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Emerging zoonotic risk: molecular detection of *Trichobilharzia franki* in Western Europe's largest artificial lake, Alqueva, Portugal

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ABSTRACT

Background: Emerging parasites pose increasing challenges at the interface of human, animal, and ecosystem health. Among these, *Trichobilharzia* spp., schistosomatid of migratory and resident waterfowl, can cause cercarial dermatitis (CD) when its larval stage (cercaria) accidentally penetrate human skin. While this parasite has been documented in several European countries, its presence in Portugal has remained unreported, leaving a critical gap in understanding potential zoonotic risks in local freshwater ecosystems. Therefore, this study aimed to investigate the presence of *Trichobilharzia* spp. in freshwater snails from Lake Alqueva, providing the first insights into its potential ecological and public health implications in Portugal.

Methods: Fieldwork was carried out on Lake Alqueva, considered the largest artificial reservoir in Western Europe, with ecological and public health relevance. Freshwater snails from shore locations in Lake Alqueva were examined for cercarial shedding, and molecular identification was performed by PCR amplification of the cytochrome c oxidase subunit 1 (*COX*1) gene and internal transcribed spacer (ITS) regions.

Results: Morphological and genetic analyses confirmed *Trichobilharzia franki* from *Radix auricularia*, showing high homology with European and Asian lineages. Moreover, human infections compatible with CD were also reported in the region.

Conclusions: These findings highlight the need for continued malacological surveillance, particularly in recreational freshwater bodies, to assess risk areas and implement mitigation strategies. Furthermore, this study expands the known geographical distribution of *T. franki* in Europe and underscores the importance of integrating ecological and public health approaches to monitor emerging zoonotic parasites.

1. Background

Certain trematode parasites are widely distributed across aquatic environments and have evolved complex life cycles that can involve waterfowl as definitive hosts and freshwater snails, fish and crustaceans as intermediate hosts [1,2]. Notably, the genus *Trichobilharzia* is significant as it is the main responsible agent for cercarial dermatitis (CD) referred as swimmer's itch in humans [1,3]. This genus thrives particularly in areas abundant with waterfowl populations and diverse freshwater ecosystems [2].

Trichobilharzia spp. are globally distributed, with several species identified in Europe, including viscerotropic species such as *Trichobilharzia szidati* and *Trichobilharzia franki*, and the neurotropic *Trichobilharzia regenti,* which differ in their pathogenic potential [3–6]. Species differentiation relies on a combination of morphological characters (e.g. eggs morphology), genetic variation, and specific host associations (see more in Horák et al. [1] and Bispo et al. [7]). Among the European species *Trichobilharzia*, *T. franki* exhibits the broadest geographic range and is most frequently associated with CD cases [6,8].

The infective stage, the cercaria, can cause this condition when it inadvertently penetrates human skin, eventually provoking an allergic reaction [5]. Although CD is not life-threatening, it can substantially impact human health, particularly in areas with high levels of recreational water use. Common clinical manifestations include intense itching, erythematous papules, and vesicular eruptions that typically appear within hours after exposure [3,5]. However, recent evidence indicates

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that these reactions are not exclusively allergic; *T. szidati* can directly activate MrgprA3-expressing sensory neurons, contributing to the rapid and intense itch observed during early exposures [9]. In sensitized individuals or after repeated exposures, symptoms may become more severe and prolonged [4,10]. Experimental infections in murine models have shown that *T. franki* can migrate to the lungs and induce inflammatory reactions [6], highlighting additional aspects of this infection that warrant further investigation.

The epidemiological characterization of CD on the Iberian Peninsula remains incomplete, with only sporadic case reports. In northwestern Spain, in the province of Salamanca, *Trichobilharzia salmanticensis* was described following human cases of CD associated with cercarial exposure in local reservoirs [11]. In Portugal, CD has not been systematically monitored; however, preliminary data from ongoing Knowledge, Attitudes, and Practices (KAP) questionnaires, administered to clinicians and pharmacists, indicate the occurrence of human infections compatible with CD, all reportedly acquired in Lake Alqueva [12]. Although *Trichobilharzia* spp. are widely distributed across Europe, their presence in Portugal has not been previously documented. In this context, the present study aimed to investigate the occurrence of etiological agents of CD, as well as their freshwater snail intermediate hosts, in Lake Alqueva, Portugal.

2. Methods

2.1. Malacological survey

Malacological surveys were carried out at Lake Alqueva (southeastern Portugal) at 25 shore locations around the Alqueva basin (locations in Table S1) between May 2023 and October 2024, encompassing the period from early spring to late summer.

At each site, visible freshwater snails present in shallow water margins were collected by handpicking with plastic forceps (e.g. Fig. 1). Sampling was conducted by two collectors, with an effort of 15 min per collector. The specimens were then transported alive, in water collected at the location, to the laboratory for further analysis. In the laboratory, snails were first placed in groups (pools) in dechlorinated water under artificial light to stimulate cercarial shedding. When cercariae were observed in a pool, individual snails from that group were subsequently tested to identify the infected specimen. The cercariae released from the infected snails were previously identified using an optical microscope, focusing on the pigmented eyespots and bifurcated tail with fins, following the dichotomous key by Faltýnková et al. [13] in order to characterize the genus. Samples of the cercariae were preserved in 70 % ethanol for subsequent molecular analysis.

2.2. Molecular analysis

The DNA was extracted from samples containing putative *Trichobilharzia* cercariae preserved in 70 % ethanol, using the DNeasy Blood and Tissue kit (QIAGEN ©, Cat. No. 69504, 06/2023 HB-2061-003 2023, Hilden, Germany), following the manufacturer's instructions. In brief, samples were centrifuged at 13,800×*g* for 5 min. Excess ethanol was removed, and the samples were left at room temperature for 10 min to evaporate any residual ethanol. DNA was extracted from *Radix* spp. after dissection of the cephalopodal and hepatopancreas regions. The extraction followed the CTAB-chloroform protocol described by Stothard et al. [14] and modified as per Bispo et al. [15]. Both regions were analyzed separately. This approach aimed to detect pre-patent infections or cases in which the infection had not yet progressed to cercarial shedding.

For all PCR protocols, each 30 μL PCR reaction included: 15 μL of Supreme NZYTaq II 2 × Green Master Mix (NZYTech ©, Portugal), 30 pmol of each primer, 3 μL of genomic DNA [3 ng], and 6 μL of distilled water, and were performed in a Biometra Tone 96G Thermal Cycler (Analytik Jena ©, Germany). The mitochondrial cytochrome c oxidase subunit 1 (*COX*1) gene of cercariae and lymnaeids cephalopodal and hepatopancreas regions were amplified using the Cox1_Schist F (5′-TCTTTRGATCATAAGCG-3′) and Cox1_Schist R (5′-TAATGCATMG-GAAAAAAACA-3′) primers [16]. The PCR profile was adapted from a previously established protocol [17], and consisted of an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 46 °C for 30 s, and extension at 72 °C for 2 min, with a final elongation at 72 °C for 7 min. For nuclear ribosomal DNA (rDNA) of cercariae samples, primers BD1 (5′-GTCGTAA CAAGGTTTCCGTA-3′) and 4S (5′-TCTAGATGCGTTCGAARTGTCGA TG-3′) were used for amplification of internal transcribed spacer (ITS) 1 region [18]. The PCR program consisted of initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min [18].

For the identification of snails' species which shed cercariae, PCR was performed using universal primers [19] LCO1490 (5′-GGTCAACA AATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGAC CAAAAAATCA-3′) to amplify *COX*1 of mitochondrial DNA. The PCR profile consisted of an initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 40 s, annealing at 50 °C for 1 min, and extension at 72 °C for 90 s, with a final elongation at 72 °C for 10 min according to previously established protocol [20].

The amplified PCR products were visualized under UV light on 1.5 % agarose gel in TAE buffer, stained with ethidium bromide and it was

Fig. 1. Sampling location at Campinho, Alentejo, Portugal (38°21′17.0″N 7°26′37.0″W). A: collection site; B: snails' habitat.

sequenced by Sanger sequencing. The sequences were deposited in the GenBank for reference.

2.3. Phylogenetic analysis

The obtained sequences were manually checked and edited in Chromas software (version 2.6.6, Technelysium, Australia). The phylogenetic relationships of the avian schistosomes in this study were analyzed using two gene regions: mitochondrial *COX*1 and nuclear ITS1-5.8S-ITS2. A dataset was created by performing a BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and the closest sequences were downloaded from GenBank™ and aligned with ClustalW in the BioEdit software (version 7.2.5, Carlsbad, CA, USA) [21]. Outgroup sequences were obtained from a separate BLAST search. Phylogenetic analysis was conducted using the Maximum likelihood (ML) method in MEGA 12 software [22], applying Tamura-Nei [23] and Kimura 2 [24] parameter model for nucleotide changes, and bootstrap analysis with 1000 replicates was used to assess the reliability of tree nodes. The final ML tree was constructed with outgroups defined by relationships from previous studies in the BLAST search, providing insights into the evolutionary relationships among the avian schistosomes sampled.

3. Results

3.1. Survey, malacological findings and cercariae identification

Over the course of eight field trips, 25 locations were surveyed around Lake Alqueva. These locations encompassed a wide range of habitat types and different forms of human use. During these surveys, various aquatic birds were observed around the lake, including domestic ducks, and other Anatidae species, as well as the invasive Egyptian geese, particularly in the Summer months. A total of 7125 snails were collected. Among these, 6414 were morphologically identified as *Physella acuta*, 660 snails as *Radix* spp., and 51 as *Gyraulus* spp. (Fig. 2, Table 1).

Among the 660 lymnaeid snails collected, 325 were suitable for molecular analysis. The remaining snails were excluded because post-collection mortality and subsequent tissue degradation rendered their material unusable for reliable DNA extraction. From each of the 325 snails, both the cephalopodal and hepatopancreas regions were dissected and processed separately. In this study, four individuals, identified morphologically and molecularly as *Radix auricularia*, shed cercariae displaying the characteristic features of *Trichobilharzia* furcocercariae (Fig. 3). These infected snails were collected at Campinho (38°21′17.0″N 7°26′37.0″W) in September 2024. The overall infection rate among *Radix* spp. was 0.6 %; however, considering only the field trip and habitat where infection was observed, the rate reached 13.8 %. Regarding the other snail species, results concerning *P. acuta* have already been published [15], while findings for the remaining species and associated parasites will be addressed in future publications.

Table 1
Number of collected snails per month at Lake Alqueva.

Month	Collected snails data		
	Gyraulus spp.	*Physella acuta*	*Radix* spp.
May 2023	1	550	23
July 2023	7	973	260
August 2023	4	1304	54
October 2023	0	625	5
April 2024	0	109	0
June 2024	23	852	187
September 2024	9	1223	118
October 2024	7	778	13
Total	**51**	**6414**	**660**

The genetic sequences obtained from the cercariae in our study exhibited a high degree of similarity—over 95 %—to reference sequences of *COX*1 for *T. franki* (lengths ranging from 899 to 974 base pairs) and 100 % homology for the ITS1 (ranging from 900 to 1000 base pairs) (Fig. 4). The sequences obtained can be found deposited in GenBank under the accession numbers PX442666-68, PV068607-08, PX442687, and PX631952-53.

Among the 650 snail tissue samples analyzed, the parasite was detected exclusively in the hepatopancreas of the four snails that had previously released cercariae after artificial light exposure (Fig. 5). No evidence of pre-patent infection was found in any of the other samples. Detection was achieved using the Cox1_Schist F and Cox1_Schist R primers targeting the *COX*1 gene, and confirmed by Sanger sequencing.

3.2. Phylogenetic analysis

Following a BLAST search, high genetic similarity was observed between the *T. franki* isolates from Lake Alqueva and those previously

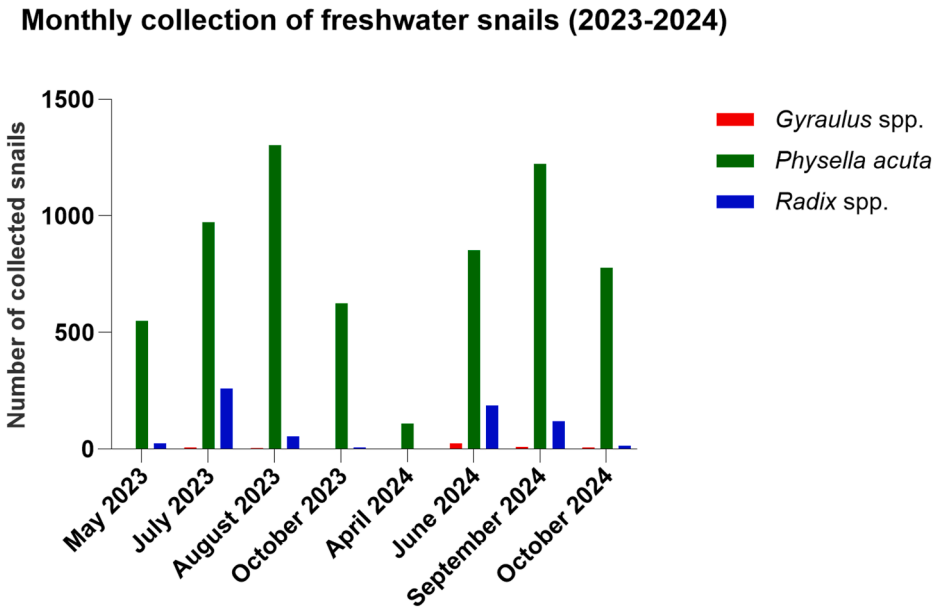

Fig. 2. Snails collected from May 2023 to October 2024.

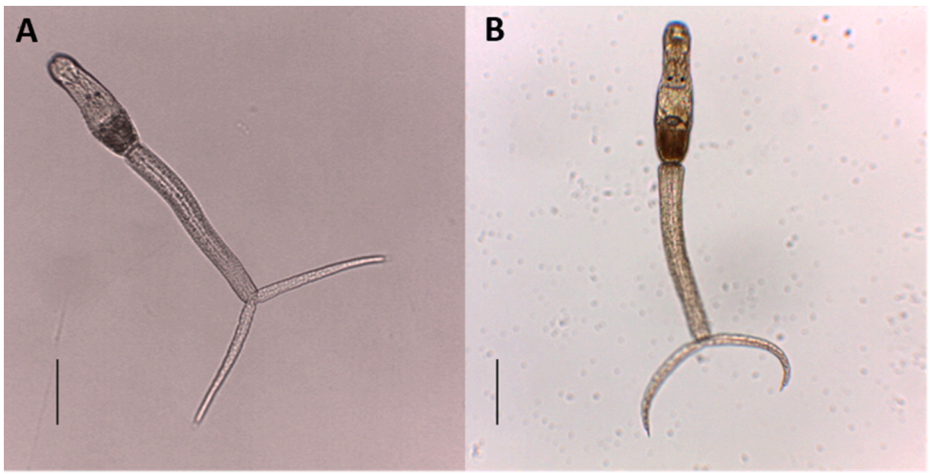

Fig. 3. Furcocercaria shed from *Radix auricularia* from Campinho, Alentejo, Portugal, identified as *Trichobilharzia franki*. A: cercaria fixed with 70 % ethanol, visualized under 40 × optic microscopy; B: cercaria fixed with Lugol Iodine under 40 × optic microscopy (scale = 100 μm).

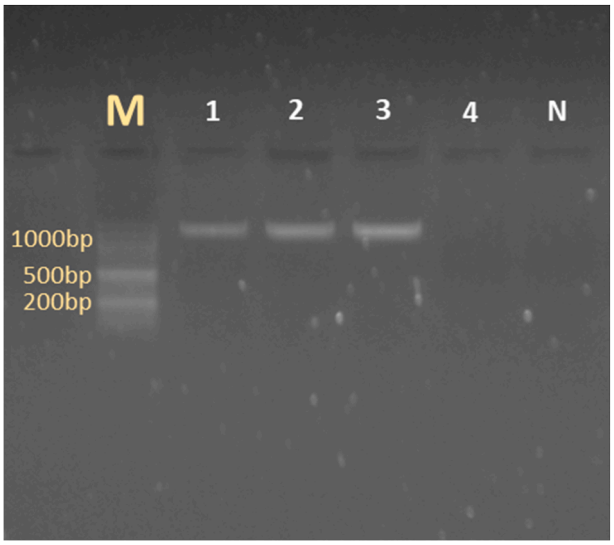

Fig. 4. PCR products of *COX*1 (amplification with Cox1_Schist F and Cox1_Schist R primers) of *Trichobilharzia* isolates. Note: M, molecular weight markers (NZYDNA Ladder VI 50–1500 bp; NZYTech, Cat. No. MB089, Lisbon, Portugal); lane 1 and 2, cercariae isolates; lane 3, snail's DNA (hepatopancreas region); lane 4, snail's DNA (cephalopodal region); N: negative control.

reported in Austria [25]. A Hungarian isolate [17] also showed substantial homology, although the query coverage was below 95 %. These relationships are illustrated in the phylogenetic analysis (Fig. 6, Fig. S1). Overall, the results highlight the genetic diversity within *T. franki* and suggest that strains from geographically distant locations may share a common ancestry, potentially facilitated by migratory routes of waterfowl.

Molecular identification confirmed that *R. auricularia* clustered phylogenetically with other European sequences of the species (Fig. 7), supporting its role as an intermediate host for *T. franki* in Lake Alqueva. Although the tree is presented, bootstrap support values were omitted due to their uniformly low scores (50–70); however, site coverage information is included.

4. Discussion

Our results represent the first identification of a *Trichobilharzia* species, *T. franki*, in Lake Alqueva, Portugal, also detected within the DNA extracted from *R. auricularia*'s tissue. This avian schistosomatid primarily infects aquatic birds and can be found in the blood vessels of the internal organs of Anatidae species, including domestic ducks [18]. It was originally described by Muller and Kimming [26], who identified morphological and physiological changes among the agents responsible for CD in Germany. Since then, *T. franki* has been reported in several European countries and parts of Western Asia [18,27,28]. Genetic studies have revealed considerable haplotype diversity within European populations, highlighting the utility of molecular tools in elucidating the complex evolutionary dynamics of the genus *Trichobilharzia* [8,18,27,29]. Although species within this genus may exhibit morphological similarities across life stages, egg morphology can assist in distinguishing some taxa, such as *T. franki* and *T. ocellata*, particularly when they share the same vertebrate host [1]. However, the amplification of the mitochondrial *COX*1 gene using the Cox1 marker remains a robust and widely used method for reliable species-level identification of trematodes, even where *COX*1 data remains relatively limited [27,28,30].

Snail populations are highly sensitive to environmental conditions, with factors such as water pollution, habitat modification, and climate variability significantly influencing their abundance [31]. Birds, as definitive hosts, play a central role in parasite dissemination, either by directly spreading the infection or by transporting infected intermediate hosts to new locations [32]. These movements can affect infection rates in snail populations, though their influence is difficult to quantify in bird infections. Nevertheless, reported average infection rates in *R. auricularia* ranged 1%–3% in previous studies [8,33]. However, it is important to note that these estimates are often based on one or two field surveys, which may not accurately capture temporal or spatial fluctuations in infection rates. Additionally, habitat characteristics, such as eutrophication, can further influence snail infection dynamics [34], highlighting the need for multiple sampling surveys across diverse habitats to obtain robust estimates of infection prevalence.

The identification of *T. franki* in Portugal suggests a potential role of migratory birds in the geographic expansion of this parasite, given the high genetic similarity between Portuguese isolates and those from distant regions. Furthermore, migratory birds often facilitate the transboundary spread of *Trichobilharzia* spp. during their seasonal movements [18,35]. Moreover, the co-occurrence of *P. acuta* in Lake Alqueva raises concern regarding the possible introduction of other species such as *Trichobilharzia physellae*, especially given its detection in Austria [36], a country along migratory routes shared with Portugal and where *T. franki* closely related to the Portuguese isolate has also been reported [25].

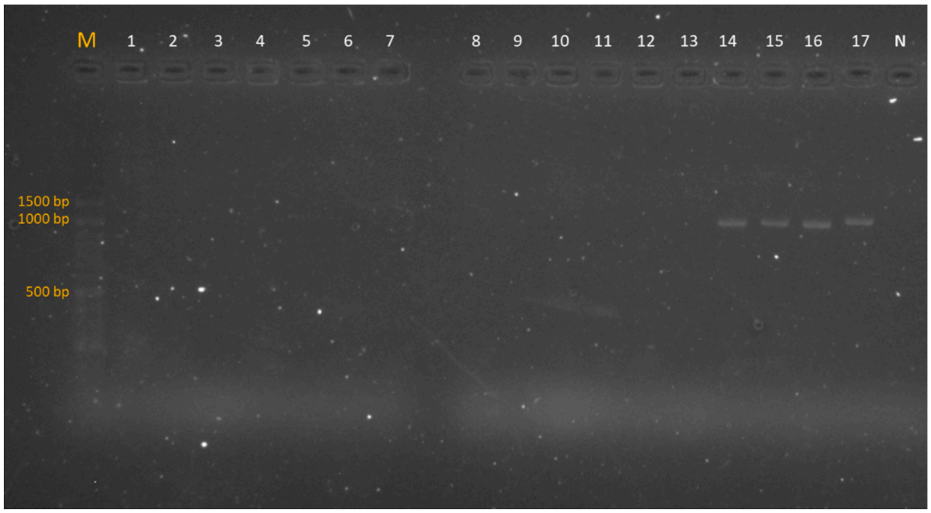

Fig. 5. PCR products of *COX*1 (amplification with Cox1_Schist F and Cox1_Schist R primers) of hepatopancreas' DNA of *Radix auricularia* collected in Campinho, Portugal, in September 2024, sequenced as *Trichobilharzia franki*. M: molecular weight markers (NZYDNA Ladder VI 50–1500 bp; NZYTech, Cat. No. MB089, Lisbon, Portugal); lane 1–13: *R. auricularia* hepathopancreas' isolates negative for shedding cercariae; lane 14–17: *R. auricularia* hepathopancreas' isolates positive for shedding cercariae; N: Negative control.

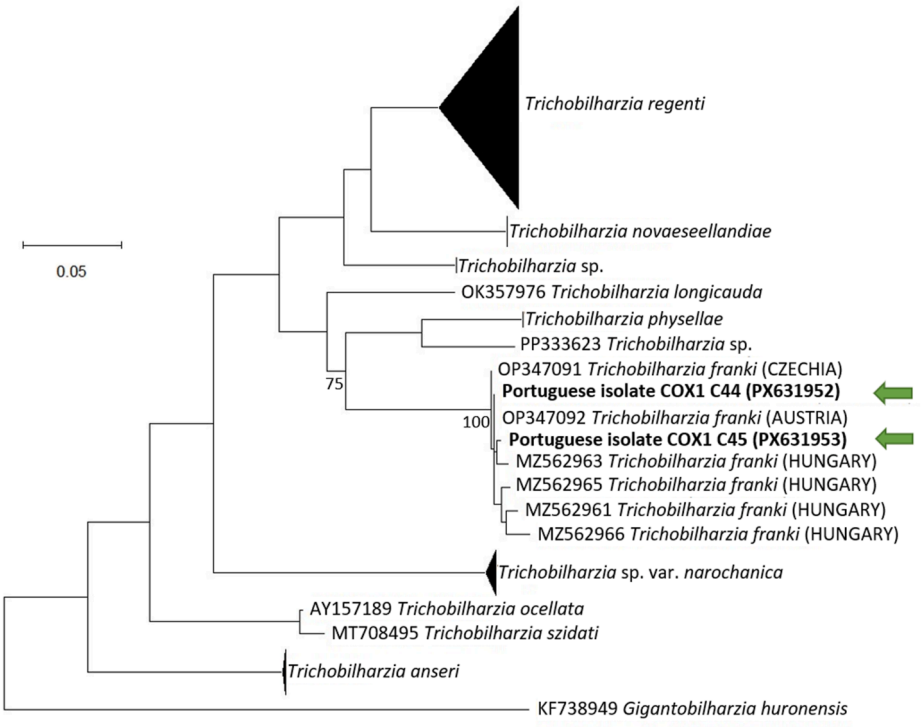

Fig. 6. Maximum likelihood tree of *Trichobilharzia COX*1 sequences. The tree was built using the Tamura-Nei model with gamma-distributed rate variation (0.20) and 1000 bootstrap replicates. The Portuguese isolates clustered with other *Trichobilharzia franki* lineages. Tree construction was performed in MEGA 12 software.

The confirmed infection of *R. auricularia* by *T. franki* reinforces the high specificity of this host-parasite relationship. Nonetheless, associations with other lymnaeid species, such as *Ampullaceana balthica* (=*Radix peregra*, *Radix ovata*, *Radix balthica*) and *Lymnaea stagnalis*, have also been documented [1,27,32,37]. Therefore, when *R. auricularia* and *A. balthica* co-occur in the same habitat, both may potentially contribute to transmission, and further investigation is needed to clarify their relative roles as intermediate hosts. Continued monitoring is necessary to track the establishment and potential spread of *T. franki* in the region. The emergence of environmental DNA (eDNA) approaches provides a valuable, non-invasive tool for detecting *Trichobilharzia* in freshwater systems, enabling surveillance even in the absence of snail collection [4,25,38].

The observed genetic homogeneity among European *T. franki* populations suggests ongoing gene flow, presumably facilitated by migratory birds capable of traveling across vast distances and multiple countries [8,18]. Similar dynamics are known for the transcontinental dispersal of *Trichobilharzia querquedulae*, found in birds migrating between Oceania, Africa, and America, demonstrating its ability to disperse across continents [35]. Some Anatidae species are recognized as crucial reservoir hosts for *Trichobilharzia*, serving as definitive hosts and significantly contributing to the spread of the parasite over broader areas [1].

The emergence of *T. franki* in Southern Europe contrasts with its well-documented presence in Central and Northern Europe. While northern regions link high infection rates to eutrophic systems [33,34],

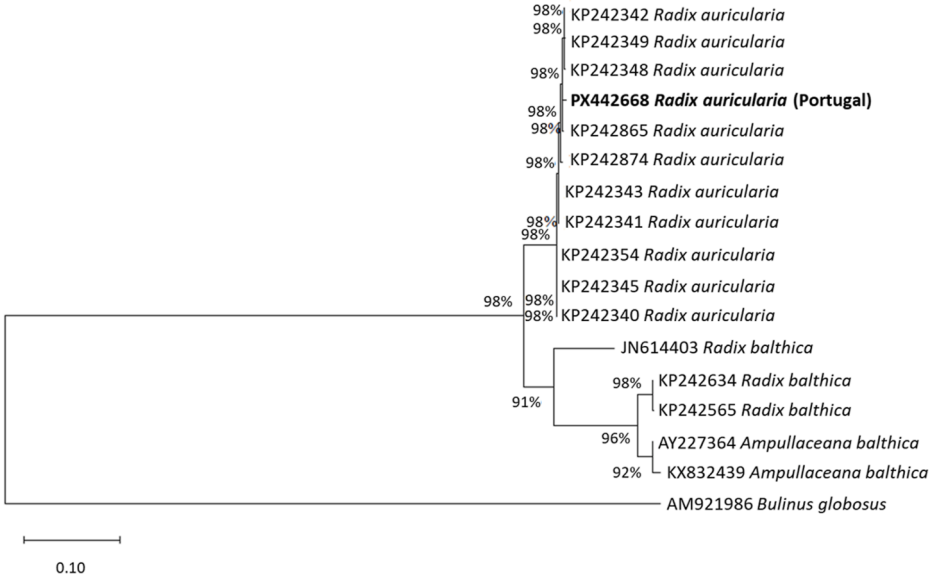

Fig. 7. Maximum likelihood tree of *Radix spp. COX*1 sequences. The tree was built using the Kimura 2-parameter model with uniform rates and 1000 bootstrap replicates. The Portuguese isolate PX442668 clustered with other *Radix auricularia* lineages. The construction of the tree was performed in MEGA 12 software.

Southern Europe offers a unique ecological context marked by warmer climates and fluctuating water levels, presenting new implications for public health in Portugal. Despite being the species implicated in the earliest Iberian CD reports, *T. salmanticensis* has not been recorded since, creating a significant gap in our understanding of its current distribution and epidemiological relevance across the Iberian Peninsula.

Lake Alqueva is a popular destination for water sports, fishing, and birdwatching, all of which increase human-water contact. Risk of cercarial exposure is heightened during warmer months, due to increased snail activity and human presence. The discomfort associated with CD, which includes severe itching and skin irritation, may discourage visitors and affect local economies reliant on water-based tourism, including hospitality and recreation sectors. In severe scenarios, public health concerns could even prompt beach closures or inhibit future tourism development plans [39]. The presence of *T. franki* raises broader questions about the distribution of *Trichobilharzia* spp. in Portugal and the potential for other aquatic systems to host infected snails. Considering the potential role of migratory birds in its introduction, regions with high bird activity may serve as future hotspots. This reinforces the importance of cross-border, multidisciplinary monitoring of wildlife-associated zoonoses to enable early detection and effective intervention strategies.

5. Conclusion

The detection of *T. franki* in Lake Alqueva represents a significant step forward in understanding the distribution of avian schistosomes in Europe. Considering the lake's increasing use for recreation, further research is needed to assess the prevalence of *Trichobilharzia* spp. and identify the environmental factors driving transmission. Monitoring bird populations and snail habitats can help mitigate CD risk, allowing timely public-health actions and local warnings when needed. Public health efforts should also prioritize awareness campaigns on CD and expand surveillance to other high-risk freshwater systems in Portugal, ensuring public health safety for residents and visitors.

CRediT authorship contribution statement

Maria Teresa Bispo: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Isabel Larguinho Maurício:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pedro Manuel Ferreira:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Silvana Belo:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Manuela Calado:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Consent for publication

All authors read and approved the final version for publication consideration.

Ethics approval and consent to participate

Not applicable.

Availability of data and materials

The data not presented in the article will be made available by the authors on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soh.2025.100143.

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