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Regional report

First record of the mermithid nematode worm *Isomermis lairdi* parasitizing black flies in Spain

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ABSTRACT

Mermithid nematodes are considered a promising biological control agent to reduce the population density of different blood-feeding vectors, i.e. black flies (Diptera: Simuliidae), which are important pests of medical and veterinary interest worldwide. Immature larvae of black flies were collected in a rill from La Rioja (Northern Spain) in the summer of 2016. *Isomermis lairdi* Mondet, Poinar & Bernadou, 1977 (Nematoda: Mermithidae) was found parasitizing eleven specimens of *Simulium cryophilum* s.l. (Rubtsov, 1959) (prevalence of 52%), which represent the first record of this nematode for Spain and the second for Europe. The confirmation of the nematode and the black fly species was carried out by both morphological and molecular approaches using the 18S ribosomal RNA and the cytochrome *c* oxidase subunit I genes. Phylogenetic analyses indicated that the collected specimens were *Isomermis lairdi* (99.4–99.9% identity with homologues from Africa) with a sequence divergence of 0.2%. The role of *Isomermis lairdi* as an alternative tool in the biological control of black flies in Spain should be further explored.

1. Introduction

Black fly outbreaks have become an emerging problem for both humans and animals in several regions of Spain in recent decades (Ruiz-Arrondo et al., 2020). Among the 55 simuliid species described in the country (Adler, 2022), *Simulium erythrocephalum* (De Geer, 1776) is considered the most important anthropophilic pest species whereas species within the subgenus *Wilhelmia* Enderlein, 1921 are recorded as the most annoying species causing severe discomfort to livestock (Ruiz-Arrondo et al., 2017).

Mermithidae nematodes comprise more than fifty genera of specialized parasites of terrestrial and aquatic invertebrates, including insect vector species such as black flies and mosquitoes (Poinar, 2018). More recently, mermithid parasitism has also been found in other vectors such as *Culicoides* biting midges (Poinar and Sarto i Monteys, 2008) and triatomine species (Martins et al., 2020). Mermithid worms typically infect the insect larvae, killing them before pupation, or the adult during its first gonotrophic cycle (Platzer, 1981).

The most common genera of mermithids found parasitizing black flies are *Mesomermis*, *Gastromermis* and *Isomermis*, while other mermithid genera have been reported less frequently (Molloy, 1981). These endoparasites have been investigated as potential biological control agents of mosquitoes and to a lesser extent in black flies (Crainey et al., 2009). For all its biocontrol potential, several problems remain unresolved and little progress has been made towards sorting out those issues over the last years.

Despite considerable information on records of parasitism is available on American and African black fly species, little effort has been made in Europe (Gradinarov, 2014) and none parasites of black flies have previously been recorded from Spain. Therefore, the main aim of this study was to report the first record of *Isomermis lairdi* in Spanish black fly fauna.

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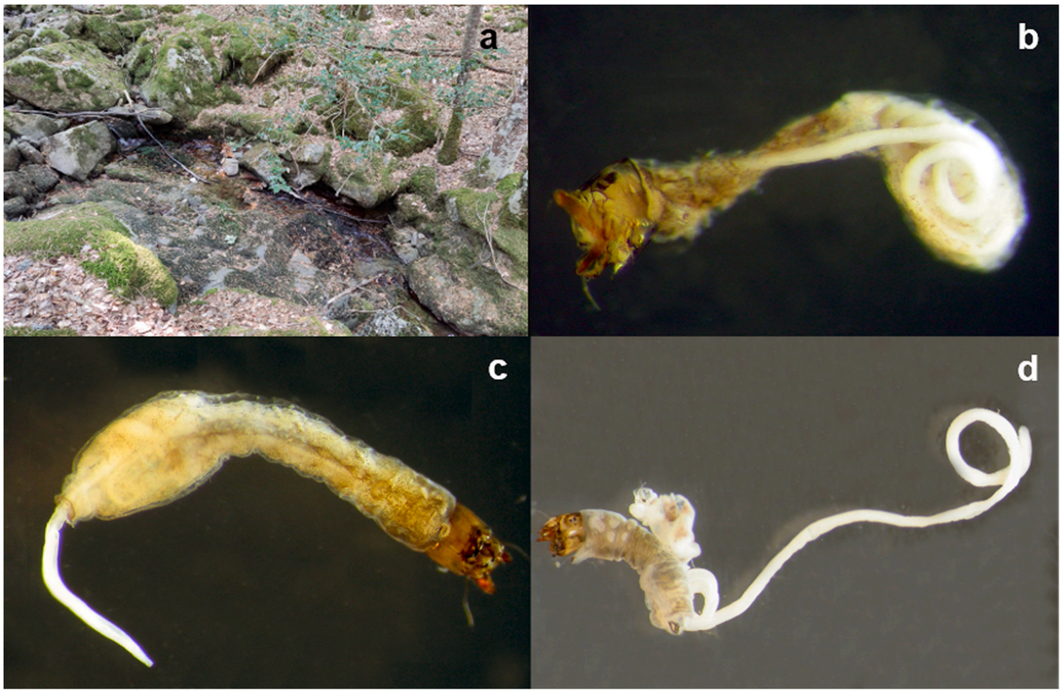

Fig. 1. View of the rill where the black flies were collected (a). Black fly larvae parasitized by *Isomermis lairdi* nematodes (b-d).

2. Material and methods

Immature stages of simuliids were collected from a rill, affluent of the river Rá (42.036919, −2.683501; 1340 m above sea level) (Fig. 1a) in the municipality of Villoslada de Cameros, within the Natural Park of Sierra Cebollera in La Rioja (Northern Spain). The specimens were manually collected from infested aquatic trailing vegetation during a single fieldwork inspection (25 July 2016). Black fly immature stages were fixed in ethanol (70%) and observed under the stereomicroscope for both species identification and search for nematode parasites.

The abdomen of the infected larvae (Fig. 1b-d) was observed under the stereomicroscope (Leica MZ8) and dissected for nematode separation. Molecular identification was needed as isolated nematodes were juvenile stages after checking their external morphology. Briefly, the DNA was extracted from the terminal section of the nematodes ($n = 11$) using Qiagen columns (DNeasy Blood & Tissue Kit). Five μl of supernatant was used for PCR amplification of the 18S ribosomal RNA (18S rRNA) gene, carried out with the forward primer 1096F 5’-GGTAATTCTGGAGCTAATAC-3′ and reverse 1912R 5’-TTTACGGTCAGAACTAGGG-3′ primers (Holterman et al., 2006). PCR was performed in a final volume of 50 μl and contained, 10 mm Tris-HCl (pH 8.3), 1.5 mm $MgCl_2$, 50 mm KCl, 200 mm each of dATP, dCTP, dGTP and dTTP and 1,5 unit of DNA polymerase (Biotools B and M labs, S.A. Madrid, Spain). The thermal profile included a preliminary denaturalization for five min at 95 °C, four cycles initial cycles consisting of 94 °C, 30s; 45 °C, 45 s; 72 °C, 45 s, followed by 34 others including 94 °C one min; 58 °C for one min, 72 °C for one min, and a final extension for five min at 72 °C. Amplified products were cloned into pCR™2.1-TOPO™ using the TOPO-TA cloning kit (Invitrogen) following the manufacturer instructions. Then, the cloned fragment was sequenced by Macrogen© using M13 standard universal primers.

Phylogenetic analyses were performed by constructing multiple alignments of nucleotide sequences including six high quality amplicon-length sequences with 841 pb ($n = 6$, lower quality/shorter sequences were not included in the phylogenetic analysis) and a wide range of nematodes (including *Aborjinia corallicola*, *Hexamermis* sp., *Isomermis lairdi*, *Mermis nigrescens*, *Limnomermis* sp., *Longidorus ferrisi*, *Pheromermis* sp. and *Thaumamermis zealandica*; $n = 22$) retrieved from GenBank. These analyses were constructed using MAFFT vs. 7 (https://mafft.cbrc.jp/alignment/server/) and subsequently edited with GBlocks (http://molevol.cmima.csic.es/castresana/Gblocks_server.html). Phylogenetic trees were built using the Maximum Likelihood (ML) method in IQ-tree v.2.2.0 (http://www.iqtree.org/) and a Bayesian approach by BEASTv1.10.4 software (Suchard et al., 2018). For both methods, the best-fitting evolutionary model was GTR + Γ + I. Bayesian analyses included two independent Markov chain Monte-Carlo (MCMC) runs carried out assuming a log-normal relaxed molecular clock, and a Birth-Death coalescent prior, until 1×10^8 states were sampled (10% of which were discarded as burnin). The final tree sample was then summarized as a maximum clade credibility (MCC) tree. Intraspecific and interspecific genetic divergences were calculated based on the Tamura-Nei model in MEGA X (Kumar et al., 2018). Sequence similarity searches were carried out through Barcode of Life Data System (https://www.boldsystems.org/index.php/IDS_OpenIdEngine) and BLASTn (MegaBlast option;https://blast.ncbi.nlm.nih.gov/Blast.cgi).

As the diagnostic morphological features were not fully developed in immature larvae of black flies, DNA was extracted from the abdomen of 11 specimens and molecular analysis was performed based on the cytochrome *c* oxidase subunit I (COI) gene to confirm the morphological identifications following the protocols of Ruiz-Arrondo et al. (2018). The remainder of the specimen was kept for further morphological examination. Detailed specimen records and sequence information of both nematodes ($n = 6$) and black fly species ($n = 4$) were submitted to the GenBank database under the accession numbers MZ920133, OP081006-OP081010 and MZ919340-MZ919343, respectively.

3. Results and discussion

In the present study, 21 simuliid larvae (only one mature) were captured, of which eleven were parasitized by these entomonematodes, representing a prevalence of 52.0%. Morphological and molecular identification was confirmed in 11 specimens: *Simulium monticola* Friederichs, 1920 ($n = 2$) and *Simulium cryophilum* s.l. (Rubtsov, 1959) ($n = 9$), being the latter parasitized by *I. lairdi* mermithids. The two black fly species captured are also new records for La Rioja province upgrading to 12 the checklist of simuliids for the region (Martinez Ruiz and Portillo

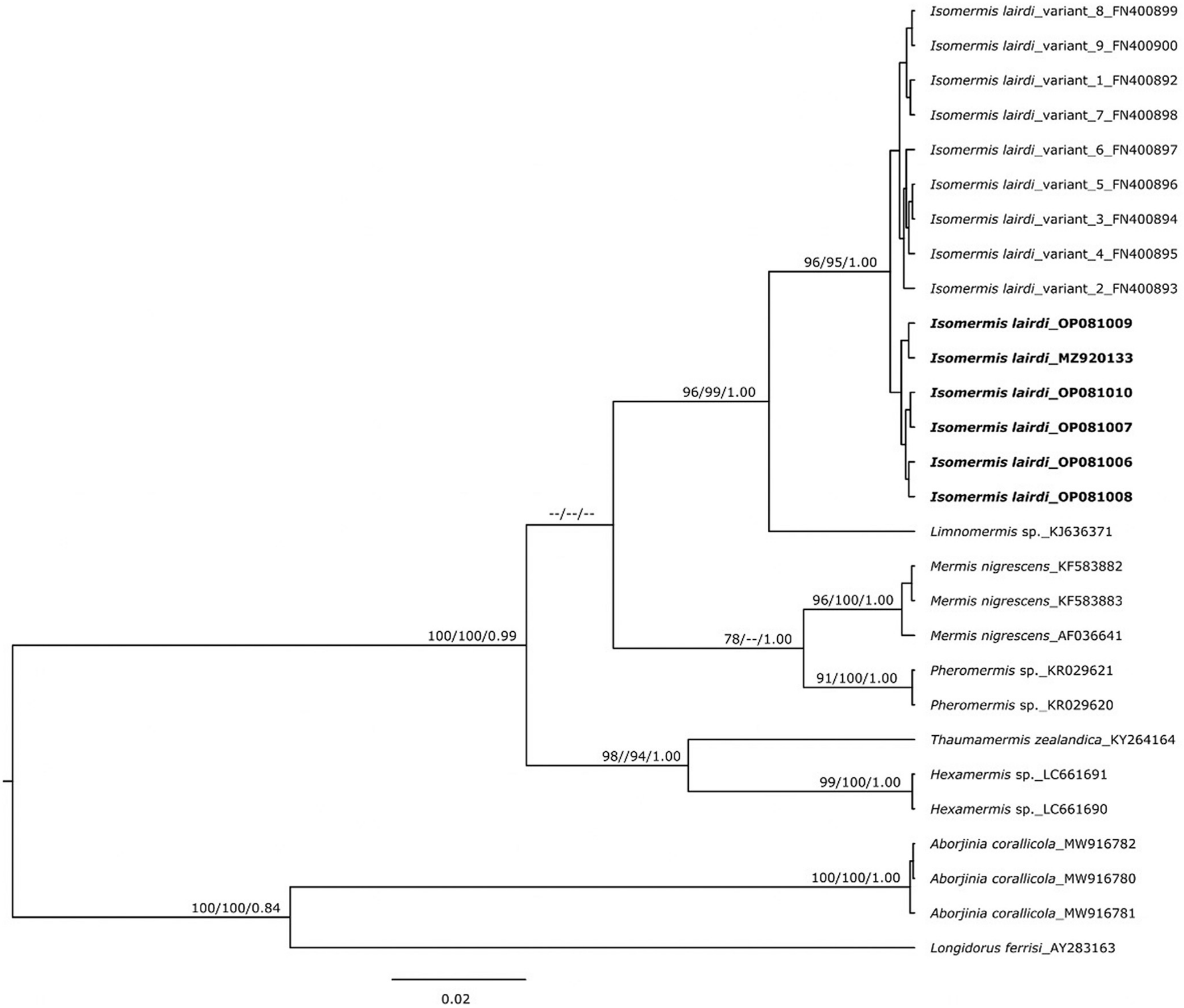

Fig. 2. Bayesian nematode mermithids 18S rRNA-based MCC phylogenetic tree. The sequences obtained in this study are shown in bold. At specific branches, the first and second values separated by "/" indicate the topological branch-support for the ML analysis (aLRT/bootstrap), with values >75% defining high stability. Likewise, the left-most value indicates posterior probability values >0.80 for the Bayesian analysis.

Rubio, 1999). It would be interesting to further explore the presence of mermithids in other common simuliid species, i.e., *Simulium erythrocephalum*, the most annoying pest species in Spain (Ruiz-Arrondo et al., 2017), with the aim of exploring the use of mermithids as a biological control strategy in the management of black fly pests.

Since the collected nematodes corresponded to juvenile stages, their morphological identification to the species level could not be confirmed and required a molecular classification approach. Based on the BLASTn (Megablast option) analysis of the obtained sequences, the mermithid nematodes ($n = 11$) parasitizing larvae of Simuliidae were identified as *Isomermis lairdi* showing 99.4–99.9% identity with homologues FN400892-FN400900 from Africa. Since the only public database records based on the 18S rRNA are African individuals, the *I. lairdi* sequences obtained in this study are, to the best of our knowledge, the first record outside Africa. Both approaches performed for phylogenetic tree reconstruction resulted in congruent tree topologies, where all of the sequences clustered according to their recognized genera in clades with high topological support. While only the Bayesian tree is shown (Fig. 2),

Table 1
Interspecific (between groups) pairwise Tamura-Nei model genetic divergence based on 18S rRNA gene.

	Isomermis lairdi	*Mermis nigrescens*	*Pheromermis* sp.	*Hexamermis* sp.	*Aborjinia corallicola*
Isomermis lairdi					
Mermis nigrescens	0.066				
Pheromermis sp.	0.065	0.025			
Hexamermis sp.	0.094	0.066	0.068		
Aborjinia corallicola	0.165	0.154	0.155	0.166	

the latter also includes results from the ML analysis. The collected mermithids clustered together with other sequences of *I. lairdi* retrieved from GenBank with high branch support, with the *I. lairdi* radiation clearly segregating away from the rest of genera in the tree (Fig. 2). Interestingly, *Limnomermis* sp. is the closest genus to *I. lairdi* but appeared separated in a different branch. *Aborjinia corallicola* and *Longidorus ferrisi* were included in a different clade from the remaining of the individuals (included in family Mermithidae) because these species correspond to the family Marimermithidae and Longidoridae, respectively. In this study, the sequence divergence was 0.2% for *I. lairdi* and 0.2% for the *I. lairdi* retrieved from GenBank. The genetic variation increased to 0.3% when all the sequences from the *I. lairdi* cluster were considered, supporting the identity of *I. lairdi* for the analysed specimens. The interspecific divergence varied between 2.5 and 16.5% (Table 1), with values generally higher than 6.0% as they are different genera of mermithids and was maximum for *Aborjinia corallicola*.

This finding constitutes the first record of the nematode *I. lairdi* from Spain. In Europe, *Isomermis rossica* seems to be well-represented (Gradinarov, 2014) whereas *I. lairdi* had been only previously known in Africa (Mondet et al., 1977; Maduabum and Iwuala, 1990) and subsequently in Bulgaria (Gradinarov, 2014). However, there are no *I. rossica* sequences in public databases, so this species cannot be included in the phylogenetic study. The reliable identification of the genus *Isomermis* (but also the whole family) is challenging (Gradinarov, 2014), especially when they are collected as juveniles. For this reason, molecular sequencing offers an unambiguous and species-specific taxonomic identification tool as a practical solution to mermithids and host range (Crainey et al., 2009). The Bayesian and ML 18S rRNA trees provided a clear understanding of the phylogenetic relationships of the Mermithidae nematodes studied. However, the lack of sequences data for the *Isomermis* genus in the databases could hamper a reliable species-level identification in some cases. *Isomermis lairdi* appears to be well adapted to a wide range of black fly host species being found parasitizing several simuliid species in West Africa (Mondet et al., 1977), particularly *Simulium damnosum* s.l*.,* the main responsible for the transmission of onchocerciasis in West Africa and *Simulium ornatum* s.l. in Bulgaria (Gradinarov, 2014), the latter considered one of the most frequent black fly species in Europe.

4. Conclusions

Isomermis lairdi might be a promising biocontrol species due to the high ecological flexibility. For this reason, coupled with the lack of knowledge in this field in Europe, further research is needed to better understand the potential of nematodes in integrated vector control strategies to reduce population densities of vector blackfly species.

Author contribution

Conceptualization: Ignacio Ruiz-Arrondo and José A. Oteo. Fieldwork, morphological and molecular identification of black flies: Ignacio Ruiz-Arrondo and Miguel Mejías-Ortiz. Morphological and molecular identification of nematodes: Susana Arcos and Oscar Soriano Hernando. Phylogenetic analysis: Ricardo Parreira. Writing - original draft preparation: Mikel A. González. Writing - review and editing: Ignacio Ruiz-Arrondo, Miguel Mejías-Ortiz and Alfonso Navas. All the commented on the previous versions and authors approved the final manuscript. Resources: José A. Oteo and Alfonso Navas; José A. Oteo is the head of Infectious Department at Hospital San Pedro-CIBIR and Alfonso Navas is the head of the Laboratory Department of Biodiversity and Evolutionary Biology at the National Museum of Natural Sciences-CSIC. Both are the ones who have obtained the resources to be able to carry out this work. Supervision: Ignacio Ruiz-Arrondo.

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Declaration of Competing Interest

The authors declare no conflict of interest related to this article.

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