SEEFOR SOUTH-EAST EUROPEAN FORESTRY

OPEN ACCESS
ISSN 1847-6481
eISSN 1849-0891

ORIGINAL SCIENTIFIC PAPER

DOI: https://doi.org/10.15177/seefor.25-08

Leaf Mine Types and Associated Parasitism in the Invasive Tree Insect Pest *Macrosaccus robiniella* (Lepidoptera: Gracillariidae): a Case Study from Slovenia and Croatia

Natalia I. Kirichenko[1,2,3*], Stanislav Gomboc[4], Oksana V. Kosheleva[5], Dinka Matošević[6], Barbara Piškur[3], Maarten de Groot[3*]

(1) Sukachev Institute of Forest, Siberian Branch of the Russian Academy of Sciences, Federal Research Center "Krasnoyarsk Science Center SB RAS", Department of Forest Zoology, Akademgorodok 50/28, RU-660036 Krasnoyarsk, Russia; **(2)** All-Russian Plant Quarantine Center (VNIIKR), Krasnoyarsk branch, st. Zhelyabova, 6/6, RU-660020 Krasnoyarsk, Russia; **(3)** Slovenian Forestry Institute, Department of Forest Protection, Večna pot 2, SI-1000 Ljubljana, Slovenia; **(4)** Gančani 110, SI-9231 Beltinci, Slovenia; **(5)** All-Russian Institute of Plant Protection (FSBSI VIZR), Laboratory of Phytosanitary Diagnostics and Forecasts, Podbelskogo 3, RU-196608 Saint Petersburg, Russia; **(6)** Croatian Forest Research Institute, Division for Forest Protection and Game Management, Cvjetno naselje 41, HR-10450 Jastrebarsko, Croatia

*** Correspondence:** e-mail : nkirichenko@yahoo.com, maarten.degroot@gozdis.si

Citation: Kirichenko NI, Gomboc S, Kosheleva OV, Matošević D, Piškur B, de Groot M, 2025. Leaf Mine Types and Associated Parasitism in the Invasive Tree Insect Pest *Macrosaccus robiniella* (Lepidoptera: Gracillariidae): a Case Study from Slovenia and Croatia. *South-east Eur for* 16(1): 15-25. https://doi.org/10.15177/seefor.25-08.

Received: 3 Feb 2025; **Revised:** 17 Apr 2025, 29 May 2025; **Accepted:** 2 Jun 2025; **Published online:** 24 Jun 2025

ABSTRACT

Since its first documentation on the European continent in 1983, the invasive micromoth *Macrosaccus robiniella* (Clemens, 1859) (Lepidoptera: Gracillariidae) was known only for its lower-side blotch mines occupying one of the leaflet halves (common mine type, hereafter mine type 1). In 2023, in abundant populations of *M. robiniella* in forest areas of Slovenia and Croatia (with more than 50% of leaves damaged), we observed that, in addition to the common mine, this invasive species is capable of forming three other mine types. One (type 2) resembles the common mine type by occupying one half of a leaflet, but the mine is situated on the upper side of the leaf. The two other mine types differ from the common mine in both appearance and position on the leaflet. These include a blotch above the midrib on the upper side of the leaflet (type 3) and a narrow blotch at the edge, causing strong downward folding (type 4). The mines of types 3 and 4 resemble the damage caused by two North American black locust herbivores that are also invasive to Europe: the gracillariid *Parectopa robiniella* Clemens, 1863 (Lepidoptera: Gracillariidae) and the gall midge *Obolodiplosis robiniae* (Haldeman, 1847) (Diptera: Cecidomyiidae), respectively. In the studied localities, the relative abundance of mine types 3 and 4 was about four times higher than that of mine type 1. The parasitism rate in these mines was 6.5 times lower compared to that in mine type 1. No statistical difference was found in the parasitism rate between mine types 3 and 4. We discuss how the leaf-mining behaviour of *M. robiniella* may provide an enemy-free space, conferring a survival advantage and supporting a high population density in forested areas.

Keywords: locust leaf-miner; biological invasion; leaf-mining behaviour; parasitoid attacks; Europe

INTRODUCTION

Behavioural adaptations are crucial for invasive species (Gulzar et al. 2024). To become a successful invader, a species must show behavioural plasticity to be able to effectively compete for space and food resources in invaded regions (Catford et al. 2018). Additionally, it must evade native enemies and/or exploit enemy-free space to thrive in the new habitat and continue expanding its range (Jeffries and Lawton 1984, Torchin et al. 2003, Xirocostas et al. 2023).

The North American *Macrosaccus robiniella* (Lepidoptera: Gracillariidae) is a leaf-mining moth trophically associated with North American black locust, *Robinia pseudoacacia* L. This tree was introduced to the European continent in the 17th century, and since that time it has widely spread in European countries, penetrating forested areas (Vítková et al. 2017). Outside its native range, *M. robiniella* was first documented in 1983 in Switzerland (Whitebread 1990). In the following decades, the leaf-miner spread across most of the European continent (Davis and De Prins 2011), including the European part of Russia (Baryshnikova 2019). In Slovenia, *M. robiniella* was detected in 1994 (Seljak 1995, Maček 1999), and in Croatia in 2001 (Mešić and Maceljski 2001). Alongside *M. robiniella*, black locust has been the host to another invasive leaf-mining moth, the North American *Parectopa robiniella* (Lepidoptera: Gracillariidae), which was first observed in 1983 in Slovenia and 2001 in Croatia (Maceljski and Igrc 1983, Maček 1984). These leaf-mining moth species are now distributed across both countries (Matošević 2007, Gomboc and Kirichenko 2022).

In their invaded range, *M. robiniella* and *P. robiniella* share the same localities, host trees and in some cases even the same leaflets (Fodor and Hâruţa 2009, Medzihorský et al. 2024). Until recently, *M. robiniella* was known for its lower side tentiform blotch mines (common mine type) occupying one of the leaflet halves, whereas *P. robiniella* always forms its mines over the midrib on the upper side of the leaflet. Thus, both species effectively shared the same trophic niche by occupying a certain side of the leaflet (Fodor and Hâruţa 2009).

In 2019, in Slovenia in the forested area around the Ljubljana Castle (Ljubljana) and in Panovec forest (Nova Gorica), we observed that *M. robiniella*, in addition to its common mine type, was able to produce mines on the upper side of leaflets, occupying one of the leaflet halves (unpublished data). To our knowledge, in Europe, a single case of upper side mine was mentioned in Switzerland (Whitebread 1990). Opportunistic surveys conducted in 2023 revealed two other mine types with unusual positions on leaflets. Some mines were situated on the upper side above the midrib, similar to the mines of *P. robiniella*, whereas other mines were positioned at the leaf edge, causing strong downward folding, resembling damage caused by another North American insect, *Obolodiplosis robiniae* (Diptera: Cecidomyiidae). This was first recorded in Europe in 2003 in Italy (Duso and Skuhravá 2004) and is also present in both Slovenia and Croatia (Duso et al. 2005, Pernek and Matošević 2009).

We investigated the presence of different mine types of *M. robiniella* in forested areas in Slovenia and Croatia, where the moth exhibited notable abundance during the study year (2023). Specifically, we (1) described for the first time the mine types occurring in Europe, (2) estimated the abundance of different mines in dense populations of *M. robiniella*; (3) clarified whether there is a correlation with the co-occurrence with *P. robiniella*; and (4) determined whether parasitism rates differ among the different leaf mine types. We hypothesized that by producing uncommon leaf mines, the invasive leaf-miner may avoid native enemies (in particular, parasitoids) and, thus, have high survival and keep high population density in forested areas.

MATERIALS AND METHODS

Study Area

The study was carried out in Slovenia and Croatia (Figure 1). In Slovenia, the forested area around the Slovenian Forestry Institute in Ljubljana (46°3'6"N, 14°29'1"E, 305 m alt.) was explored.

In Croatia, sampling was conducted in a forested area in Krapinsko-zagorska županija (Krapina-Zagorje County), at two locations near the town of Pregrada: Kunagora (46°9'44"N; 15°44'43"E; 279 m alt.) (Figure 2) and Vinagora (46°10'25"N; 15°42'17"E; 262 m alt.).

Ljubljana is characterized by a continental climate with cold winters, humid springs and autumns, and warm summers. The average annual precipitation is about 1,400 mm, with the majority occurring between June and October (Slovenian Environment Agency 2025). The hottest months are July and August (on average around 21°C), while the coldest month is January (on average around 0°C).

Krapina-Zagorje County has a similar climate type but is slightly warmer with a moderately rainy climate. The temperature in July and August is about 22°C, while in winter the temperature can drop to –3°C (Izvješće 2018). Precipitation is also fairly evenly distributed throughout the year, with winter being the driest season. In Pregrada, annual precipitation ranges from 900 to 1,100 mm (Klima Hrvatske 2000).

Field Sampling

The fieldwork was conducted between October 1 and 14, 2023, during the period when mature mines from

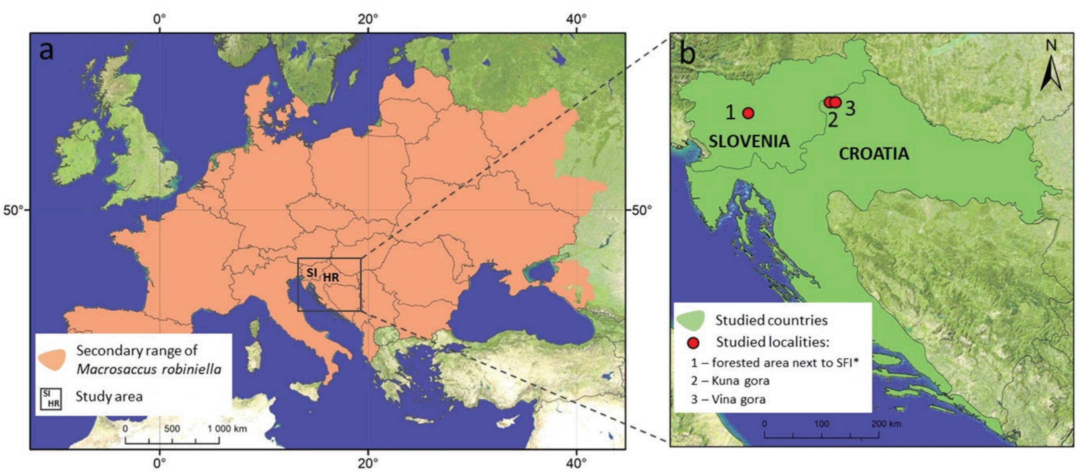

Figure 1. The area of study: **(a)** current schematic distribution of the invasive *Macrosaccus robiniella* in Europe and **(b)** studied localities in Slovenia and Croatia. Legend: *SFI – Slovenian Forestry Institute, Ljubljana.

Figure 2. Sampled locality in Croatia, near Kunagora, 2023: **(a)** *Robinia pseudoacacia* trees attacked by *Macrosaccus robiniella* on both sides of the road; **(b, d)** blotch mines above the midrib (*Parectopa*-like mines); **(c)** *Parectopa robiniella* mines (shown for comparison).

the second, most numerous generation of the moth, were present on the leaves. In the studied localities in Slovenia and Croatia, the populations of *M. robiniella* were noticeably abundant (with >50% of the leaves damaged and a proportion of 0.05 to 0.59 of the leaf compounds per attacked leaf (Table 1), which can also be referred to as a heavy infestation level. We surveyed *R. pseudoacacia* trees growing in open areas, i.e., at forest edges (Figure 2a). A total of thirteen trees aged 3–5 years were examined: eight in Croatia and five in Slovenia. The trees were chosen randomly. On each tree, four branches in the lower part of the tree were examined. All leaves on the branches were counted, and five leaves per branch were haphazardly detached and packed into zip-lock plastic bags for transport to the Department of Forest Protection of the Slovenian Forestry Institute (Ljubljana) for subsequent analysis. In total, 260 compound leaves and approximately 4,050 leaflets were examined across all localities (Figure 2a–d, Table 1). Data from the two countries were pooled, as the moth populations had similar densities (infestation level: >50% of all mined leaves on the sampled trees (Table 1)).

On each leaf, the number of leaflets was counted, and both the upper and lower sides were inspected for the presence and type of *M. robiniella* mines (Figure 3). Four mine types were identified (see Results). When several mine types were found on a single leaf, they were counted according to type, and the occurrence of different mine types on a single leaflet was also recorded. Furthermore, the number of *P. robiniella* mines and their combinations with *M. robiniella* mines were also recorded on individual leaves and leaflets.

Parasitism Documentation

Parasitism was estimated for all mine types, except for upper-side blotch mines situated on one of the leaflet halves, as such mines were rarely observed. Up to 35 mines of the three most common types were randomly selected from the examined leaflets for parasitoid rearing (mine type 1: 34 leaflets; mine type 2: 15 leaflets; mine type 3: 24 leaflets). Only leaflets with a single mine type were

Table 1. Summary of the locations, number of trees and leaves, number of leaf compounds per leaf, and number of *Macrosaccus robiniella* and *Parectopa robiniella* mines on leaf compounds.

Location	Date	Tree ID	Branch ID	Leaf compounds per leaf			*M. robiniella*			Av. proportion of attacked leaf compounds per leaf	*P. robiniella*		
				Av.	Min.	Max.	Av.	Min.	Max.		Av.	Min.	Max.
Croatia, Pregrada	01.10.2023	1	1	18.8	17	21	3.6	2	7	0.19	1.5	1	2
			2	18.0	15	19	4.6	1	11	0.26	0.0	0	0
			3	16.8	14	19	4.4	2	6	0.26	1.3	1	2
		2	1	17.0	13	18	9.6	7	14	0.56	0.0	0	0
			2	18.6	15	21	9.2	1	15	0.49	2.5	2	3
			3	18.0	17	21	7.8	3	18	0.43	0.0	0	0
		3	1	19.0	18	20	9.6	2	18	0.51	3.0	3	3
			2	17.0	15	19	10.0	6	18	0.59	1.5	1	2
			3	16.6	13	19	5.8	2	10	0.35	11.0	1	1
Croatia, Vinagora	14.10.2023	1	1	15.6	14	17	2.0	1	4	0.13	2.0	1	3
			2	20.2	19	21	3.2	1	5	0.16	4.8	1	9
			3	20.6	19	21	2.4	2	3	0.12	7.6	5	12
		2	1	16.2	15	17	2.4	1	5	0.15	3.0	2	5
			2	16.8	14	19	3.8	2	6	0.23	1.3	1	2
			3	17.8	16	19	3.8	2	6	0.21	11.0	1	1
		3	1	18.0	15	19	3.2	1	7	0.18	1.5	1	2
			2	16.4	16	17	1.4	1	2	0.09	2.2	1	4
			3	11.8	10	14	2.2	1	6	0.19	4.0	4	4
		4	1	16.0	15	18	2.4	2	4	0.15	2.3	1	5
			2	16.2	12	19	2.4	0	5	0.15	3.3	1	6
			3	16.4	15	18	4.8	2	8	0.29	3.5	2	5
		5	1	15.2	12	17	3.2	2	5	0.21	1.5	1	3
			2	16.6	13	19	3.0	1	8	0.18	3.0	1	4
			3	17.2	16	19	3.4	1	6	0.20	4.8	3	7
Slovenia, Slovenian Forestry Institute	13.10.2023	1	1	14.0	11	17	2.6	1	6	0.19	0.0	0	0
			2	13.6	10	17	2.0	0	5	0.15	5.0	3	6
			3	12.8	11	16	0.8	0	1	0.06	3.4	2	5
		2	1	10.0	9	11	1.8	1	3	0.18	11.0	1	1
			2	14.4	7	17	1.4	1	2	0.10	1.7	1	2
			3	13.0	9	15	1.4	1	2	0.11	0.0	0	0
		3	1	14.4	12	18	2.0	0	5	0.14	1.7	1	2
			2	14.8	12	18	1.8	1	2	0.12	5.5	4	7
			3	16.8	15	20	1.6	1	2	0.10	3.0	2	5
		4	1	15.2	9	18	0.8	0	2	0.05	2.8	1	6
			2	19.4	17	21	1.2	0	3	0.06	6.8	4	10
			3	16.8	13	21	1.2	1	2	0.07	3.5	1	6
		5	1	13.2	12	17	1.2	0	2	0.09	5	1	9
			2	14.0	12	17	1.6	0	4	0.11	7.5	2	13
			3	19.0	17	20	1.6	0	4	0.08	6.2	1	10

Av. – average, Min. – minimum, Max. – maximum.

used. When multiple mines were present, the target mine, along with a surrounding green area (5–10 mm around the mine), was excised from the leaflet. Leaflets with mines or the excised mines were individually placed in plastic Petri dishes (60 mm in diameter) to allow parasitoid adults to emerge. The dishes with the mines were kept indoors in stable conditions (23°C, 50% humidity) for one month. At the end of this period, the dishes were inspected, and the emerged parasitoids and moths were counted. Parasitoid adults were preserved in 96% ethanol, and moths were pinned for collections. Mines from which nothing emerged were dissected under an Olympus SZ51 stereomicroscope (Tokyo, Japan, Olympus Corporation). In these mines, the number of parasitoid pupae and dead *M. robiniella* larvae or pupae (without signs of parasitism) was counted. When the cause of larval and pupal death was not identified, these individuals were all categorized as “died from unknown causes”.

Data Analysis

We used a contingency table with a Chi-square test to compare differences between the number of mine types with competition compared to the total number of mines, and parasitism (parasitoid emerged, adult moth emerged and unknown cause of death). A Chi-square test was also used to analyze the distribution of mine types with and without competition from *P. robiniella*, compared to a random distribution of mine types. Furthermore, the relative abundance was calculated per average leaf by dividing the number of mines by the number of leaflets and multiplying by 16 (the average number of leaflets per leaf). Differences in relative abundance and mine types were analyzed using a general linear mixed model (GLMM) with a zero-inflated negative binomial error distribution using the “glmmTMB” library (Brooks et al. 2017). Location, tree ID and branch ID were nested and included as random effects. A post hoc Tukey test was conducted using the “emmeans” library (Lenth 2023). The analyses were performed in the R (R Core Team 2022) and PAST (Hammer et al. 2001) statistical programs.

A map showing the current distribution of *M. robiniella* in Europe was created based on the data from Baryshnikova (2019) and De Prins and De Prins (2024), with an update from iNaturalist (2025). Countries where the moth was recorded are indicated in their entirety, except for Sweden, where only the southern part is shaded.

RESULTS

Description of Mine Types

Four mine types were observed in *M. robiniella* in the studied forested areas in Slovenia and Croatia (Table 2).

The lower-side blotch mine, or mine type 1, is an oval tentiform blotch situated on the lower side of the leaflet on one of the leaflet halves (Figure 3a, 3b).

The upper-side blotch mine, or mine type 2, resembles type 1 in appearance (a blotch mine occupying one half of a leaflet) but is situated on the upper side of the leaflet (Figure 3c, 3d). The upper-side midrib mine (*Parectopa*-like mine), or mine type 3, is an elongated blotch located above the midrib on the upper side of the leaflet, causing upward folding of the leaflet in the middle (Figure 3e–h). In terms of its location on the leaflet, this mine type resembles *P. robiniella* damage. Mine type 4 (i.e., lower-side edge mine, or *Obolodiplosis*-like mine) is an elongated blotch situated on the lower side of leaflet next to the leaflet edge, causing strong downward folding and resembling damage

Table 2. Summary of the different mine types in different locations.

Location	Leaf mine type*	Leaf mines				Average number of compounds per leaf
		Av.	Min.	Max.	Total	
Croatia, Pregrada	1	0.47	0	2	21	17.76
	2	0.33	0	4	15	17.76
	3	5.09	0	14	229	17.76
	4	2.18	0	5	94	17.76
Croatia, Vinagora	1	1.93	0	6	82	16.73
	2	0.03	0	2	2	16.73
	3	1.95	0	8	81	16.74
	4	1.15	0	5	86	16.73
Slovenia, Slovenian Forestry Institute	1	0.05	0	1	4	14.76
	2	0.11	0	2	8	14.76
	3	1.12	0	6	84	14.76
	4	0.48	0	2	36	14.76
Total		**0.63**	**0**	**14**	**742**	**16.21**

*Leaf mine types: 1 – lower-side blotch, 2 – upper-side blotch, 3 – upper-side blotch above the midrib (*Parectopa*-like mine), 4 – lower-side edge mine (*Obolodiplosis*-like mine); Av. – average, Min. – minimum, Max. – maximum.

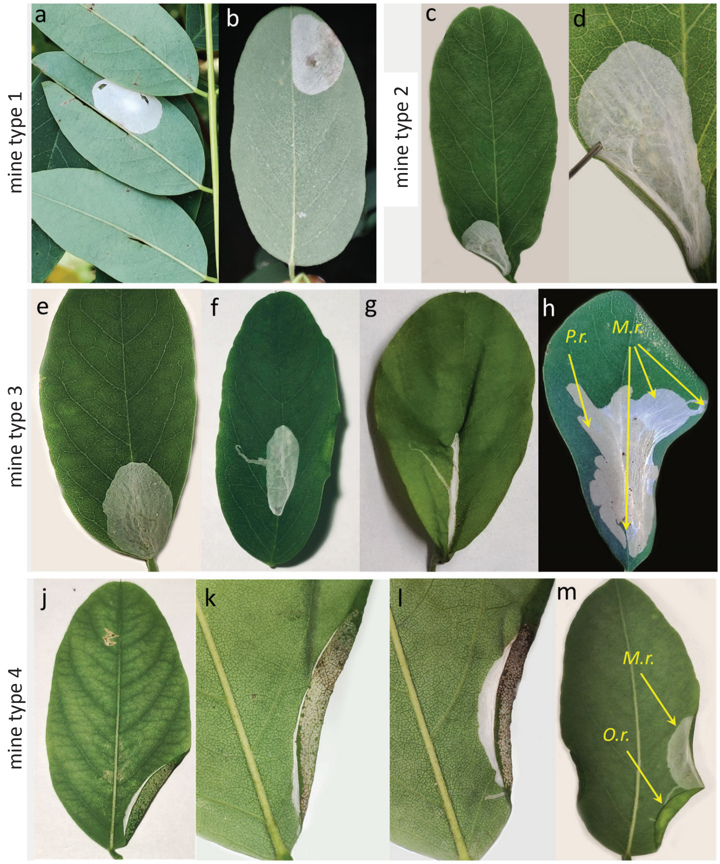

Figure 3. Different leaf mine types of *Macrosaccus robiniella* documented in Slovenia and Croatia: **(a, b)** lower-side blotch mines situated on one of the leaflet halves (mine type 1); **(c, d)** upper-side blotch mine situated on one of the leaflet halves (mine type 2); **(e, f, g)** upper-side mines situated above the midrib, or *Parectopa*-like mines (mine type 3); **(h)** overlapping mines of *M. robiniella* [*M.r.*] and *P. robiniella* [*P.r.*]; **(j, k, l)** lower-side edge mines causing strong downward folding, or *Obolodiplosis*-like mines (mine type 4); **(m)** co-occurrence of *M. robiniella* [*M.r.*] mine and *Obolodiplosis robiniae* [*O.r.*] damage on one leaflet. In figs **l, m**, the mines were slightly unbent.

caused by the invasive North American locust gall midge *O. robiniae* (Figure 3j–m). All mine types often begin with a short epidermal tunnel. However, as the mine grows and transitions into a blotch, the preceding tunnel often becomes indistinguishable.

Abundance of Different Mines

In Croatia and Slovenia, the abundance of leaf mines varied significantly between mine types 1–4 (Figure 4, Chi^2 =262.51, df = 3, p<0.001).

The highest relative abundance (median of 1.99 per leaf) was observed for mine type 3 (*Parectopa*-like mine), followed by mine type 4 (*Obolodiplosis*-like mine) (mean of 1.12 per leaf). Mine types 1 and 2 were the least abundant (mean of 0.53 and 0.14 per leaf, respectively), occurring about four times less frequently than mine types 3 and 4.

Co-occurrence of *M. robiniella* and *P. robiniella* Mines

A significantly higher number of *M. robiniella* mines of all four types were found on leaves without *P. robiniella* compared to those with *P. robiniella*, more than would be expected by random chance (Chi^2=323.96, df=1, p=1.98e-72) (Figure 5).

A similar pattern was observed for mine type 3 (Chi^2=280, df=1, p<0.0001). When comparing the percentage of mine type 3 between the total number of leaflets and leaflets where both species (*M. robiniella* and *P. robiniella*) occurred, no significant difference in ratios was found (Chi^2 = 3.14, df=3, p=0.37) (Figure 6).

However, when focusing solely on mine types on leaflets where both species (*M. robiniella* and *P. robiniella*) co-occurred, there was a significant difference between mine types, which was much greater than would be expected by random chance (Chi^2=50.89; df=3; p<0.0001). Mine type 3 had the highest occurrence (n=35), followed by mine type 4 (n=31), mine type 1 (n=5) and mine type 2 (n=1).

Parasitism in *M. robiniella* Mines

We recorded a statistically lower parasitism rate in mine types 3 and 4 compared to the commonly known mine

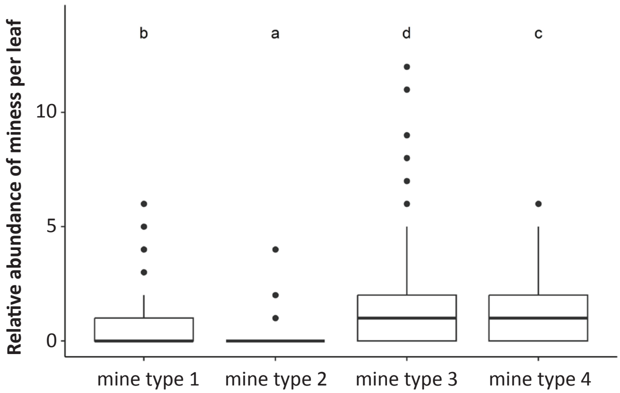

Figure 4. Boxplots showing the relative abundance of mines of different types per average leaf (16 leaflets). Significantly different groups ($p<0.05$) are indicated by different letters above the boxes. The boxplots show the median, first and third quartiles, and the calculated maximum and minimum with extreme values.

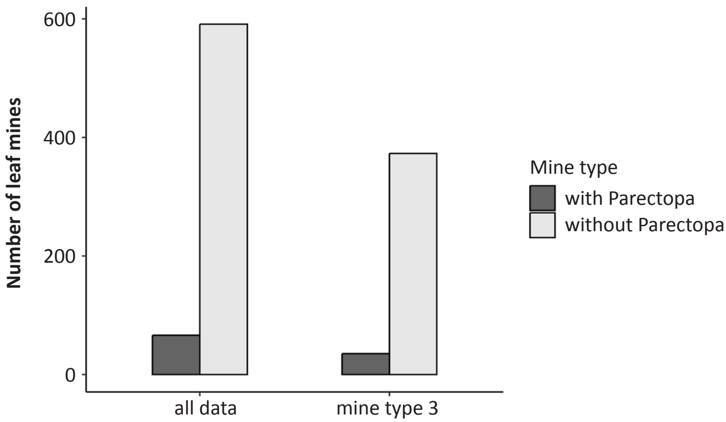

Figure 5. Number of leaflets showing the absence and presence of co-occurrence of mines of *Macrosaccus robiniella* and *Parectopa robiniella* for all data available, including only those with *M. robiniella* mines above the midrib (mine type 3).

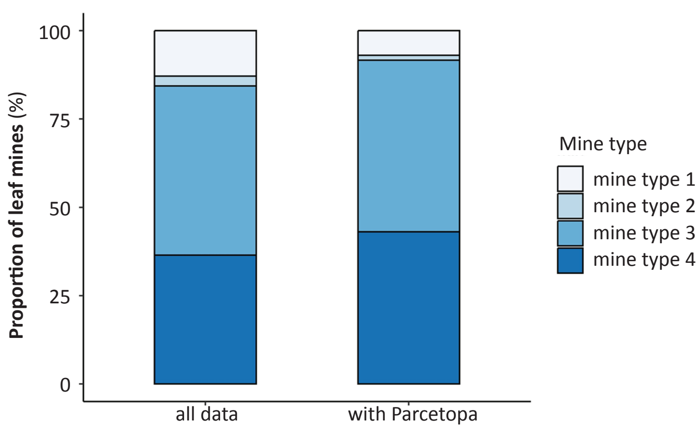

Figure 6. The different positions of *Macrosaccus. robiniella* mines on the leaflet with the number of co-occurrences of *Parectopa robiniella* and *M. robiniella* (N=72) and the total number of sampled leaflets (N=779) based on field observations in Slovenia and Croatia. Leaf mine types: 1 – lower-side blotch, 2 – upper-side blotch, 3 – upper-side blotch above the midrib (*Parectopa*-like mine), 4 – lower-side edge mine (*Obolodiplosis*-like mine).

type 1 (Chi^2=6.96, df=2 p=0.03; Chi^2=17.44, df=2, p=0.0002) (Figure 7). Parasitism in mine types 3 and 4 was about 6–7%, which is 6.5 times lower than in mine type 1. There was no difference in parasitism between mine types 3 and 4 (Chi^2=2.78, df=2, p=0.248).

In total, 16 adult parasitic wasps were recorded in the studied *M. robiniella* mines in Slovenia and Croatia, belonging to two eulophid species: *Pnigalio pectinicornis* (L.) (12 adults) and *Pediobius saulius* (Walker) (4 adults) (Hymenoptera: Eulophidae). Both species were present at a 1:1 ratio in mine type 1 (4 adults each of *Pn. pectinicornis* and *Pe. saulius*). In contrast, only *Pn. pectinicornis* was present in mine type 3 (5 parasitoid adults) and mine type 4 (3 adults).

DISCUSSION

Our study, conducted in forested areas in Slovenia and Croatia, identified four distinct leaf mine types of *M. robiniella* on *R. pseudoacacia*. Until now, this moth was largely known in Europe by its lower-side blotch mine (mine type 1) (Whitebread 1990, Seljak 1995, Maček 1999, Ellis 2024); exceptionally a single case when the moth's larva produced upper side blotch mine situated between the main vein and the leaf edge was recorded in Switzerland (Whitebread 1990). Interestingly, in the studied high-density populations, *M. robiniella* showed a tendency to mine the upper leaf surface rather than create the typical lower-side blotch mines. In such cases, *M. robiniella* predominantly situated mines above the midrib, similar to another invasive leaf-mining moth, *P. robiniella.* However, unlike *P. robiniella,* whose mines remain flat throughout larval development, the midrib mines of *M. robiniella* cause an upward contraction of the leaflet along the midrib, resulting in a volumetric structure that is less accessible from the outside.

Indeed, the larvae of some gracillariid moths, particularly (but not exclusively) representatives of the genera *Phyllonorycter* and *Macrosaccus*, produce silk threads that attach to the epidermis inside their blotch mines. These silk threads dry and cause the epidermis to fold, making the blotch mines somewhat tentiform, or volumetric. This folding creates a larger internal space, preventing the mine from tearing during expansion (Hering 1951, Maček 1999, Davis and De Prins 2011). Furthermore, the tentiform shape appears to reduce accessibility to natural enemies, thereby protecting the larvae or pupae from being parasitized. Our observations suggest that the upper-side midrib mines of *M. robiniella* are more strongly folded compared to the lower-side blotches situated on one of the leaflet halves, likely due to greater silk production on the epidermis inside the mine. Another issue that can make *M. robiniella* to intensively use upper side of leaflets is a strong intraspecific competition as this moth species is known to notably increase population density in the invaded areas (Fodor and Hâruţa 2009).

Another finding is that *M. robiniella* appears to mimic damage caused by the invasive North American locust gall midge *O. robiniae.* The main difference is that the curled leaf edge in *M. robiniella* mines is not thickened, as it is in *O. robiniae*, which induces galls by manipulating the physiology of the host plant's leaf, causing it to curl while the larva feeds beneath the curled leaflet edge (Staszak et al. 2023). In lower-side edge mines, *M. robiniella* larvae feed within the mine, beneath the strongly folded epidermis, causing downward curling of the leaflet edge. This silk-induced folding makes the mine less conspicuous.

Despite creating upper-side midrib mines in areas typically occupied by *P. robiniella*, *M. robiniella* does not show clear evidence of competing with *P. robiniella* for space on leaflets. However, we found that in such mines, the parasitism rate of *M. robiniella* is significantly lower than that in lower-side blotch mines.

From the limited sampling set available to us from Slovenia and Croatia, only two parasitoid species, *Pnigalio pectinicornis* and *Pediobius saulius*, were reared from the leaf mines of *M. robiniella.* Both are generalist parasitoids native to Europe and commonly associated with various native Gracillariidae species (Csóka et al. 2009). Notably,

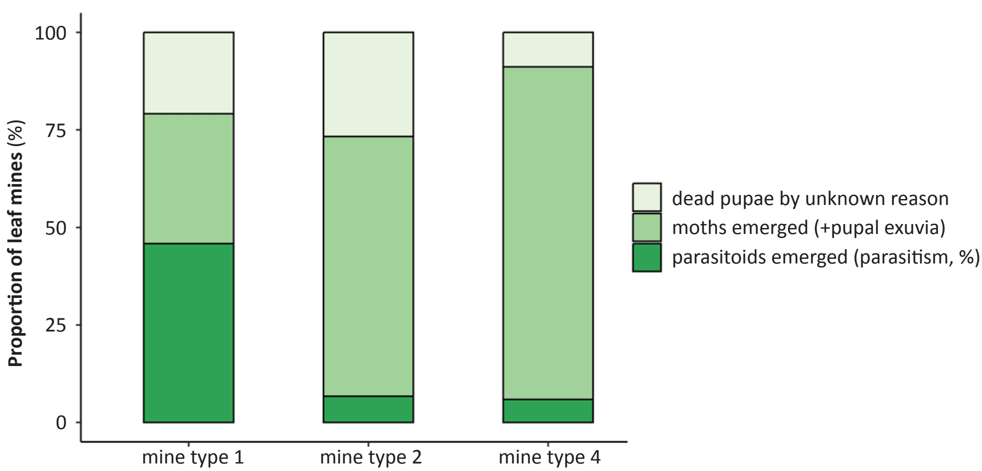

Figure 7. Parasitism of larvae and pupae of *Macrosaccus robiniella* in different leaf mine types. Leaf mine types: 1 – lower-side blotch, 3 – upper-side blotch above the midrib (*Parectopa*-like mine), 4 – lower-side edge mine (*Obolodiplosis*-like mine).

Pn. pectinicornis and *Pe. saulius* were present in lower-side blotch mines, for which *M. robiniella* is commonly known, whereas only *Pn. pectinicornis* was reared from *Parectopa*-like and *Obolodiplosis*-like mines. These two parasitoids exhibit different life strategies: *Pn. pectinicornis* is an ectoparasitoid, while *Pe. saulius* is an endoparasitoid of leaf-mining lepidopteran and coleopteran larvae (Bouček and Askew 1968). Notably, only *Pn. pectinicornis* was found in upper-side midrib and lower-side edge mines, i.e. mines with strongly folded epidermis resulting in mine "closure". Representatives of *Pnigalio* do not lay eggs in the larva but near it or even at some distance (e.g., Duncan and Peña 2000, Bernardo et al. 2008). After hatching, the parasitoid larva makes a hole in the cuticle of the host larva and feeds through it while remaining on the host larva's body. Even if the egg is laid at some distance, the hatched larva actively crawls into the mine to find the host larva and start feeding (Yegorenkova and Yefremova 2012). For *Pe. saulius*, the infection of the host appears to be a more complex procedure. This species needs to inspect the mine surface, locate the host larva or young pupa hidden inside, pierce it and lay eggs inside the host's body (Viggiani 1964). Attacking *Parectopa*-like and *Obolodiplosis*-like mines could be an energy-consuming task for *Pe. saulius*, if realizable at all, which may explain why we did not record this parasitoid in strongly contracted mines of *M. robiniella*. This suggests that *M. robiniella* may avoid attacks of parasitoids (at least some of them) that it recruited from native gracillariids in Europe.

In Europe, at least 37 hymenopteran species of parasitoids from five families (Braconidae, Chalcididae, Encyrtidae, Eulophidae, Pteromalidae) are known to attack *M. robiniella* (Davis and De Prins 2011, De Prins and De Prins 2024). In Hungary, where the parasitoid communities of *M. robiniella* and *P. robiniella* have been extensively studied, it was shown that all parasitoid species were recruited by these two alien moths from native Gracillariidae, mostly those associated with oaks (Csóka et al. 2009). Interestingly, the parasitoid community of *M. robiniella* is slightly richer than that of *P. robiniella* (19 vs. 12 parasitoids), and parasitism rates are higher for *M. robiniella* (47% vs. 15%, respectively) (Csóka et al. 2009). An early study in Croatia showed that both North American leaf miners are attacked by seven European generalist parasitoid species with slight differences in parasitoid composition, with significantly higher parasitism in *M. robiniella* (Matošević and Melika 2012). This suggests that, after invading Europe, *M. robiniella* may have encountered increased parasitism compared to *P. robiniella*. The heightened pressure from parasitism may have driven *M. robiniella* to develop adaptations aimed at locating enemy-free space and evading parasitoid attacks. Notably, the North American locust gall midge *O. robiniae* does not share parasitoids with gracillariids and is only attacked by the specialized parasitoid *Platygaster robiniae* (Hymenoptera: Platygastridae), which is believed to have arrived in Europe with its host from North America (Buhl and Duso 2008) and does not parasitize gracillariids. Thus, mimicking *O. robiniae* damage could be beneficial for *M. robiniella*.

Interestingly, in Europe, *M. robiniella* has been known solely for its lower-side blotch mines (Whitebread 1990, Seljak 1995, Maček 1999, Davis and De Prins 2011). Matošević (2007) conducted a detailed study of the biology, ecology and parasitoids of *M. robiniella* in Croatia between 2004 and 2006 and observed exclusively lower-side blotch mines, with no upper-side midrib mines recorded. In contrast, in the native range (North America), *M. robiniella* has also been documented to produce upper-side midrib mines (*Parectopa*-like mines) (Braun 1908, Weaver and Dorsey 1967) and *O. robiniae*-like mines.

Our study has several limitations. The survey was conducted only in forested areas and only in two countries within the *M. robiniella*-invaded range, and it is possible that different mine types may also occur in urbanized areas, including in other European countries. Only high-density populations were considered, and thus, it remains unclear how low-density populations of this invasive moth behave in Europe. The number of parasitoid individuals reared from different mine types of *M. robiniella* was rather small, resulting in the identification of only two eulophid species, whereas the parasitoid assemblage of *M. robiniella* in the invaded range is much larger (Csóka et al. 2009, Matošević and Melika 2012, De Prins and De Prins 2024). Further studies involving larger samples of leaf mines and parasitoid rearing over multiple years are needed to confirm *M. robiniella*'s strategy of seeking refuge from parasitoids by exploiting different parts of leaflets for mining. Finally, it would be important to clarify whether constructing alternative mines (mimicking the damage of other endophagous insects) is a strategy observed over time in the invaded range or whether it is also evolving in the native range (some parts of North America). Long-term documentation of damage patterns would help elucidate the life history and evolutionary adaptations of *M. robiniella* in both its native and invaded ranges. Citizen science platforms, such as iNaturalist, which store photographs of leaf mines from both continents over time, could provide valuable data for exploring this question.

CONCLUSIONS

Four leaf mine types were described from highly dense populations of the North American leaf-miner *Macrosaccus robiniella* in Slovenia and Croatia in 2023. Mine type 1 (i.e., lower-side blotch occupying one of the leaflet halves) is the damage by that the species was known since its first detection in Europe. Mine type 2 is similar to mine type 1, but the mine is located upper side of one of the leaflet halves. Mine type 3 (blotch above the midrib on the upper side of the leaflet) and 4 (narrow blotch at the edge, causing strong downward folding) resemble the damage of two other invasive insects from North America, i.e., the gracillariid *Parectopa robiniella* and the gall midge *Obolodiplosis robiniae*, respectively. The mine types 1 and 2 were least present in the studied populations. Mine types 3 and 4 were up to four times more abundant than mine types 1 and 2. Parasitism rate was 6.5 times lower in mine types 3 and 4 compared to mine type 1.

Author Contributions

NIK, SG and MdG conceived and designed the research, NIK and SG carried out the field measurements, NIK and OVK performed laboratory analysis, NIK and MdG processed the data and performed the statistical analysis, NIK, MdG and BP secured the research funding, supervised the research and helped to draft the manuscript, NIK, SG, OVK, DM, BP, and MdG wrote the manuscript.

Funding

The study was supported by the core research group Forest Biology, Ecology and Technology (P4-0107) of the Slovenian Research Agency and the Administration for Food Safety, Veterinary Sector and Plant Protection at the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia [field work], as well as by the Russian Science Foundation (grant No. 22-16-00075-P) [parasitism assessment].

Acknowledgments

The authors thank Jan Nagel for carefully checking the English in the manuscript and for suggesting improvements.

Conflicts of Interest

The authors declare no conflict of interest.

REFERENCES

Baryshnikova SV, 2019. Gracillariidae. In: Sinev SYu (ed.) Catalogue of the Lepidoptera of Russia, Edition 2.Zoological Institute RAS, St. Petersburg, Russia, pp. 36-43.

Bernardo U, Monti MM, Nappo AG, Gebiola M, Russo A, Pedata PA, Viggiani G, 2008. Species status of two populations of *Pnigalio soemius* (Hymenoptera: Eulophidae) reared from two different hosts: an integrative approach. *Biocontrol* 46(3): 293-303. https://doi.org/10.1016/j.biocontrol.2008.05.009.

Bouček Z, Askew RR, 1968. Palaearctic Eulophidae excl. Tetrastichinae. *Index of Entomophagous Insects* 3: 1-260.

Braun AF, 1908. Revision of the North American species of the genus *Lithocolletis* Hübner. *T Am Entomol Soc* 34: 269-357.

Brooks ME, Kristensen K, van Benthem KJ, Magnusson A, Berg CW, Nielsen A, Skaug HJ, Maechler M, Bolker BM, 2017. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *The R Journal* 9(2): 378-400. https://doi.org/10.32614/RJ-2017-066.

Buhl PN, Duso C, 2008. *Platygaster robiniae* n. sp. (Hymenoptera: Platygastridae) parasitoid of *Obolodiplosis robiniae* (Diptera: Cecidomyiidae) in Europe. *Ann Entomol Soc Am* 101(2): 297-300. https://doi.org/10.1603/0013-8746(2008)101[297:PRNSHP]2.0.CO;2.

Catford JA, Bode M, Tilman D, 2018. Introduced species that overcome life history tradeoffs can cause native extinctions. *Nat Commun* 9: 2131. https://doi.org/10.1038/s41467-018-04491-3.

Csóka G, Pénzes Z, Hirka A, Mikó I, Matošević D, Melika G, 2009. Parasitoid assemblages of two invading black locust leaf miners, *Phyllonorycter robiniella* and *Parectopa robiniella* in Hungary. *Period Biol* 111(4): 405-411.

Davis D, De Prins J, 2011. Systematics and biology of the new genus *Macrosaccus* with descriptions of two new species (Lepidoptera, Gracillariidae). *Zookeys* 98: 29-82. https://doi.org/10.3897/zookeys.98.925.

De Prins J, De Prins W, 2024. Global Taxonomic Database of Gracillariidae. *Macrosaccus robiniella* (Clemens, 1859). Available online: https://www.gracillariidae.net/species/1549 (20 December 2024).

Duncan RE, Peña JE, 2000. Fecundity, host stage preferences and the effects of temperature on *Pnigalio minio* (Hymenoptera: Eulophidae), a parasitoid of *Phyllocnistis citrella* (Lepidoptera: Gracillariidae). *Proc Flo State Hort Soc* 113: 20-24.

Duso C, Fontana P, Tirello P, 2005. Spread of the gall midge *Obolodiplosis robiniae* (Haldeman) injurious to black locust in Italy and Europe. *Inf Fitopatol* 5: 30-33.

Duso C, Skuhravá M, 2004. First record of *Obolodiplosis robiniae* (Haldeman) (Diptera Cecidomyiidae) galling leaves of *Robinia pseudoacacia* L. (Fabaceae) in Italy and Europe. *Frustula Entomol* 25(38): 117-122.

Ellis WN, 2024. Plant Parasites of Europe leafminers, galls and fungi. Available online: https://bladmineerders.nl (20 December 2024).

Fodor E, Hâruţa O, 2009. Niche partition of two invasive insect species, *Parectopa robiniella* (Lepidoptera; Gracillariidae) and *Phyllonorycter robiniella* (Clem.) (Lepidoptera: Gracillariidae). *Res J Agric Sc* 41(2): 261-269.

Gomboc S, Kirichenko NI, 2022. An overview of Gracillariidae leaf mining moths in Slovenia with new records for the country. *Diversity* 14(10): 811. https://doi.org/10.3390/d14100811.

Gulzar R, Hamid M, Hassan T, Rashid I, Khuroo AA, 2024. Different sets of traits determine transition of alien species along the invasion continuum. *Sci Total Environ* 912: 169137. https://doi.org/10.1016/j.scitotenv.2023.169137.

Hammer O, Harper DAT, Ryan PD, 2001. PAST: Paleontological Statistics software package for education and data analysis. *Palaeontol Electron* 4(1): 1-9. http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

Hering EM, 1951. Biology of the leaf miners. Berlin, Germany, 420 p. https://doi.org/10.1007/978-94-015-7196-8.

iNaturalist, 2025. *Macrosaccus robiniella*. Available online: https://www.inaturalist.org/taxa/508151-Macrosaccus-robiniella (20 January 2025).

Izvješće o stanju okoliša Krapinsko-Zagorske županije 2014-2017 godine, 2018. Krapinsko-zagorska županija Upravni odjel za prostorno uređenje, gradnju i zaštitu okoliša. Bureau Veritas Croatia, Croatia, Zagreb. Available online: https://kzz.hr/dokumenti/izvjesce-o-stanju-okolisa-kzz-2014-2017-godine/ (20 December 2024). [in Croatian].

Jeffries MJ, Lawton JH, 1984. Enemy free space and the structure of ecological communities. *Biol J Linn Soc* 23: 269-286. https://doi.org/10.1111/j.1095-8312.1984.tb00145.x.

Klima Hrvatske, 2000. Državni hidrometeorološki zavod. Available online: https://meteo.hr/klima.php?section=klima_hrvatska&param=k1_7 (15 December 2024).

Lenth R, 2023. 'emmeans': estimated marginal means, aka least-squares means. R package version 1.9.0. Available online: https://CRAN.R-project.org/package=emmeans (22 December 2024).

Maček J, 1984. Robinijev listni zavrtač (*Parectopa robiniella* Clemens) nov škodljivec drevesnih vrst Slovenije. *Gozdarski Vestnik* 42(2): 73-75. [in Slovenian].

Maček J, 1999. Hiponomološka favna Slovenije. Slovenska akademija znanosti in umetnosti, Razred za naravoslovne vede, Classis IV: Historia naturalis, 37. [in Slovenian].

Maceljski M, Igrc J, 1983. *Parectopa robiniella* Clemens – novi štetni insekt nearktičnog porijekla u Jugoslaviji – prethodno saopćenje. *Zašt Bilja* 34(3): 427-430. [in Slovenian].

Matošević D, 2007. Lisni mineri drvenastog bilja u Hrvatskoj i njihovi parazitoidi. PhD Thesis, Faculty of Forestry and Wood Technology, University of Zagreb, Zagreb, Croatia, 195 p. [in Croatian].

Matošević D, Melika G, 2012. Diversity of parasitoid assemblages of native and alien leaf miners in Croatia. *Sumar List* 7-8: 367-376. [in Croatian].

Medzihorský V, Mally R., Trombik J., Turčáni M, Medzihorská M, Shoda-Kagaya, Martin GD, Sopow S, Kochi K, Liebhold AM, 2024. The demise of enemy release associated with the invasion of specialist folivores on an invasive tree. *Ecography* 2024(5): e07082. https://doi.org/10.1111/ecog.07082.

Mešić M, Maceljski A, 2001. *Phyllonorycter robiniella* Clemens a new insect pest in Croatia. *Agriculturae Conspectus Scientificus* 66(4): 225-230. https://acs.agr.hr/acs/index.php/acs/article/view/285.

Pernek M, Matošević D, 2009. Bagremova muha šiškarica (*Obolodiplosis robiniae*) – novi štetnik bagrema i prvi nalaz parazitoida *Platygaster robiniae* u Hrvatskoj. *Sumar List* 3-4, CXXXIII: 157-163. [in Croatian].

R Core Team, 2022. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available online: https://www.R-project.org/ (17 December 2024).

Seljak G, 1995. *Phyllonorycter robiniella* (Clemens), another new Lithocolletidae of the *Robinia* in Slovenia. *Gozdarski Vestnik* 53: 78-82. [in Slovenian]

Staszak AM, Ratajczak E, Leśniewska J, Piotrowska-Niczyporuk A, Kostro-Ambroziak A, 2023. A broad spectrum of host plant responses to the actions of the gall midge: case study of *Robinia pseudoacacia* L. and *Obolodiplosis robiniae* (Haldeman). *BMC Plant Biol* 23: 19. https://doi.org/10.1186/s12870-022-03914-0.

Slovenian Environment Agency, 2025. Slovenia. Available online: https://meteo.arso.gov.si/met/sl/climate/diagrams/ljubljana/ (23 January 2025).

Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ, Kuris AM, 2003. Introduced species and their missing parasites. *Nature* 421: 628-630. https://doi.org/10.1038/nature01346.

Viggiani G, 1964. Contributi alla conoscenza degly insetti fitophagi minatori e loro simbionti. IV. Morpho-biologia del *P. saulius* Walk. (Hym. Eulophidae) e considerazioni sulle altre specie congeneri europee. *Boll Lab Entomol Agrar Portici [ZDB]* 22: 205-244. [in Italian].

Vítková M, Müllerová J, Sádlo J, Pergl J, Pyšek P, 2017. Black locust (*Robinia pseudoacacia*) beloved and despised: a story of an invasive tree in Central Europe. *Forest Ecol Manag* 384: 287-302. https://doi.org/10.1016/j.foreco.2016.10.057.

Weaver JE, Dorsey CK, 1967. Larval mine characteristics of five species of leaf-mining insects in black locust, *Robinia pseudoacacia*. *Ann Entomol Soc Am* 60(1): 172-186. https://doi.org/10.1093/aesa/60.1.172.

Whitebread SE, 1990. *Phyllonorycter robiniella* (Clemens, 1859) in Europe (Lepidoptera, Gracillariidae). *Nota Lepidopterologica* 12(4): 344-353.

Xirocostas ZA, Ollerton J, Tamme R, Peco B, Lesieur V, Slavich E, Junker RR, Pärtel M, Raghu S, Uesugi A, Bonser SP, Chiarenza GM, Hovenden MJ, Moles AT, 2023. The great escape: patterns of enemy release are not explained by time, space or climate. *P Roy Soc B-Biol Sci* 290: 20231022. https://doi.org/10.1098/rspb.2023.1022.

Yegorenkova E, Yefremova Z, 2012. The preimaginal stages of *Pnigalio gyamiensis* Myartseva & Kurashev, 1990 (Hymenoptera, Eulophidae), a parasitoid associated with *Chrysoesthia sexguttella* (Thunberg) (Lepidoptera, Gelechiidae). *Zookeys* 214: 75-89. https://doi.org/10.3897/zookeys.214.3266.