DNA barcoding of the endemic Polish populations of the genus *Reisseronia* Sieder, 1956 (Lepidoptera, Psychidae)

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Abstract. The genus *Reisseronia* Sieder, 1956 (Lepidoptera, Psychidae, Epichnopteriginae) comprises 18 species, which are usually distributed in a limited number of very small areas in Europe and the Middle East. The genus *Reisseronia* was first reported in Poland in 2005; after detailed investigations, the Polish populations were described as separate species, namely *R. imielinella* Malkiewicz, Sobczyk & Larysz, 2013 and *R. annae* Larysz, 2017. Later, another population of bagworm moths belonging to the genus *Reisseronia* was found in Komańcza (Bieszczady Mountains). We carried out the first genetic studies of these parthenogenetic species from Poland. Sequence analysis of the DNA barcode region of the mitochondrial cytochrome *c* oxidase subunit I (COI) revealed slight differences between three Polish populations of the genus *Reisseronia*.

Introduction

The genus *Reisseronia* Sieder, 1956 (Lepidoptera, Psychidae, Epichnopteriginae) comprises 18 species distributed throughout Europe and the Middle East (Turkey) (Sobczyk and Werno 2021). Until 2000, only nine species of this genus were known; the remaining nine have been described in recent years. The genus is characterised by brachypterous females and parthenogenetic reproduction, which has been reported in three species. The distribution is often endemic and restricted to very small areas (Sobczyk and Werno 2021).

The genus *Reisseronia* was reported for the first time in Poland in 2005, when several psychid larvae were collected in Imielin near Katowice in Upper Silesia (Larysz 2007). The collected specimens were classified as *Reisseronia gertrudae* Sieder, 1962. However, due to some differences in morphology in the adults, pupae and life history, the specimens were described as a separate species, namely *R. imielinella* Malkiewicz, Sobczyk & Larysz, 2013 (Malkiewicz et al. 2013) (Fig. 1a). In the following years, another population was found in the Janów district of Katowice (Upper Silesia). The specimens were described as a new species, *R. annae* Larysz 2017 (Fig. 1b), based on morphological differences in the imagines, pupae and female genitalia (Larysz 2017). Another population of an unidentified species of *Reisseronia* was found in 2017 in Komańcza (Bieszczady Mountains) (Fig. 1c). In all three cases, the occurrence was limited to very small areas and parthenogenesis was confirmed.

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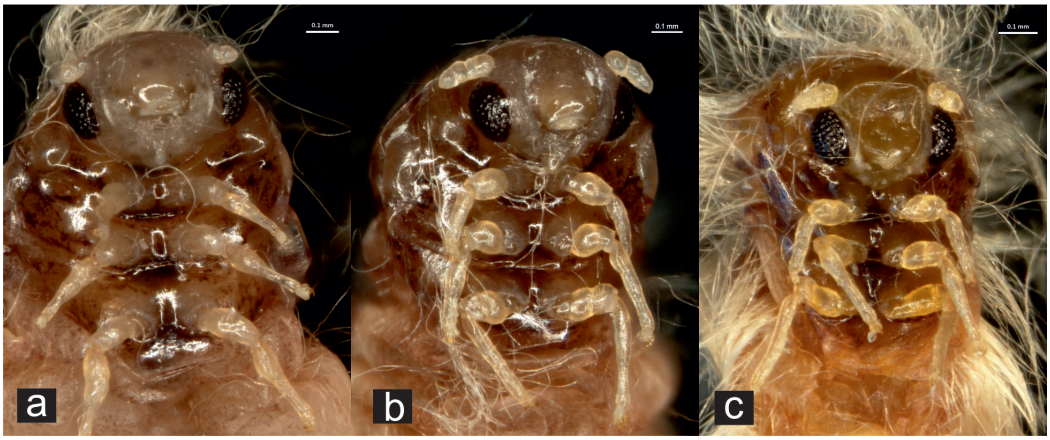

Figure 1. Head and thorax ventral view of females. **a**. *Reisseronia imielinella* (Imielin near Katowice); **b**. *Reisseronia annae* (Katowice); **c**. *Reisseronia sp.* (Komańcza, Bieszczady Mountains). Scale bar: 0.1 mm.

Due to the lack of molecular data on this genus from Poland, we decided to perform a molecular analysis based on DNA barcode sequences of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene. We used the resulting data to better understand the relationships within the Polish members of the genus *Reisseronia* and their relationships within the other members of this genus.

Materials and methods

Sample collection

The examined material (larval cases with mature larvae or pupae) was collected in three locations in Poland (see Table 1 for details). All used samples were removed from larval cases and placed in 96% ethanol.

DNA extraction and amplification

The total genomic DNA was extracted from thorax muscle tissues using a Sherlock AX kit (A&A Biotechnology, Gdańsk, Poland) following the manufacturer's recommended protocol. The polymerase chain reaction (PCR) amplifications were performed using 25 µl of ready-to-use PCR Mix Plus (A&A Biotechnology), 2 µl of template DNA, 1 µl of each primer (10 µM) and ultrapure water to a final volume of 50 µl. The COI gene was amplified using the primers LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and HCO2198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer et al. 1994). The PCR conditions as proposed by Elzinga et al. (2013) involved denaturation at 95 °C for 3 min; 30 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s; and a final extension at 72 °C for 5 min. PCR product purification and sequencing were performed at Genomed S.A. (Warsaw, Poland). The obtained sequences were deposited in GenBank under accession numbers PP766000–PP766010.

Molecular methods

The sequences were aligned using ClustalW (with default parameters) in the MEGA 11 software (Tamura et al. 2021). The genetic distance between individuals was calculated using the Kimura two-parameter model (K2P) in MEGA 11 software. A Neighbor-Joining (NJ) tree was constructed

Table 1. Collecting data, voucher ID and NCBI GenBank accession numbers of analysed specimens.

No.	Species	Collecting data	Voucher	Acession number
1.	*Reisseronia annae*	Poland, Katowice-Janów, 50°15'14"N, 19°04'56"E, 270 m, 7.v.2017, A. Larysz leg. (larva L4)	Ra 1	PP766003
2.	*Reisseronia annae*	Poland, Katowice-Janów, 50°15'14"N, 19°04'56"E, 270 m, 7.v.2017, A. Larysz leg. (larva L4)	Ra 2	PP766004
3.	*Reisseronia annae*	Poland, Katowice-Janów, 50°15'14"N, 19°04'56"E, 270 m, 7.v.2017 (emerged 27.v.2017), A. Larysz leg.	Ra 3	PP766005
4.	*Reisseronia annae*	Poland, Katowice-Janów, 50°15'14"N, 19°04'56"E, 270 m 7.v.2017, A. Larysz leg. (larva L4)	Ra 4	PP766006
5.	*Reisseronia imielinella*	Poland, Imielin, 50°08'48"N, 19°11'08"E, 260 m, 6.vi.2020 (emerged 22.vi.2020), A. Larysz leg.	Ri 1	PP766007
6.	*Reisseronia imielinella*	Poland, Imielin, 50°08'48"N, 19°11'08"E, 260 m, 6.vi.2020. (emerged 23.vi.2020), A. Larysz leg. (larva L4)	Ri 2	PP766008
7.	*Reisseronia imielinella*	Poland, Imielin, 50°08'48"N, 19°11'08"E, 260 m, 6.vi.2020, A. Larysz leg. (larva L4)	Ri 3	PP766009
8.	*Reisseronia imielinella*	Poland, Imielin, 50°08'48"N, 19°11'08"E, 260 m, 6.vi.2020, A. Larysz leg. (larva L4)	Ri 4	PP766010
9.	*Reisseronia sp.*	Poland, Komańcza, 49°20'12"N, 22°04'16"E, 470 m, 11.vii.2017 (emerged 21.iii.2018), A. Larysz leg.	RK	PP766000
10.	*Reisseronia sp.*	Poland, Komańcza, 49°20'12"N, 22°04'16"E, 470 m, 11.vii.2017 (emerged 20.iii.2018), A. Larysz leg.	RK 1	PP766001
11.	*Reisseronia sp.*	Poland, Komańcza, 49°20'12"N, 22°04'16"E, 470 m, 28.v.2017, A. Larysz leg. (larva L4)	RK 2	PP766002

using MEGA 11 based on the K2P model for nucleotide substitutions. An additional 19 COI freely available sequences of various *Reisseronia* obtained from the BOLD System (accessed: 1st of May 2025) were used to better understand the taxonomy position of the specimens from Poland. The detailed information is provided in the Table 2.

Results

The comparison with other *Reisseronia* species revealed no distinct differences between *R. imielinella* and *R. annae* as well as *R. gertrudae* and population from Komańcza. A close relationship with *Reisseronia tarnierella* (Bruand, 1851) is also noticeable (Fig. 2).

The pairwise genetic distances between *Reisseronia* species, generated using the Kimura two-parameter method, are presented in Suppl. material 1. The pairwise divergence between these two species and the population from Komańcza and from *R. gertrudae* is only 0.9%. Surprisingly, no differences were noted between the specimens from Komańcza and the *R. gertrudae* sequence. The distance to other members of the genus *Reisseronia* ranges from 3.4% for *R. tarnierella* (BOLD ID: POESE455-22) to 13.7% for two undetermined species from Greece (BOLD ID: PHLAI1016-14 and LEATC042-13) (Suppl. material 1).

Discussion

DNA barcoding based on genetic variation of a 658 base pair (bp) fragment of the 5′ end of the mitochondrial COI gene is a widely used tool for species discrimination. However, the DNA barcoding thresholds for species delimitation are variable. On the one hand, many studies have

Table 2. List of additional species of *Reisseronia* obtained from the BOLD System and used in this study.

No.	Species	BOLD ID:	Locality:
1.	*Reisseronia gertrudae*	TIPSY895-19	Slovenia: Notranjska
2.	*Reisseronia magna*	POESE054-15	Greece: Peloponnese, Gorani
3.	*Reisseronia malickyi*	LPALE1656-23	Greece: Crete
4.	*Reisseronia malickyi*	LEASX093-21	Greece: Crete
5.	*Reisseronia nigrociliella*	POESE059-15	Bulgaria: Sandanski, Liljanovo
6.	*Reisseronia satanella*	LEAST1534-18	Italy
7.	*Reisseronia* sp.	POESE453-22	Greece: Attica, Elafonisos
8.	*Reisseronia* sp.	POESE452-22	Greece: Ionian Islands, Kefalonia; Andriolata church
9.	*Reisseronia* sp.	LEATC041-13	Greece
10.	*Reisseronia* sp.	LEATC040-13	Greece
11.	*Reisseronia* sp.	POESE056-15	Greece: Ithaki, Stavros
12.	*Reisseronia* sp.	POESE053-15	Greece: Kefalonia, Enos
13.	*Reisseronia* sp..	PHLAI1017-14	Greece: Ionian Islands
14.	*Reisseronia* sp.	PHLAI1016-14	Greece
15.	*Reisseronia* sp.	LEATC042-13	Greece
16.	*Reisseronia tarnierella*	LON6875-18	Croatia: Zadar County
17.	*Reisseronia tarnierella*	GBLAB1717-14	Germany: Saarland
18.	*Reisseronia tarnierella*	TIPSY893-19	Slovenia: Kraski rob
19.	*Reisseronia tarnierella*	POESE455-22	Serbia: Surdulica, Vlasina

determined that the threshold for K2P genetic distance between two species is 2–3% (Hebert et al. 2003; Hajibabaei et al. 2006; Ross et al. 2008; Strutzenberger et al. 2012; Zahiri et al. 2014). On the other hand, values lower than 2% are typical for intraspecific variation or young sister-species (Huemer and Hausmann 2009; Hausmann et al. 2011; Mutanen et al. 2012; Dincă et. 2013). There are also a number of reports suggesting that species identification based solely on COI barcoding may be inaccurate. This problem may be caused by mitochondrial genome introgression, which is also present in Lepidoptera (Jiggins 2003; Zakharov et al. 2009; Mutanen et al. 2016; Cong et al. 2017), or by the occurrence of nuclear mitochondrial pseudogenes (Song et al. 2008). Therefore, based on the DNA barcoding approach only (K2P 2% threshold), we are dealing with a single *Reisseronia* species in Poland, which belongs to the parthenogenetic species *R. gertrudae* (TL: Austria, South Styria, Kitzeck in Sausal), whose wider distribution was recently confirmed (Predovnik et al. 2020). Moreover, the differentiation of closely related species complexes based only on DNA barcode COI sequences can be ambiguous, as reported in some cases of lepidopterans (van Nieukerken et al. 2012; Alipanah et al. 2022).

The differences in the female genitalia, leg morphology, antennae, pupae and pupal head plates suggest the validity of their species status. Additionally, a lack of correlation between morphological variability and variability in mitochondrial DNA sequences has been found in many Lepidoptera (e.g. Kato and Yagi 2004; Vandewoestijne et al. 2004; Korb et al. 2016; Domagała and Lis 2022). In the case of butterflies and moths, attention is paid to the variability of the wing pattern, but it cannot be excluded that the variability of other anatomical features is not reflected in the mtDNA sequence – for example, intraspecific variation in genitalia is known in several lepidopteran species (e.g. Mutanen and Kaitala 2006; Goulson 2008).

The correct classification of bagworm moths can be difficult. In the case of males, morphological identification is in many cases based on the morphometrics of the genitalia and analysis of the

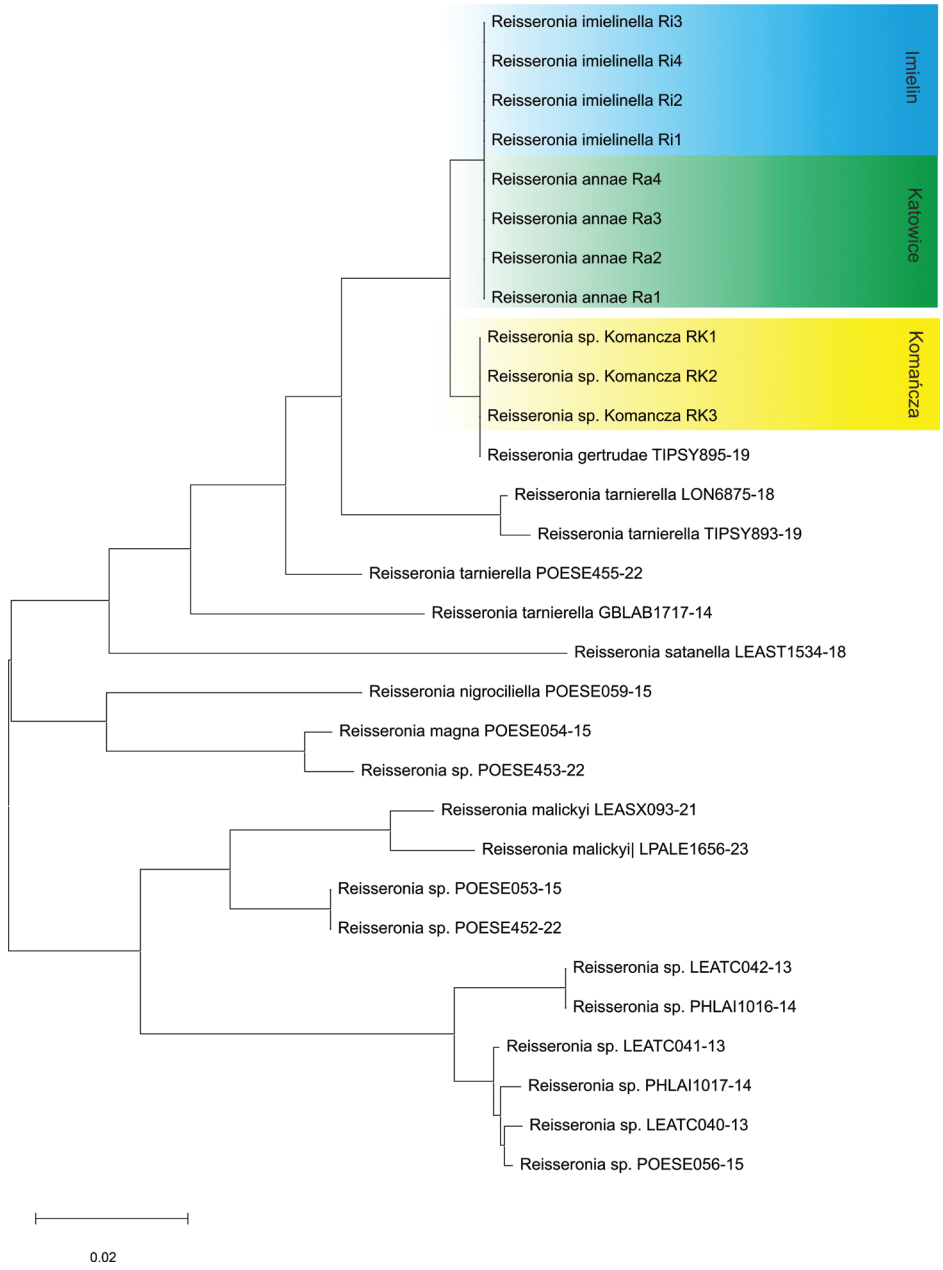

Figure 2. An unrooted Neighbor-joining tree, generated under the Kimura 2-parameter model, for the species of genus *Reisseronia*. Branch lengths represent the number of substitutions per site as percentage. The specimens from Poland are shade in colour.

shape of the wing scales, but the identification of females is more difficult due to the lack of wings (Chevasco et al. 2014). According to Chevasco et al. (2014), molecular methods are an effective way to delimit species of bagworm moths, but they should be combined with the analysis of geometric

morphometrics of male genitalia. On the one hand, in the case of the Polish members of *Reisseronia*, parthenogenesis has been confirmed, and so far, only females are known. On the other hand, according to Mutanen et al. (2012), a compromise approach should be applied for species delimitation, including cases exceeding the DNA barcoding threshold (e.g., arbitrarily 2%) but also indicating differences in ecological and morphological characters or differences in an unrelated nuclear markers.

Conclusion

In conclusion, our preliminary study based on a small number of individuals showed slight differences in COI sequences among three Polish populations of *Reisseronia*. At present, on the one hand we cannot exclude that we have a single *Reisseronia* species in Poland (*Reiseronia gertrudae*). On the other hand, we are dealing with parthenogenetic populations where the analysis of maternally inherited COI may be ambiguous. We will refrain from making any taxonomic decisions until we carry out additional analyses in the future. Whole-genome analyses will ultimately provide a better understanding of the relationships between these species as well as other *Reisseronia* members.

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Supplementary material 1

The pairwise genetic distances among *Reisseronia* species generated using the Kimura-2-Parameter method

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Data type: docx

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