifoliella* in Russia [9–11,26–32]. However, the phylogeography of this pest associated with its non-native host plant remains

unstudied. Furthermore, the potential for the early occurrence of this moth in North America, where *P. balsamifera* is native, has not been investigated yet.

The goal of this study was to explore the phylogeography of *Ph. populifoliella* in the Palearctic, with a focus on North Asia, where the moth significantly damages the North American balsam poplar. For that, we assessed the genetic polymorphism of the moth across its vast native range based on the analysis of the COI gene of mtDNA, estimated haplotype diversity, and distribution in the Palearctic. Furthermore, we used next-generation sequencing (NGS) to investigate larval and pupal remnants found in the leaf mines in old herbarium specimens of poplars to verify the presence of *Ph. populifoliella* in the Nearctic, particularly in the USA and Canada, where its favorable host plant (*P. balsamifera*) is naturally present. The latter question was especially important to address after we discovered many characteristic leaf mines on the herbarium specimens of *P. balsamifera* from the USA (dated 1850–1974).

2. Materials and Methods

2.1. Study Region

This study covered two biogeographic zones: the Palearctic (native range of *Ph. populifoliella*) and the Nearctic (native range of *P. balsamifera*) (Figure 1A). In the Palearctic, the study was focused on North Asia (i.e., the Asian part of Russia) (Figure 1B).

North Asia, or the Asian part of Russia, is a macroregion located east of the Ural Mountains, bordering Kazakhstan, Mongolia, and China to the south and bounded by the Arctic Ocean to the north and the Pacific Ocean to the east [33]. It is characterized by a continental climate [34], with extreme temperature fluctuations throughout the year. Winters are long (5–7 months), while summers are relatively short (2–3 months) and hot [35]. In the continental part, January temperatures range from −16 °C in the south to −48 °C in the north, while July temperatures vary from +5 °C in the north to +23 °C in the south. During the summer, maximum daytime temperatures can exceed +35 °C. Annual precipitation varies from 350 mm in the north to 2000 mm in high altitudes [35,36].

In North Asia, sampling was conducted in 11 regions (from the Tyumen Region to the Khabarovsk Territory, Russia; see Figure 1), primarily along the Trans-Siberian Railway. The specimens were also collected in the European part of Russia, specifically in Moscow and Saint Petersburg, where outbreaks of the moth have been occasionally reported [11,26]. Additionally, opportunistic sampling was conducted in Bulgaria and Slovenia. Furthermore, the genetic data of *Ph. populifoliella* from other European countries (Belarus, Germany, Lithuania, Finland, and France) were included in the study. From our experience, in European countries, *Ph. populifoliella* has a low population density and it is difficult to find its leaf mines. Additionally, data from India, where the moth has been detected in significant numbers [3], were included in this study. Overall, the material for the study originated from nine countries and 37 localities (Figure 1; Table S1). The study area from where the herbaria of *Populus* spp. originated and the approach to study the herbarized specimens are both described in Section 2.3.

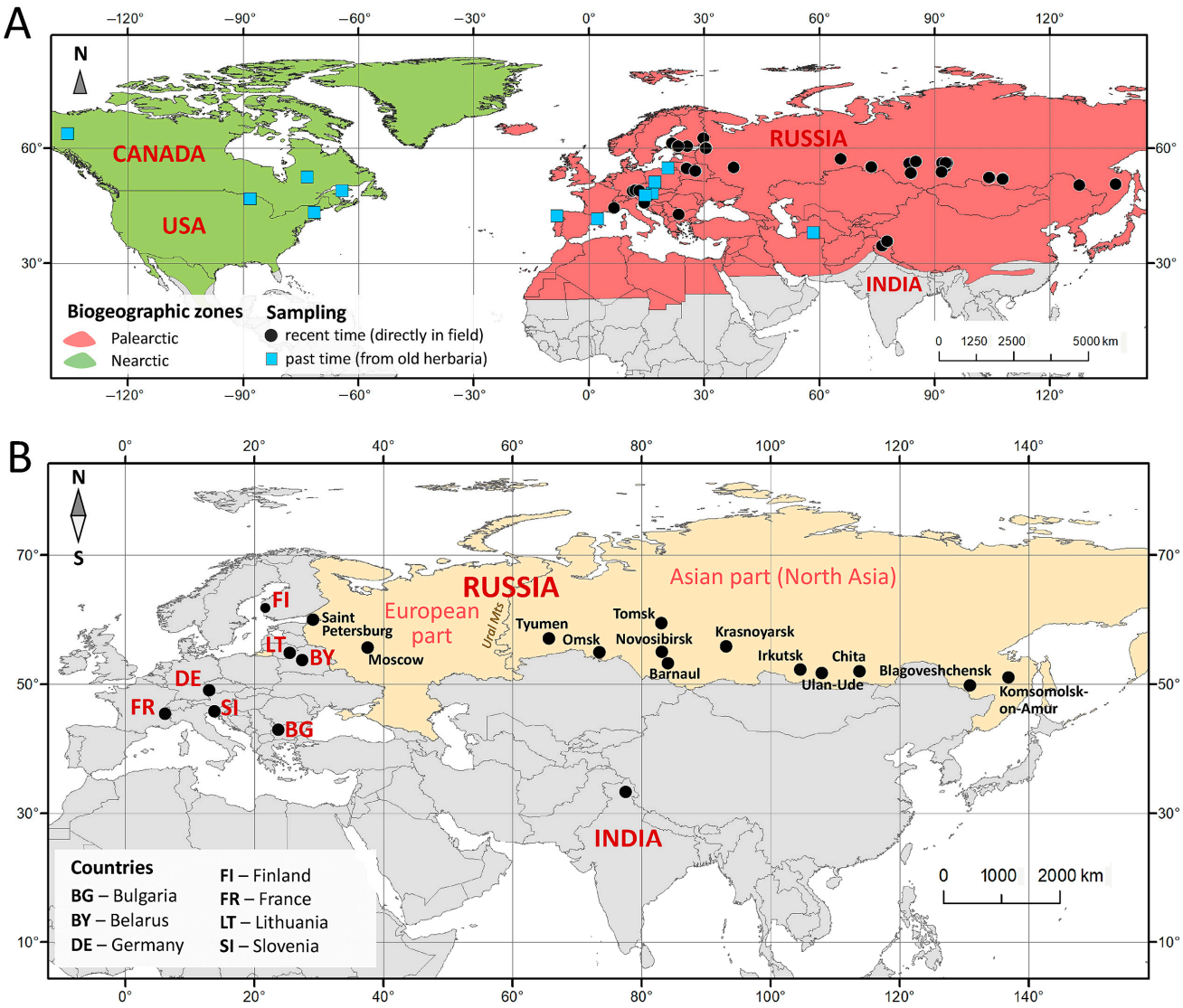

Figure 1. Studied biogeographic zones, countries, and localities. In the Palearctic, the localities where the insects were sampled directly in the field in the recent time (2006–2022) are indicated by black circles (**A**); in the Palearctic and Nearctic, the localities where the insect remnants were dissected from old herbaria collected in the past time (1850–1974) are shown by blue rectangles (**A**). In map (**B**), only the countries in which sampling was conducted in the recent time are shown; in Russia, the main cities of the regions where sampling was performed are specified. The Ural Mountains surveying the natural border between the European and the Asian parts of Russia are indicated in map (**B**). The maps were produced using ArcGIS 10.6.1 software [37].

2.2. Field Sampling

The specimens of *Ph. populifoliella* were collected in the Palearctic in 2006–2022 (Table S1). In the study, this period is regarded as 'the recent time' to highlight its recentness compared to 'the past time' (1850–1974) when herbarium specimens were originally collected, from which we dissected insect remnants (see Section 2.3).

In North Asia, the sampling of *Ph. populifoliella* was carried out in urban plantings (parks, gardens, alleys, etc.; see Figure 2A–C). Up to 10 trees of balsam poplar, black poplar (*P. nigra* L.), and their hybrid, referred to in some literature sources as *Populus* × *sibirica* (G.V. Krylov & G.V. Grig. ex A.K. Skvortsov, 2007 [15,18,38]), were examined. From these trees, around 50 leaves carrying mines were randomly collected from the lower part of the

tree crowns in mid- to late July or in late August, a time when later-instar larvae or pupae of the first and the second generations accordingly were present in the mines.

Figure 2. Sampling of leaves from *P. balsamifera* trees heavily attacked by *Ph. populifoliella* in North Asia. (**A**)—trees with damaged leaves and some newly grown apical leaves (not yet attacked by the moth), Irkutsk, mid-July 2015; (**B**,**C**)—leaves with numerous mines, Krasnoyarsk, July 2020; (**D**)—an adult moth; (**E**)—freshly herbarized leaves with mines. Photo: N.I. Kirichenko. Photo A is published with permission from the photographed coauthor, V.Y. Kuzevanov.

In the laboratory, larvae and pupae were dissected from the leaf mines. The specimens were preserved in 95% ethanol in 1.5 mL vials (Axygen, Glendale, Arizona, USA) and stored at a temperature of −20 °C. In a few instances in Russia, when sampling required identification confirmation through adult morphology, the mines containing larvae and pupae were placed in 100 mL plastic containers for obtaining the adult, following rearing techniques [39–41]. Hatched moths (Figure 2D) were immobilized with ethyl acetate vapor. They were identified to the species level based on forewing pattern (or, whenever needed, confirmed by examining male genitalia) [4,5]. During the mass hatching of moths, a part of the material was placed in 95% ethanol. Spare specimens of leaves with mines were herbarized (Figure 2E). The specimens from Russia (insects and leaves with mines) are stored in the Sukachev Institute of Forest SB RAS (Krasnoyarsk, Russia).

In Bulgaria, leaf mines were opportunistically collected from *P. nigra* in early August 2013, and in Slovenia, in 2022. The specimens from India were collected on balsam poplar hybrids in 2018. Data originating from Belarus, Germany, Lithuania, Finland, and France were used from publicly accessible datasets in the Barcode of Life Data System (BOLD) [42]. The specimen data and the collectors are listed in Table S1.

2.3. Sampling Insect Remnants from Old Herbaria

A total of 890 herbarium specimens, stored on individual sheets in the herbarium collections at the National Museum of Natural History (Paris, France), the Natural History Museum (Vienna, Austria), and the Berlin Botanic Garden and Botanical Museum (Berlin, Germany), were examined (Figure 3).

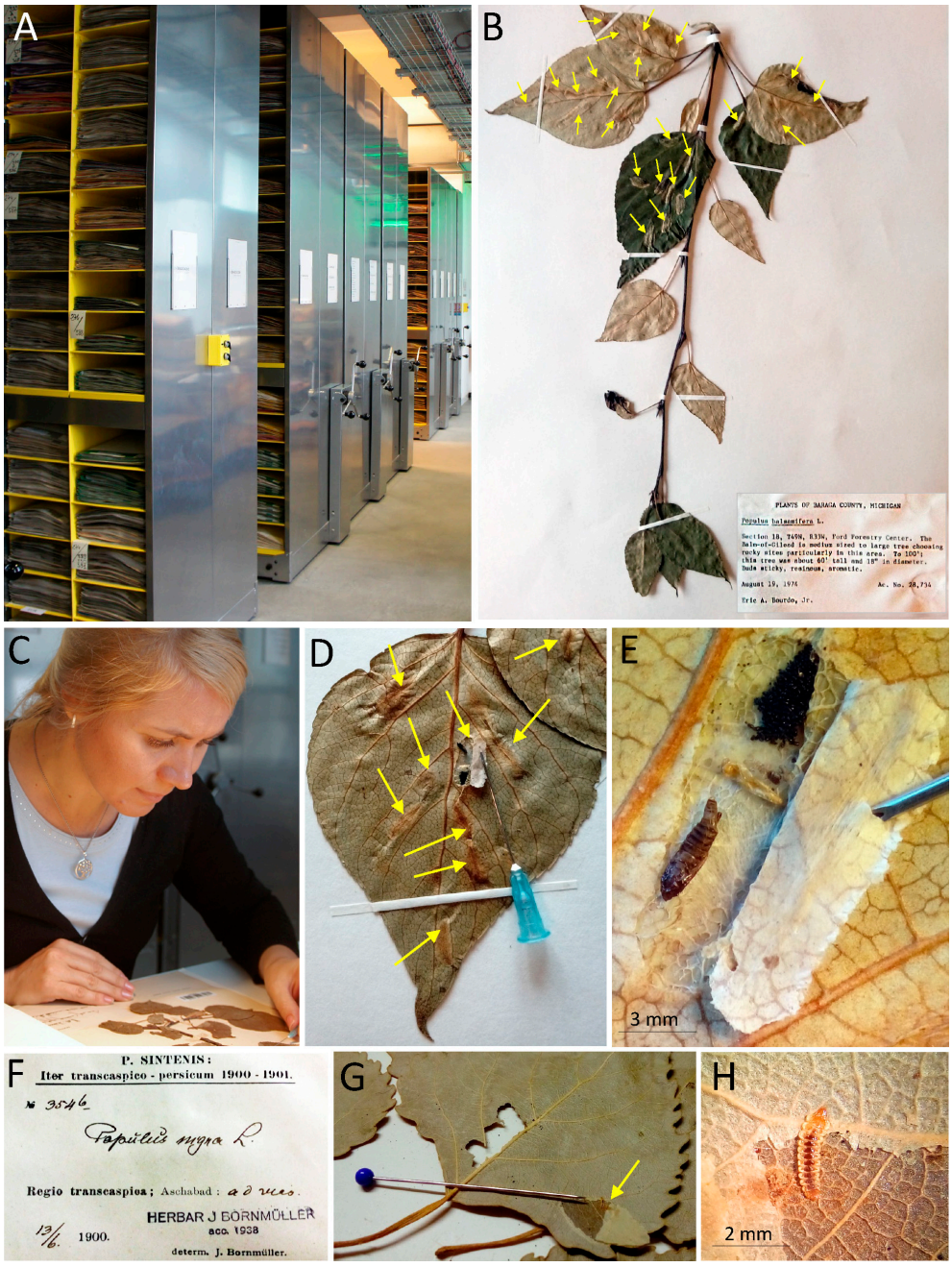

Figure 3. Sampling in herbarium depositaries. (**A**)—herbarium of the National Museum of Natural History (Paris); (**B**)—herbarized specimen of *P. balsamifera* carrying many mines typical of *Phyllonorycter*; (**C**)—dissecting the leaf mine; (**D**)—opened mine on the leaf of *P. balsamifera* containing pupa; (**E**)—close-up of the pupa; (**F**)—the herbarium specimen label (*P. nigra*); (**G**)—an opened mine on the leaf of *P. nigra* containing young larva; (**H**)—close-up of the larva. In photos (**B**,**D**,**G**), the leaf mines are indicated by yellow arrows. Photo: N.I. Kirichenko. Photo C is published with permission from the photographed coauthor, N.I. Kirichenko.

The herbarized specimens were represented by twigs with leaves or just by leaves from three poplar species: balsam, black, and white poplars (*P. alba* L.). They were collected in the Palearctic (specifically, Europe and Central Asia) and/or the Nearctic (Canada and the USA) in 1850–1974 (Figures 1A and 3). The leaves were examined for the presence of the characteristic blotch mines. Only the leaf mines measuring 1.0–1.8 mm in length were included in the analysis, as they contained older larvae or pupae providing more DNA material compared to young (minute) larvae from juvenile mines.

With the agreement of the herbarium curators, insects found in the mines (larvae and pupae) were dissected from the leaf mines by syringe by opening the epidermis covering the mine. To avoid contamination, the scalpel used to cut the mines and the forceps used to pick up the insects were properly washed in 95% ethanol after each manipulation. The use of flame for sterilizing tools was prohibited in the herbarium depositaries. Overall, 14 insects (larvae of pupae) were collected, i.e., by one individual from one herbarium specimen. Insect remnants were placed in Eppendorf tubes (Life sciences, Hamburg, Germany) with labels and stored in a freezer prior to molecular genetic analysis. Images of the herbarium specimens were captured using a Sony Alpha NEX-5 digital camera (Sony Group Corporation, Tokyo, Japan) and subsequently adjusted using Adobe Photoshop 24.0.1 software (Adobe Inc., San Jose, CA, USA).

2.4. DNA Barcoding

In total, DNA barcodes from 83 *Ph. populifoliella* specimens sampled in the recent time were analyzed in this study. Of these, 47 specimens were sequenced as part of the current study (45 specimens from Russia, and 1 specimen from both Bulgaria and Slovenia). For comparative analysis, DNA barcodes for 25 specimens (from Europe: Belarus, Germany, Lithuania, Finland, and France) were retrieved from BOLD (publicly accessible data), while 11 DNA barcodes (all from North India) were used from our previously published dataset [3].

Freshly sampled insects preserved in 95% ethanol in microtubes were transferred to a genetic plate with 96 wells (Eppendorf, Sample Submission Kit, BOLD System, CCDB, Guelph, Canada) filled with 0.1 µL of 95% ethanol. To prevent cross-contamination during the transfer of insects from the field tubes to the wells of the genetic plate, the forceps were washed in 95% ethanol, and their tips were sterilized over a flame after each handling. The plate was sent to the Canadian Center for DNA Barcoding (CCDB) at the University of Guelph (Guelph, ON, Canada) for DNA barcoding. Sanger sequencing was performed using the primer set C LepFolF/C LepFolR [43].

The remnants of 14 larvae and pupae of *Phyllonorycter* spp. found in leaf mines within archival herbarium specimens of *Populus* spp. (dated 1850–1974) were used in the molecular genetic study. This effort was particularly aimed at testing the hypothesis of the introduction of *Ph. populifoliella* to North America in the past time, where its favorable host plant, *P. balsamifera*, naturally grows. Since *Ph. populifoliella* can occasionally be found on other poplar species in the Palearctic (such as *P. nigra* and *P. alba*) [44], the leaves of these plants were also examined in herbarium collections.

The insect remnants were transported in microvials to the CCDB for sequencing. In the insect remnants, up to 15 overlapping fragments (ranging from 100 to 300 nucleotides) of the mtDNA COI gene region (658 bp) were sequenced following a previously published NGS protocol [45] adapted for the Sequel platform (Pacific Biosciences, PacBio, Menlo Park, CA, USA). The rationale for choosing this method was its high accuracy and efficiency when applied to old specimens [46]. Multiple fragments were aligned to the reference sequence using Codon Code Aligner V.3.7.1 (CodonCode Corporation, Centerville, MA, USA) to assemble nucleotide contigs. Alignment was carried out in BIOEDIT ver. 7.2 [47].

All DNA-barcoded specimens were identified using the identification system in BOLD [42] and/or in GenBank [48] via BLAST (Basic Local Alignment Search Tool) [49]. The Barcode Index Number (BIN) [50] was obtained from BOLD for all examined specimens.

A total of 102 sequences were used in the study, including reference and outgroup sequences (Table S1). One sequence (GMBMP280-18: Belarus, 05.VIII.2016, T. Lipinksay coll.) was used in the analysis of intraspecific variability and also served as reference sequences in the analysis of herbarium data (Table S1). The COI sequences, electropherograms, specimen data, and images of the insects used in the analysis have been deposited in BOLD [42] and GenBank (National Center for Biotechnology Information). The sequences are accessible in BOLD through the following link: https://dx.doi.org/10.5883/DS-POPMOTH (accessed on 17 January 2025).

2.5. Data Analysis

Two phylogenetic trees were constructed: one for *Ph. populifoliella* specimens collected in the recent time in the Palearctic and another one for *Phyllonorycter* spp. dissected from leaf mines found in old herbaria from the Palearctic and Nearctic. The maximum value of intraspecific genetic variability and frequencies of genetic distances in *Ph. populifoliella* were estimated based on Kimura's two-parameter (K2P) model and the bootstrap method with 2500 iterations in the MEGA X program [51]. The phylogenetic trees were constructed using the Maximum likelihood (ML) estimation algorithm and K2P model and the bootstrap analysis (2500 iterations) in the MEGA X. A topology of basal branches in the phylogenetic trees was considered reliable if the bootstrap value exceeded 70. The ML tree of *Ph. populifoliella* collected in the recent time was rooted in a sequence of a related poplar moth, *Ph. pastorella* (Russia, Novosibirsk, collected in 2009 on *P. balsamifera*). The ML tree of the poplar moths' remnants dissected from herbarium specimens was rooted with a sequence of *Phyllocnistis unipunctella*, a species, whose remnants were also found in herbarized specimen (Russia, Kaliningrad, collected in 1892 on *P. canadensis*).

The parsimony algorithm was used to construct the median network of haplotypes in PopArt 4.8.4 [52]. Haplotype diversity was assessed with DnaSP v. 5.10.1 [53] and compared between Asia and Europe using the non-parametric Mann–Whitney U-test ($p < 0.05$) [54]. The relationships between the number of haplotypes and sampling size and between the lengths of the COI gene sequences obtained from remnants of poplar moths (*Phyllonorycter* spp). found in herbarium specimens and the age of herbarium specimens were estimated using Spearman's rank correlation ($p < 0.05$) in Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA). A map of haplotype distribution across the Palearctic was created using ArcGIS 10.6.1 [37].

3. Results

3.1. Genetic Variability and Phylogeography of Ph. populifoliella in the Palearctic

In BOLD, the DNA barcodes of all 83 specimens from Europe and Asia involved in the study were assigned to a single BIN (BOLD:AAD8619) corresponding to *Ph. populifoliella*. In the ML tree, the specimens from North Asia and Europe clustered together, showing no clear geographic structure. The cluster from North India, consisting of 11 identical sequences, was the most distant (Figure 4, the cluster highlighted in yellow).

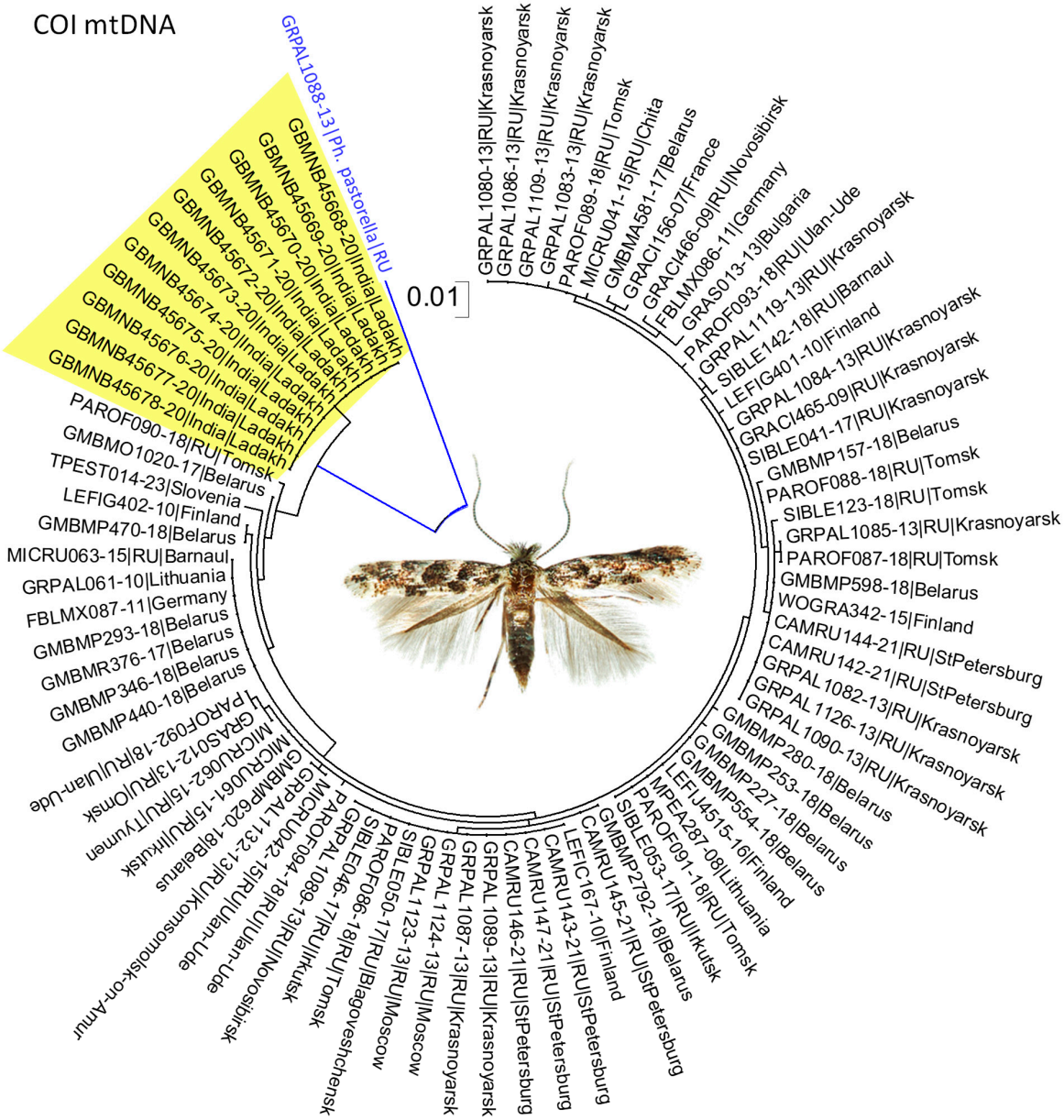

Figure 4. Maximum likelihood tree of *Ph. populifoliella* from different regions of the Palearctic. The most distant cluster consisting solely of the specimens from North India is highlighted in yellow. The rooting sequence is indicated in blue.

The genetic variability of *Ph. populifoliella* reached 3.73%. However, when the Indian cluster was excluded from the analysis, and, thus, only North Asian and European populations were considered, the maximum intraspecific variation dropped to 2.1%. This value was observed between the specimen from Krasnoyarsk, Russia (Process ID: GRPAL1119-13), and one from Sophia, Bulgaria (Process ID: GRAS013-13). For the majority of Asian and European specimens (47% of all analyzed samples), intraspecific genetic variation ranged between 0.3% and 0.79% (Figure 5).

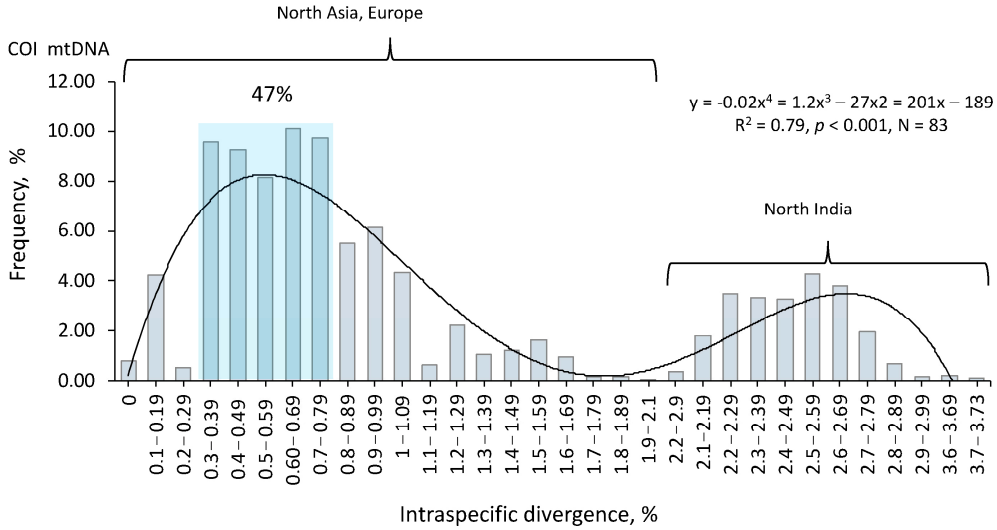

Figure 5. Frequency distribution of genetic distances in *Ph. populifoliella* in the Palearctic, described by quartic polynomial function ($p < 0.05$). The sum of all bars is 100%. The most common genetic distances observed in the species range are highlighted in blue.

In total, 30 COI haplotypes were identified among 83 moth specimens, meaning that one in every three specimens carried a new haplotype (Figure 6, Table S2). In Asia, 21 haplotypes were recorded (in 48 specimens), while in Europe, 13 haplotypes were found (in 35 specimens). Notably, only four haplotypes, H3 (dark blue), H6 (red), H7 (orange), and H27 (grey), were shared between Asia and Europe, representing 13% of all haplotypes detected (Figure 6, Table S2). Thus, the absolute number of haplotypes was higher in Asia compared to Europe.

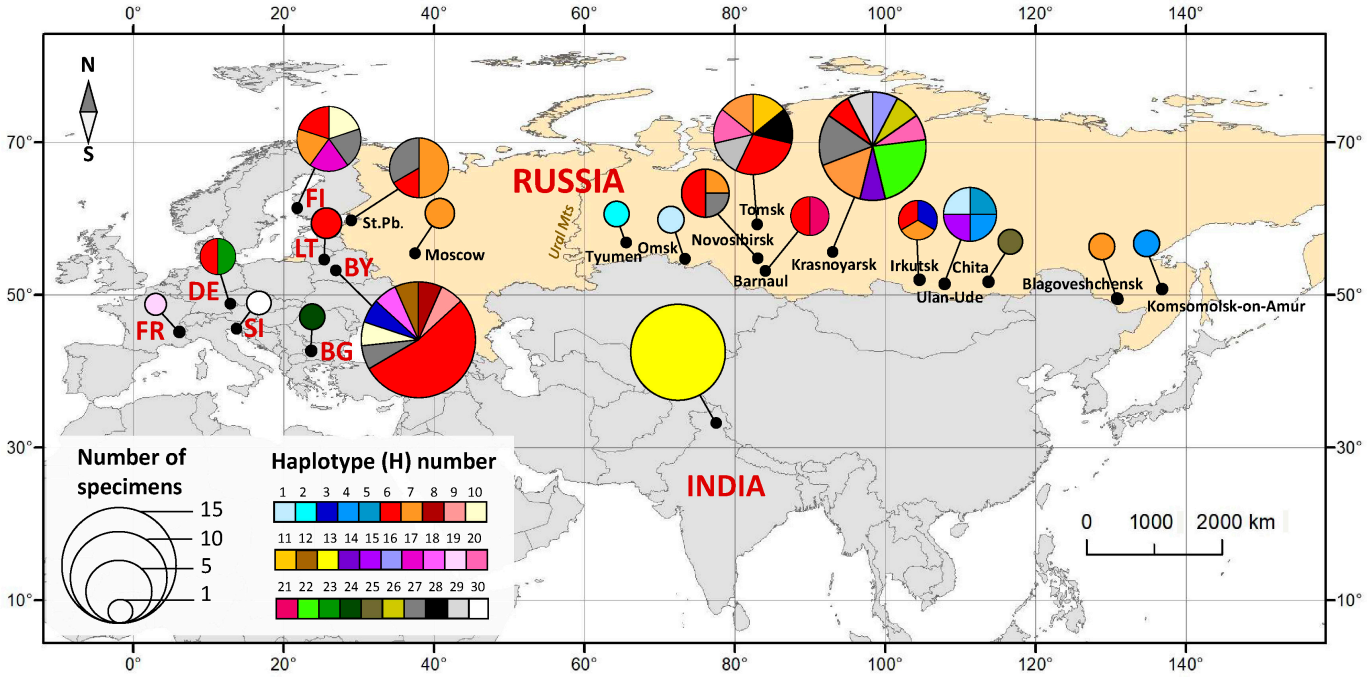

Figure 6. The distribution of *Ph. populifoliella* haplotypes in the Palearctic. Haplotype composition is shown in pie charts for European countries and India; in Russia, haplotype composition is indicated for the main cities. Abbreviated countries: BG—Bulgaria, BY—Belarus, DE—Germany, FI—Finland, FR—France, LT—Lithuania, SI—Slovenia, and Russia (European part). The size of the pie chart indicates the number of specimens studied (see legend). The colored sectors represent different haplotypes (30 in total; see legend). The distribution of haplotypes by country is provided in Table S2.

Taking into account the sample size, the relative haplotype diversity of *Ph. populifoliella* was not significantly different between the continents: 0.44 ± 0.08 in Asia vs. 0.37 ± 0.07 in Europe (Mann–Whitney U-test, Z = –0.71, N_{Asia} = 48, N_{Europe} = 35, $p > 0.05$). The nucleotide diversity of *Ph. populifoliella* was n = 0.0095, Tajima's D = –0.348 at p = 0.77. A significant positive correlation was observed between haplotype diversity and sampling efforts (y = 1.4x + 0.2; R^2 = 0.66; $p < 0.05$).

In the median haplotype network, two main clusters (A and B) were identified, separated by seven mutation steps (Figure 7). Cluster A, the largest, included 29 haplotypes and encompassed all specimens from Asia (mostly North Asia) and Europe, with no clear geographic resolution. In this cluster, neighboring haplotypes were linked by 1–2 mutation steps. The dominant haplotype, H6 (red), accounted for 24% of all studied specimens and was distributed across the Palearctic (Figure 7, Table S2). In Europe, H6 was found in five out of eight studied countries: Germany, Lithuania, Belarus, Finland, and Russia (the European part). In North Asia, it was found in 5 out of the 11 studied regions (Table S2). Cluster B was comprised exclusively of specimens from India and represented a single haplotype, H13 (Figure 7).

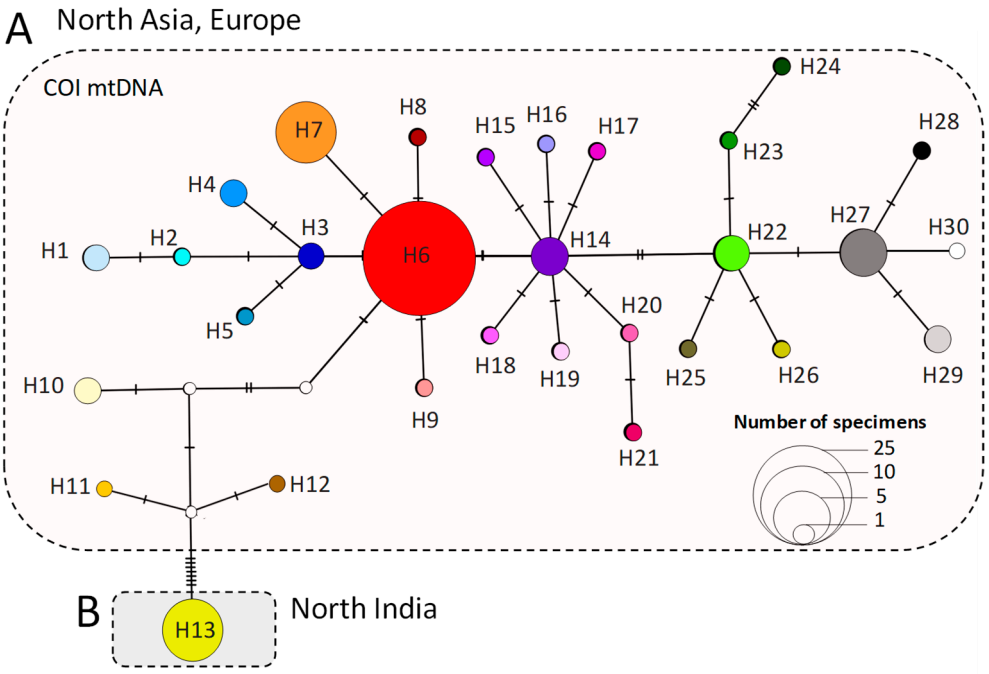

Figure 7. Median haplotype network of *Ph. populifoliella* in the Palearctic. Clusters (**A**,**B**) are framed in dotted-line rectangles. Colored circles represent different haplotypes (H1–H30), with the circle size proportional to the number of specimens (see legend). Short transverse lines and small white circles (○) indicate hypothetical haplotypes (undetected in the study).

3.2. The DNA-Based Identification of Poplar Moths from Old Herbaria (Palearctic, Nearctic)

Typical leaf mines were found in 14 out of 890 herbarium specimens, or in 1.5% of the cases. These 14 herbarium specimens were collected between 1850 and 1974, i.e., from 50 to 174 years ago. Among them, seven herbarium specimens were from Europe (four from Austria, two from Spain, and one from Poland; dated 1879–1925), one from Asia (Turkmenistan; 1901), and six from North America (by three from the USA and Canada; 1850–1974). In Michigan and Connecticut (USA), noticeable damage was revealed on the leaves of *P. balsamifera* in five herbarium specimens dated 1850–1974 (Figures 3B and 8). These specimens contained up to 10 leaf mines per leaf (Figure 8), resembling the damage caused by *Ph. populifoliella* to leaves of balsam poplar in North Asia (Figure 2).

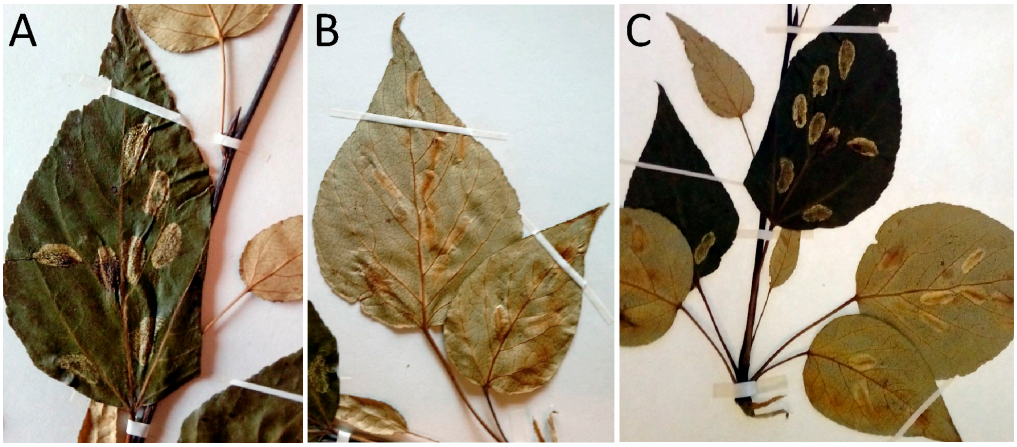

Figure 8. Herbarium specimens of *P. balsamifera* with the leaf mines typical of the genus *Phyllonorycter*. (**A**,**B**)—USA, Michigan, 19 August 1974; (**C**)—USA, Connecticut, 1 July 1850. Specimens are stored in the herbarium collection of the Natural History Museum of Vienna (Austria). Photo: N.I. Kirichenko.

Using NGS, COI sequences were obtained for 14 *Phyllonorycter* larvae or pupae remnants dissected from herbarium specimens dated 1974–1850. The length of recovered COI fragments ranged from 145 bp (specimen LMINH159-19 from the USA, Connecticut, 1850) to 658 bp (four specimens: LMINH157-19 and LMINH158-19 from the USA, Michigan, 1931, and LMINH160-19 and LMINH161-19 from Austria, 1886).

Relatively long sequences, i.e., greater than 60% (>395 bp) of the targeted length (658 bp) were obtained from 11 out of 14 insect specimens (Figure 9). Among them, five individual insects were dissected from the herbarium specimens collected in the Palearctic more than a century ago (i.e., back in time from 123 to 145 years) (Figure 9). For the insect specimens dissected from the oldest herbarium sample, collected 174 years ago (in 1850), the length of the obtained COI sequence was 145 bp (i.e., 22% of the targeted length). The length of sequences showed a significant negative correlation with the specimens' ages (R^2= 0.46, n = 14, $p < 0.05$) (Figure 9).

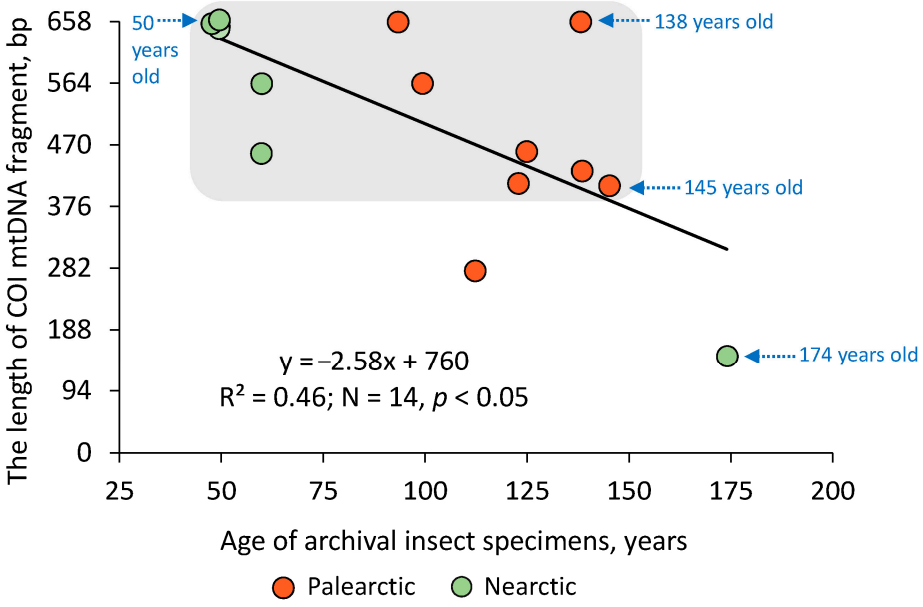

Figure 9. The relationship between the length of the COI sequences and the age of archival specimens of *Phyllonorycter* spp. dissected from herbarium collected in the Palearctic and Nearctic in 1850–1974. The insect specimens with sequence lengths over 60% are shown in the grey rectangular.

Overall, 13 out of 14 sequences (including the oldest from a 174-year-old herbarium specimen) were identified to the species level using the identification system of BOLD. Seven sequences obtained for the archival insects from the Palearctic corresponded to three moth species: *Ph. populifoliella* (three sequences), *Ph. comparella* (three), and *Ph. pastorella* (one). The oldest specimen of *Ph. populifoliella* was found in the mines on herbarized leaves sampled in 1879 in Austria.

On the phylogenetic tree, the sequences clustered according to their respective species and showed high similarity to the reference DNA barcodes from BOLD (Figure 10). In the ML tree, the specimens of *Ph. populifoliella* dissected from the herbarium collected in Austria and Poland (in 1879–1931) grouped with the reference specimen from Belarus (Figure 10). The specimens of *Ph. comparella* found in herbarium from Spain and Austria (1886–1925) clustered with the reference specimen from Germany, and *Ph. pastorella* from Turkmenistan (1901) grouped with the reference specimen from Finland (Figure 10).

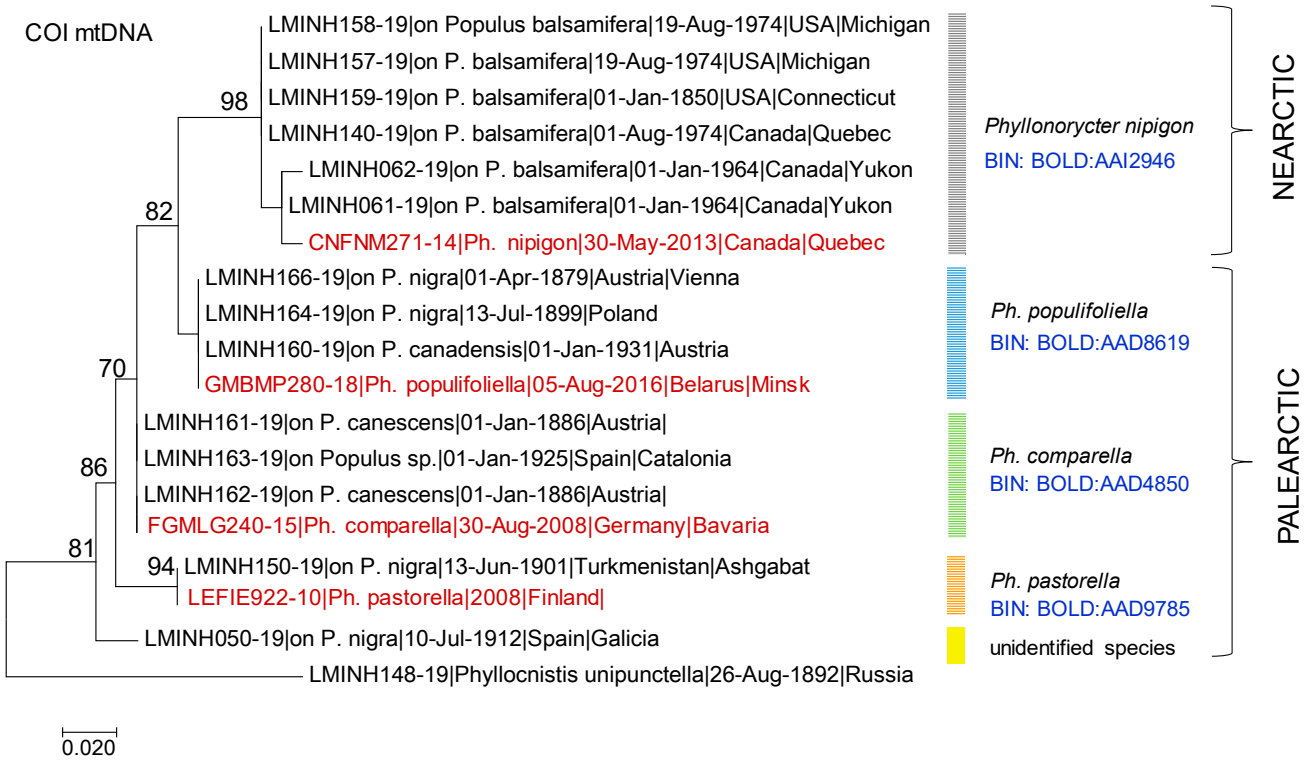

Figure 10. Maximum likelihood tree of *Phyllonorycter* spp. specimens dissected from herbarium collected in the Palearctic and Nearctic in 1974–1850. The reference sequences are highlighted in red. The tree was built with the K2P nucleotide substitution model and bootstrap method (2500 iterations). For each specimen, the following data are provided: Process ID | host plant (P.—*Populus*) | sampling date | country | region. Each genetic cluster corresponds to a *Phyllonorycter* species and is defined by Barcode Index Number (BIN). Branch lengths are proportional to the number of nucleotide substitutions per site.

In the Nearctic, six archival individual insects (three from herbarized specimens from the USA and Canada, collected in 1850–1974) were unmistakably identified as the North American poplar moth *Ph. nipigon* (Freeman). These archival specimens were grouped with the reference sequence of *Ph. nipigon* from Canada (CNFNM271-14, Quebec, 1974; Figure 8). Four of these archival specimens (three from the USA and one from Canada) had identical COI sequences. The maximum intraspecific genetic divergence (1.4%) was observed between the archival specimens of *Ph. nipigon* from Canada (LMINH-062-19, Yukon, 1964) and the reference sequence from Canada, Quebec (Figure 10).

One poplar moth larva dissected from a herbarium specimen of black poplar collected in Spain (Galicia) in 1912 failed to be identified to the species level (Figure 10).

Among three archival specimens of *Ph. populifoliella*, the one from Austria (LMINH-160-19, sampled in 1931, sequence length 658 bp) represented the *Ph. populifoliella* haplotype H7, which is also found in recent specimens from Finland and Russia (Figure 6, Table S2). The sequences of the other two archival specimens of *Ph. populifoliella* (LMINH-166-19, Austria, 1879 and LMINH-164-19, Poland, 1899) were shorter, 408 and 450 bp, respectively. They could represent two early haplotypes potentially derived from haplotypes H18 and H9, which were recorded in recent specimens from Belarus (Figure 6, Table S2). Due to the incomplete sequences of these two archival specimens, it was not possible to determine their exact positions in the haplotype network built for recent specimens (Figure 7).

4. Discussion

In the Northern Hemisphere, 19 *Phyllonorycter* species are known to develop on poplars (*Populus* spp.), among which 13 species are distributed in the Palearctic and 7 in the Nearctic, with 1 species (*Ph. apparella*) present on both continents (Figure 11).

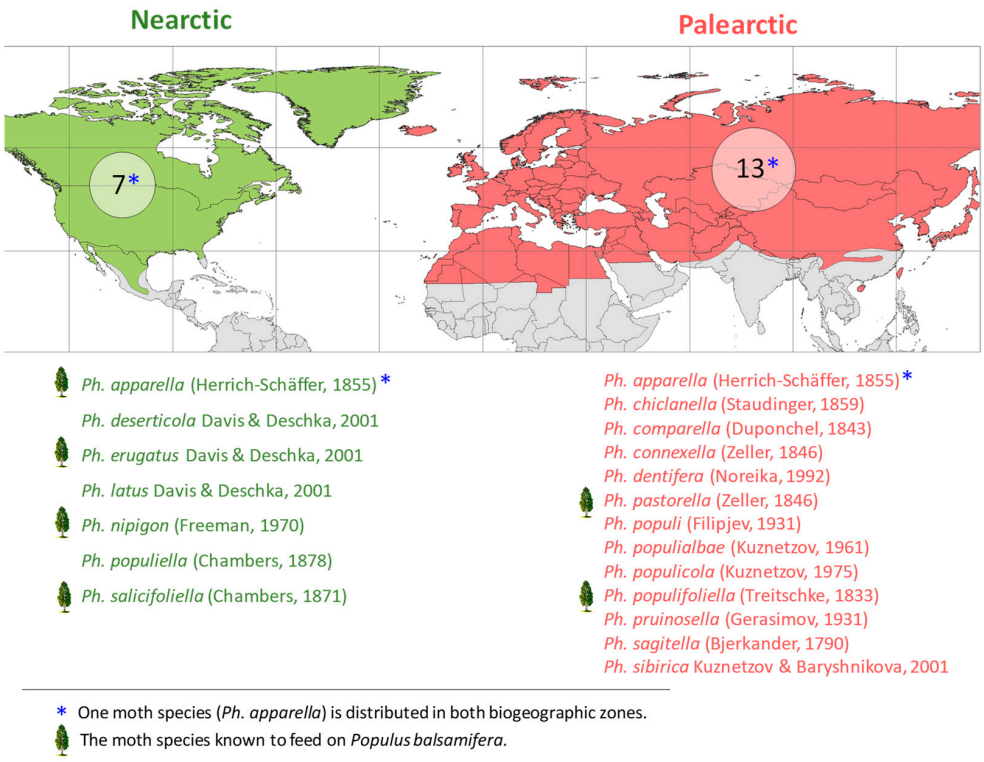

Figure 11. *Phyllonorycter* species feeding on poplars (*Populus* spp.) in the Northern Hemisphere [1,2,6,7,55]. Note: in the Palearctic, *Ph. hilarella* (Zetterstedt, 1839) was also mentioned on *Populus tremula* in [55] with reference to [56]. However, in [2], only willows, *Salix* spp., are indicated. We did not include this species on the map, as its trophic association with a poplar species would need to be confirmed.

The majority of these *Phyllonorycter* species (i.e., 18 out of 19) are trophically associated with more than one poplar species [1,2,6,7,55]. Notably, six *Phyllonorycter* species feed on *P. balsamifera* (Figure 11). In the Nearctic, four native moths (*Ph. apparella*, *Ph. erugatus*, *Ph. nipigon*, and *Ph. salicifoliella*) develop on this native plant. In the Palearctic, where the plant is alien, it serves as a host for *Ph. pastorella* and *Ph. populifoliella* (Figure 11). Among these two trans-Palearctic moth species, only *Ph. populifoliella* benefits from the novel trophic association with the North American balsam poplar. In North Asia, where this plant and

its hybrids are common in urban areas and other anthropogenic landscapes [19,20], this moth provides spectacular long-lasting outbreaks [10].

Our study, despite being based on a limited number of *Ph. populifoliella* specimens (83 specimens), allowed for the detection of high intraspecific genetic variability (3.73%) and defined 30 haplotypes in the Palearctic. Interestingly, the majority of these haplotypes (20 out of 30, or 67%) were found solely in North Asia. This could be linked to the trophic association of the native moth with the alien plant and the host availability. In North Asia, *P. balsamifera* and its hybrids are widely planted [19,20] and support high population densities of the moth [10]. Long-lasting outbreaks may provide the environment for rapid evolution, leading to the rich genetic diversity of *Ph. populifoliella*. In Europe, the number of detected haplotypes was lower than in North Asia, and only 13% of haplotypes were shared between European and Asian populations of *Ph. populifoliella*. However, this result should be treated with caution, as it can be impacted by the limited sampling. Indeed, our analysis revealed a statistically significant positive correlation between the number of detected haplotypes and the sampling size. Such a trend was also recorded for other gracillariids [8,57,58]. This suggests that as the sample size increases in the moth's populations, a greater number of haplotypes could be identified. In our case, additionally, the unique haplotype detected in India could also be discovered in other parts of the Palearctic with increased sampling efforts.

Until our recent study [3], *Ph. populifoliella* was not known from India. Numerous leaf mines were found in 2017 in the north of the country in the Ladakh region at a high altitude (between 2932 and 3400 m a. s. l.) [3]. Notably, the moth damaged unidentified introduced poplar species (supposedly from the balsam poplar section). It was hypothesized that the species might have expanded from North Asia, where it is abundant and outbreaks regularly [3]. However, in the present study, we could not detect the haplotype H13 (found in India) in North Asia to support this hypothesis. Thus, it remains unclear whether or not the Indian population represents a significant evolutionary divergence or an outlier.

Besides clarifying modern ranges of insects, it is essential to learn more about their past distributions to have a better understanding of their biogeography. Historical herbaria may serve as valuable sources of data for that [59,60]. Even though botanists prefer to collect uninjured plant samples to herbaria [61], they may overlook or disregard damage left by miniature endophagous insects, such as leaf mines, as their mines do not harm leaf lamina integrity (compared to external-feeding insects). Larvae and pupae of leafminers found in historical herbaria are excellent objects for molecular genetic analysis to address various ecological, biogeographic, and taxonomic questions [58,62,63].

Earlier, we demonstrated the successful use of NGS in obtaining DNA barcodes for remnants of larvae and pupae of the lime leafminer *Ph. issikii* dissected from up to 162-year-old herbaria [58]. In the present study, using NGS (Sequel platform, PacBio), we were able to obtain DNA barcodes for larval and pupal remnants of *Ph. populifoliella* dissected from up to 174-year-old herbaria. Based on the molecular genetic data, we were able to identify three *Phyllonorycter* species from herbaria collected in the Palearctic. They were *Ph. populifoliella* (from Austria and Poland), *Ph. pastorella* (Turkmenistan), and *Ph. comparella* (Austria and Spain). In these countries, these moths are known as native species [64–66]. Notably, *Ph. populifoliella* was not detected in archival herbarium samples collected in North America in 1850–1974. Instead, the indigenous poplar moth *Ph. nipigon* was identified based on six DNA-barcoded larvae and pupae dissected from herbaria from the USA and Canada. A significant number of *Ph. nipigon* leaf mines were found on herbarium samples of *P. balsamifera* dated 1886–1974, indicating that this balsam poplar is also susceptible to attacks by the native North American leafminer.

Upon introduction to the Palearctic, *P. balsamifera* turned out to be highly vulnerable to the Palearctic *Ph. populifoliella*. It is possible that *Ph. populifoliella* could eventually spread to North America, where its favorable host is naturally present. Indeed, the transatlantic movement of leafmining moths, which can occur through various pathways, including air, water, or land transport, is not an uncommon event [67,68]. Conversely, the spread of *Ph. nipigon* to the Palearctic cannot be excluded, given the widespread distribution of *P. balsamifera* in this region.

5. Conclusions

Our study clarified the phylogeography of *Ph. populifoliella* in the Palearctic and revealed high genetic diversity, with the highest absolute number of haplotypes found in North Asia. In this macroregion, the moth experiences regular outbreaks on the widely distributed North American balsam poplar (*P. balsamifera*) and its hybrids. Such trophic association between the native moth and the alien host plant is beneficial for *Ph. populifoliella* as it can promote the fast evolution of the moth resulting in its rich genetic diversity and the selection of vigorous haplotypes. This hypothesis needs to be studied more deeply with greater sampling in North Asia.

Furthermore, our research emphasized the multidisciplinary value of archival herbaria and their particular application to ecological studies. It provided an excellent example of the successful use of NGS for the accurate identification of insect remnants preserved in old herbaria. This approach also proved effective for exploring the historical ranges of endophagous insects and testing hypotheses about their early introductions to new biogeographic regions.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/f16020190/s1: Table S1: Biogeographic data of *Ph. populifoliella* collected in the Palearctic in the recent time and the poplar moths *Phyllonorycter* spp. dissected from old herbaria from the Palearctic and Nearctic; Table S2: The haplotypes of *Ph. populifoliella* detected in Asia and Europe.

Author Contributions: Conceptualization, N.I.K.; methodology, N.I.K., P.T., V.Y.K. and E.N.A.; software, N.I.K., M.A.R. and A.A.E.; formal analysis, N.I.K., P.T., S.V.B., S.Y.S. and M.A.R.; investigation, N.I.K., P.T. and M.A.R.; resources, N.I.K., S.V.B., S.Y.S. and E.V.Z.; data curation, N.I.K.; writing—original draft preparation, N.I.K.; writing—review and editing, N.I.K., P.T., M.A.R., A.A.E., S.V.B., S.Y.S., V.Y.K., E.N.A., P.R.S., A.V.S., E.V.Z., K.V.K. and D.L.M.; visualization, N.I.K. and M.A.R.; supervision, N.I.K. and D.L.M.; project administration, N.I.K.; funding acquisition, N.I.K. All authors have read and agreed to the published version of the manuscript.

Funding: The study was carried out with the support of an RSF grant (No. 22-16-00075) (molecular genetic study), the basic project of the Sukachev Institute of Forest SB RAS (No. FWES-2024-0029) (herbarium study), and the partial support of the core research group Forest biology, Ecology, and Technology (No. P4-0107, Slovenian Research Agency) (specimens sampling and data analysis). S.V.B. and S.Y.S. were supported by the state research project (No. 122031100272-3) (morphological identification), and A.V.S. was supported by an RSF grant (No. 24-16-00092) (national literature review).

Data Availability Statement: The genetic data used in the study are publicly accessible in BOLD using the following link: https://dx.doi.org/10.5883/DS-POPMOTH (accessed on 16 January 2025).

Acknowledgments: We thank Nikita Mamaev (St. Petersburg, Russia) and Sergey Rudykh (Ulan-Ude, Russia) for providing few insect specimens for DNA barcoding; Irina Mikhaylova (Krasnoyarsk, Russia) for help with mapping; the curators and staff at herbarium depositaries, including Vanessa Invernón (the herbarium (code P) at National Museum of Natural History, Paris, France), Peter Hein and Sarah Bollendorff (the herbarium (code B) at Berlin Botanic Garden and Botanical Museum, Berlin, Germany), and Christian Bräuchler (the herbarium (code W) at Natural History Museum,

Vienna) for allowing us to access the collections and dissect the leaf mines of herbarized specimens; the team at the Biodiversity Institute of Ontario, University of Guelph (Ontario, Canada), for their assistance in producing the DNA barcodes for our study; and Stanislav Gomboc (Slovenia) for his thoughtful reading of the manuscript and wise suggestions.

Conflicts of Interest: The authors declare no conflicts of interest.

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