



# First insights into the ectoparasitic helminth fauna of freshwater fish on an Adriatic island

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## Short Communication

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### Abstract

Surface flow of freshwater on Adriatic islands is rare due to the extreme permeability of the karst terrain. Hence, most helminthological studies of freshwater fishes in the Adriatic drainage have focused on mainland freshwater systems, while data from islands are scarce. We collected minnow, *Phoxinus lumaireul* (Schinz, 1840), specimens in the Suha Ričina stream on Krk Island and screened them for helminth ectoparasites. Identification of metacercariae cysts (black spots) was carried out by sequencing part of the 28S rDNA gene, and a single monogenean worm was identified by sequencing part of the internal transcribed spacer 2 (ITS2). To estimate the level of infection, the number of black spots was counted to calculate prevalence, mean abundance, and mean intensity. Phylogenetic inference based on maximum likelihood and Bayesian inference revealed a rare black spot disease taxon, genus *Uvulifer*, which represents one of the first records in Europe on fish (second intermediate host), a first record from a *Phoxinus* host, a first record for the Adriatic drainage and Croatia, and a first record for Southern Europe in general. Furthermore, the monogenean was identified as *Gyrodactylus* sp., potentially representing a new species.

## Introduction

Platyhelminth parasites of fish comprise the Trematoda (Digenea and Apidogastrea), the Monogenea and Cestoda (tapeworms and allies), with monogeneans and trematodes being responsible for the majority of infections (Cribb *et al.* 2002). Of these taxa, the majority of monogeneans and larvae of some digeneans are ectoparasites on fins, skin, or gills. Monogenea have a direct life cycle without intermediate hosts (Bakke *et al.* 2007), with *Gyrodactylus* von Nordmann, 1832, being one of the most common genera infecting freshwater fishes, while digenean trematodes have complex life cycles involving a series of hosts (Klinger and Floyd 1998). Diplostomidae Poirier, 1866, is a family of digenean trematodes infecting freshwater snails and fish as intermediate hosts, and mammals and fish-eating birds as definitive hosts (Niewiadomska 2002). Metacercarial stages of some diplostomid species are known for inducing black spot disease, a common syndrome among freshwater fish (Post 1983), easily recognized by melanin cysts formed around the parasite (Niewiadomska 2002). With the introduction of allochthonous fishes to European freshwater systems, their parasites have also been introduced, including taxa that induce black spot disease in fishes (Stoyanov *et al.* 2017). So far, helminthological studies of freshwater fish in the Adriatic drainage have been focused on mainland freshwater ecosystems (Benovics *et al.* 2021a, 2021b, 2021c; Dezfuli *et al.* 2024; Mladineo *et al.* 2009; Řehulková *et al.* 2020; Stojanovski *et al.* 2020; Zrnčić *et al.* 2009) and coastal lagoons (Dezfuli *et al.* 2014; Giari *et al.* 2020), with a single record of a black spot disease parasite detected in *Rutilus prespensis* (Karaman, 1924) (Stojanovski *et al.* 2020).

Islands in the eastern Adriatic are a part of a larger Outer Dinaric range, characterized by intense karstification processes (Terzić *et al.* 2010), and are built out of limestone and dolomite (Bonacci 2015). Because of extreme permeability of the karst terrain, infiltration of water is fast. Therefore, surface flow on Adriatic islands is rare and appears only after heavy precipitation events (Bonacci 2015). Consequently, only few freshwater fishes are found on these islands, and next to nothing is known about their biology or parasite fauna. On Krk Island, in the northeastern Adriatic, a population of the minnow *Phoxinus lumaireul* (Schinz, 1840) (Actinopterygii: Leuciscidae) inhabits the Suha Ričina stream. Genetic data – lack of haplotype sharing between the island and neighboring mainland populations (Reier *et al.* 2022) – indicate that this population is an autochthonous population that got separated from the mainland populations in the course of the sea level rise following the last glacial maximum and was not introduced by humans. In the present study, we aimed at characterizing the helminth ectoparasite fauna of this unique island population of *P. lumaireul* from Krk Island to get first insights into the ectoparasite diversity these fish are exposed to.

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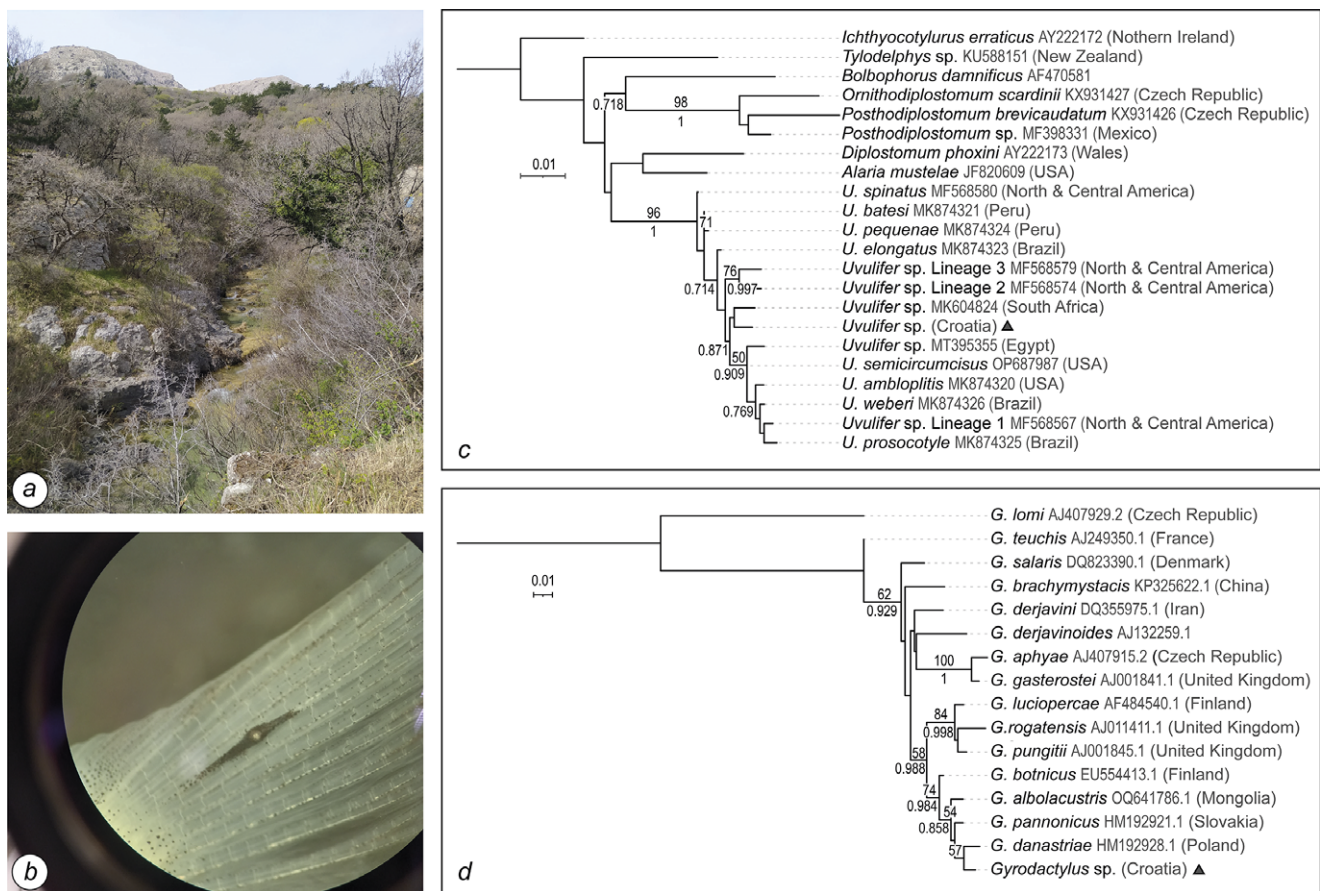
## Materials and methods

A total of 38 *P. lumaireul* was collected (Supplementary Figure S1) at three locations along a 5-km stretch of the Suha Ričina stream on Krk Island, Croatia, in the northeastern Adriatic (Supplementary Figure S2), in April and October 2022 (44°59'57.66" N, 14°42'32.94" E; 45°0'49.86" N, 14°41'56.16" E; 45°01'16.76" N, 14°40'59.09" E). Fish were caught using backpack electrofishing (AGK, model: IG200-1, power: 5 kW). Suha Ričina is the longest (12 km) freshwater stream on any of the Adriatic islands with a basin area of 26 km<sup>2</sup> (Rubinić and Ožanić 1998). During dry season, as the water level lowers, there is almost no surface flow, and the stream primarily flows underground, where its flow is maintained throughout the year (Figure 1) (Valković *et al.* 2014). Suha Ričina stream and the majority of Krk Island are a part of Natura 2000 areas (HR2001357 and HR1000033). Sampling was conducted by licensed operators with a collecting permit (UP/I-324-02/22-01/1) from the government and local level authorities.

Fish samples were initially stored in 1:1 mixture of distilled water and un-denatured 96% ethanol in the field, and afterwards transferred to un-denatured 96% ethanol. Standard length of each fish was measured in millimeters and weight in grams prior to fixation. Fish were visually screened for ectoparasites using a stereomicroscope (Figure 1). To determine helminth infection parameters following Bush *et al.* (1997), the number of metacercariae cysts (black spots) was counted to calculate prevalence

(percentage of infected hosts), mean abundance (mean of the number of individuals of a particular parasite species per host examined), and mean intensity (mean of the number of individuals of a particular parasite species per infected host in a sample). The relationship between the number of parasites and host parameters (standard length and weight) was evaluated through the non-parametric Spearman's *r<sub>s</sub>* correlation test using PAST 4.10 (Hammer and Harper 2001). Parasites were removed using forceps and scalpel and individually stored in 96% ethanol until further processing.

Genomic DNA was extracted from individual worms, three digenean and one monogenean, using a *Quick-DNA*<sup>TM</sup> MiniPrep Plus Kit (Zymo Research) following the manufacturer's *Solid Tissues* protocol with minor modifications; specifically, elution was repeated two times after initial incubation for 10 minutes instead of five. PCR amplification and sequencing of parts of the 28S ribosomal RNA for digenean parasites (primers C1: 5'-ACC CGC TGA ATT TAA GCA T-3' and D2: 5'-TGG TCC GTG TTT CAA GAC-3' (Chisholm *et al.* 2001)), and the internal transcribed spacer 2 (ITS2) for *Gyrodactylus* (primers ITS4.5: 5'-CAT CGG TCT CTC GAA CG-3' and ITS2: 5'-TCC TCC GCT TAG TGA TA-3' (Matejusová *et al.*, 2001)) followed Kobl Müller *et al.* (2024), with the exception of the exact PCR cycling conditions (Supplementary Figure S3). Sequences were visualized on an ABI 3500 capillary sequencer (Applied Biosystems).



**Figure 1.** a) Suha Ričina flowing through Baška valley. b) Metacercariae cyst (black spot) on the caudal fin of a *P. lumaireul* seen through a stereomicroscope. c) Maximum likelihood tree (1,000 bootstrap replicates; TPM3u+F+R2 model; 785 base pairs) of the digenean dataset. d) Maximum likelihood tree (1,000 bootstrap replicates; TVM+F+R2 model; 489 base pairs) of the monogenean dataset. ML bootstrap values (cutoff at 50%) are depicted above branches and BI posterior probabilities (cutoff at 0.7) below branches. Haplotypes of specimens collected on Krk Island are marked with a triangle.

Sequences were checked and edited manually in MEGA 11.0 (Tamura *et al.* 2021). BLAST search against GenBank was used for tentative species identification. The newly generated DNA sequences have been deposited at GenBank under the accession numbers PP918946 (*Uvulifer* sp.; all samples that worked had identical sequences) and PP918958 (*Gyrodactylus* sp.). For phylogenetic placement of our samples, ITS2 sequences of monogenean and 28S sequences of digenean parasites from GenBank (Supplementary Table S1) were added to the alignments. Sequences were aligned with MUSCLE and p-distances calculated (Supplementary Table S3; Supplementary Table S4) using MEGA 11.0. The best-fit models of evolution were determined with ModelFinder (Kalyaanamoorthy *et al.* 2017) using the BIC criterion. Phylogenetic trees were inferred in Phylosuite v1.2.2. (Zhang *et al.* 2020) using maximum likelihood (ML) with IQTREE (Nguyen *et al.* 2015) and Bayesian inference (BI) with MrBayes 3.2.6. (Ronquist *et al.* 2012). For both sets of alignments (groups of parasites), BI analyses were run with six Markov chain Monte Carlo (MCMC) chains in two parallel runs for 3,000,000 generations and sampled every 1,000 generations. The initial 25% of sampled data was discarded as burn-in after ensuring that the average standard deviation of split frequencies was <0.01 (indicating run convergence). Node support in the ML tree was assessed with 1,000 standard bootstrap replicates for both groups of parasites.

## Results and discussion

A total of 38 specimens of *P. lumaireul* was collected, with a standard length of 30–68 mm and weighing 0.411–5.53 g (Table 1, Supplementary table S2). In addition, three specimens of European eel, *Anguilla anguilla* (Linnaeus, 1758) were also recorded at two of the sites (one at 45°0'49.86" N, 14°41'56.16" E and two at 45°01'16.76" N, 14°40'59.09" E). Of the 38 individuals screened, 17 *P. lumaireul* were found with metacercariae cysts on the fins and subepidermal tissue (207 metacercariae cysts in total; Table 1), while a single monogenean (genus *Gyrodactylus*) was found on the skin. We found no significant correlation between intensity of the black spot disease and host parameters (standard length: Spearman's  $r_s = 0.1349$ ,  $p = 0.4194$ ; weight: Spearman's  $r_s = 0.1403$ ,  $p = 0.4007$ ).

Based on BLAST search, maximum likelihood, and Bayesian inference analysis, the 28S sequences indicate that the metacercariae cysts (the three samples had identical sequences) found on *P. lumaireul* from Krk Island belong to *Uvulifer* Yamaguti, 1934, a genus of diplostomid digeneans (Figure 1). The *Uvulifer* sample from Krk Island was resolved as the sister group of *Uvulifer* from South Africa, but the large genetic distances (Figure 1, Supplementary Table S3) suggest that these samples are unlikely to belong to the same species. Currently, a single species, *U. denticulatus* Rudolphi, 1819, is recognized in Europe (López-Jiménez *et al.* 2018), and based on our phylogenetic analysis, there is

no clear match with another *Uvulifer* species; therefore, it can be speculated that the specimens from Krk Island belong to this particular species. Unfortunately, no 28S sequence data of unambiguously identified *U. denticulatus* are available on GenBank, preventing robust species-level identification. By employing molecular and morphological methods, recent studies (Achatz *et al.* 2019; López-Jiménez *et al.* 2018) have uncovered previously unknown species richness within *Uvulifer* in the Neotropics. Similarly, molecular data indicate cryptic diversity in European *Apo-phallus* Lühe, 1909, another genus of diplostomid digeneans inducing black spot disease in cyprinids (Sándor *et al.* 2017). Only a few records of *Uvulifer* parasites in Europe have been documented thus far in the literature. Most of these records are from definitive hosts (kingfishers) (Baylis 1939; Sitko *et al.* 2006) and first intermediate hosts (snails) (Faltýnková *et al.* 2008) with a single record from second intermediate hosts (fish) (Knaack 1971), which is why the taxon is considered as rare (Faltýnková *et al.* 2008). Hitherto, only *Posthodiplostomum cuticola* (von Nordmann, 1832) Dubois, 1936 has been reported as a black spot disease parasite from the Adriatic drainage (Stojanovski *et al.* 2020). Thus, the *Uvulifer* specimens from Krk represent one of the first records of the parasite found on fish in Europe, a first record from a *Phoxinus* host, a first record for the Adriatic drainage and Croatia, and a first record for Southern Europe in general, since all the other records originate from Central Europe (Faltýnková *et al.* 2008; Knaack 1971; Sitko *et al.* 2006) and the UK (Baylis 1939).

The metacercarial stages of the *Uvulifer* and other black spot disease genera are known to be pathogenic to their fish intermediate hosts. In cases of high infection intensities, deleterious effects and mortalities are possible (Hoffman and Putz 1965; Hunter and Hunter 1938; Lemly and Esch 1984; McAllister *et al.* 2013), especially in juvenile fish, which can experience blood loss, physiological stress, and mortalities (Krull 1934; Lemly and Esch 1984). To estimate the effect of *Uvulifer* parasites on *P. lumaireul* in an isolated habitat such as Suha Ričina stream on Krk Island, detailed population based studies are required.

Based on BLAST search, maximum likelihood, and Bayesian inference analysis, the ITS2 sequence of the single monogenean (*Gyrodactylus*) found on *P. lumaireul* from Krk Island is most closely related (p-distances: 1.25–1.88%; Supplementary Table S4) to *Gyrodactylus panonicus* Molnár, 1968; *G. albolacustris* Lumme, Ziętera and Lebedeva, 2017; *G. botnicus* Lumme, Ziętera and Lebedeva, 2017; and *G. danastriae* Lumme, Ziętera and Lebedeva, 2017, all of which parasitize on *Phoxinus*. As genetic distances between the *Gyrodactylus* from Krk Island and any of these other four species exceed the genetic distance between *G. albolacustris* and *G. panonicus* (Supplementary Table S4), it can be argued that the *Gyrodactylus* from Krk Island could represent a distinct, undescribed species following the logic of Lumme *et al.* (2017). Several more species of *Gyrodactylus*, belonging to different genetic lineages, have been reported from *Phoxinus* in Europe (Pettersen *et al.* 2016; and references therein). In most of the previous studies on *Phoxinus* parasites, the host was identified as *P. phoxinus*. A recent revision of the genus, however, revealed that it comprises a complex of several originally largely allopatrically distributed species (Palandačić *et al.* 2017). The majority of monogeneans are highly host-specific (Bakke *et al.* 2002), and even though one host species might be parasitized by several *Gyrodactylus* species, this large number of *Gyrodactylus* species apparently specialized on *P. phoxinus* might actually indicate that different *Gyrodactylus* species are specific to different *Phoxinus* species. Hence, host–parasite relationships in the *Phoxinus*–*Gyrodactylus* system need

**Table 1.** Mean standard length and mean mass of the host *Phoxinus lumaireul* (N=38) from Krk Island (Croatia), prevalence, mean abundance, and mean intensity of metacercarial cysts of *Uvulifer* sp

SL [mm] mean ± SD range [mm]]	Weight [g] mean ± SD range [g]]	Prevalence (%)	Abundance mean ± SD	Intensity mean ± SD
42.5 ± 9.16 (30–68)	1.37 ± 1.11 (0.41–5.53)	44.7	5.45 ± 10.48	12.2 ± 12.91

to be reevaluated based on recent taxonomic changes/additions in both the hosts and parasites.

Characterizing host–parasite interactions, especially those of vulnerable host species, is crucial not just for estimating biodiversity loss, but also because of the key roles in the ecosystem functioning that parasite species may serve (Ingelbrecht et al. 2024). In this study, we present important new data on the ectoparasitic helminth diversity in southern Europe. We provide the first record for the genus *Uvulifer* in the Adriatic drainage, and in southern Europe in general, and find indication for a hitherto undescribed *Gyrodactylus* species parasitizing *Phoxinus lumaireul* on Krk Island. Detailed morphological work is needed to clarify the species identity of our new records. Further, whether these two species are common and widely distributed in southern Europe or have, especially in the case of *Gyrodactylus* sp., a restricted distribution is still unclear and requires further studies focusing on these taxa.

**Supplementary material.** The supplementary material for this article can be found at <http://doi.org/10.1017/S0022149X24000889>.

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**Competing interest.** None

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