VETERINARIA ITALIANA
RIVISTA DI SANITÀ PUBBLICA VETERINARIA

Paper

First molecular identification of the zoonotic parasite Anisakis pegreffii (Nematoda: Anisakidae) in Albania

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Veterinaria Italiana, Vol. 62 No. 1 (2026) DOI: 10.12834/VetIt.3855.37828.2

Abstract

For the first time, Anisakidae larvae in commercially caught fish from the southwestern Ionian Sea off the Albanian coast were identified using molecular techniques. Atlantic horse mackerel (*Trachurus trachurus*) from the Vlora district were examined for parasitic infections. Enzymatic digestion revealed the presence of *Anisakis* spp. larvae, which were subsequently analysed at ISS Rome by multiplex PCR and PCR-RFLP. Molecular results confirmed the larvae as *Anisakis pegreffii*. This study provides the first molecular confirmation of *Anisakis* species in Albanian waters. The detection of *Anisakis* larvae highlights potential risks for seafood safety and public health, underlining the importance of regular monitoring and risk assessment in the region.

Keywords

Anisakis pegreffii, Ionian Sea, Albanian coast, Atlantic horse mackerel, food safety, molecular identification

Introduction

Industrial fisheries play a significant role in the Albanian economy, with the city of Vlora serving as a key hub due to its tourism and economic potential (Brokaj, 2014). Anisakiasis is a zoonotic infection caused by the larval stages of nematodes from the genus Anisakis (Dujardin, 1845). The life cycle of Anisakis involves small crustaceans as first intermediate hosts, fish and squid as secondary intermediate or paratenic hosts, and marine mammals as definitive hosts. In fish, larvae are typically found on the surfaces of visceral organs, within the body cavity, and occasionally in muscle tissue (Mattiucci & Nascetti, 2008).

The growing popularity of raw fish dishes, such as Japanese sushi, in Albania has raised concerns about seafood safety. Since 2014, national surveillance programs have incorporated control measures for Anisakis to protect consumers in Albania. Previous reports have documented the presence of Anisakis larvae in various fish species in southern Albania, with a particularly high prevalence (68.18%) in Atlantic horse mackerel *(Trachurus trachurus)* (Ozuni et al., 2021). The genus Anisakis includes several recognized species, but only Anisakis simplex sensu stricto (s.s.) and Anisakis pegreffii have been confirmed as zoonotic pathogens capable of infecting humans (Cipriani et al., 2015; Zhu et al., 1998).

The zoonotic implications of these parasites are of significant concern, particularly due to the consumption of raw or insufficiently processed seafood that may harbour live larvae (Audicana & Kennedy, 2008; Chai et al., 2005; Mattiucci et al., 2018). In humans, the larvae do not mature but cause anisakiasis, a condition where the larvae migrate from the gastrointestinal tract and become embedded in the mucosa and submucosa, leading to symptoms such as gastric pain and vomiting. This can result in eosinophilic granuloma (Ishikura & Namiki, 1989), and allergic reactions linked to Anisakis infections have been increasingly reported (Audicana & Kennedy, 2008). Particularly, two species of the Anisakis simplex complex are responsible for human infections: *A. simplex s.s.* and *A. pegreffii* (Audicana & Kennedy, 2008; Mattiucci & Nascetti, 2008; Mattiucci et al., 2018).Anisakid nematodes can be differentiated based on

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their morphological and molecular characteristics (Berland, 1961; D'Amelio et al., 2000; Nadler et al., 2005; Zhu et al., 1998) although members of the *Anisakis simplex* complex are morphologically indistinguishable. The identification of Anisakis larvae in Albania has traditionally relied on morphological methods. Molecular techniques, particularly PCR-based methods, provide an accurate means of differentiation (Mattiucci et al., 2011) and a reliable approach for confirming species identity following morphological examination.

This study aimed to identify *Anisakis spp.* larvae isolated from *T. trachurus* caught off the southeastern coast of Albania, using PCR-RFLP and sequence analyses.

Materials and methods

Parasite Sampling

Atlantic horse mackerel (*T. trachurus*) was collected from the Vlora district as part of the National Monitoring Plan of fish and fish products in Albania. Fish samples were collected monthly from Veterinary inspectors throughout the entire year, from fish processing facilities, fish farms, and fishing vessels in the Vlora district (Figure 1) and submitted to the Food Safety and Veterinary Institute for analysis. A total of 158 fish samples were collected from Vlorë and visually inspected for the presence of Anisakis larvae in the viscera.

A total of five nematode larvae (third-stage larvae) were extracted from a single fish and analyzed individually (Figures 2, 3, and 4). The parasites were morphologically identified under a stereomicroscope (Optika SZM Led 1,2, Ponteranica, Italy) according to Mozgovoy's classification (1953). The larvae were washed thoroughly in distilled water and preserved in 70% ethanol for subsequent molecular analysis. Molecular identification was performed at the European Union Reference Laboratory for Parasites, Istituto Superiore di Sanità (ISS), Rome, Italy.

Figure 1. Map of sampling area (Vlora district, Albania) (source: © d-maps.com).

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Figure 2. Samples brought to the laboratory as part of the Anisakis monitoring plan

Figure 3. Collection of Anisakis larvae from the fish abdominal cavity

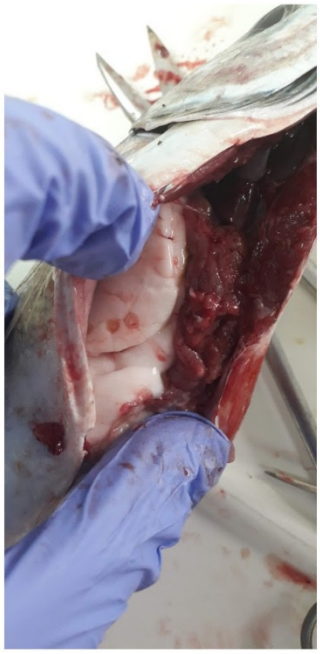

Figure 4. Visual inspection showing the presence of Anisakis larvae during fish examination

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DNA Extraction and PCR-RFLP Results

DNA extraction was conducted using the DNA IQ™ System kit (Promega) according to the manufacturer's instructions. The ITS region (ITS1, 5.8S rDNA gene, and ITS2) approximately 1000 bp, was amplified using the primers NC5 (forward: 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (reverse: 5′-TTAGTTTCTTCCTCCGCT-3′) described by Zhu et al. (1998). PCR was carried out in a final volume of 50 µL, consisting of 2 µL DNA template, 25 µL 2× PCR Master Mix HotStart (Qiagen GmbH, Hilden, Germany), 0.5 µM of each primer, and nuclease-free water. The thermal profile was: 95°C for 2 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s; with a final extension at 72°C for 7 min. Amplicons were visualized by capillary gel electrophoresis (Qiaxcel, Qiagen GmbH, Hilden, Germany). The amplicons, approximately 1000 bp, were subjected to PCR-RFLP analysis with two restriction enzymes, HinfI and HhaI, for the species identification of the A. simplex complex, following the genetic key described by D'Amelio et al. (2000). Two different reaction mixes, for the PCR-RFLP assay, were prepared for each enzyme: 10 µL of each PCR product was digested with 5 U of HinfI (5′ G/ANTC 3′) and 5 U of HhaI (5′ GCG/C 3′) (Thermo Fisher Scientific Inc., MA, USA), 2 µL of 10× Restriction Buffer (Tango® Buffer), and nuclease-free water, in a final volume of 20 µL. The mixtures were incubated at 37 °C for 2 h. Restriction fragments were visualized and analyzed by capillary gel electrophoresis (Qiaxcel, Qiagen GmbH, Hilden, Germany). Expected PCR/RFLP fragments sizes (in bp) for each Anisakidae species are reported in Table 1. The size of the bands was determined by comparing them with a DNA size marker (50–1000 bp).

To confirm the restriction patterns produced by digestion, Sanger sequencing was performed by GENEWIZ (Leipzig, Germany) using the same primer pairs as ITS PCR. The sequences obtained from the ITS rDNA region were aligned using MEGA version 11 (Tamura et al., 2021). ITS rDNA sequences were also analysed in GenBank in order to detect fixed diagnostic nucleotide positions capable of discriminating the species identified.

ITS - PCR/RFLP fragments		
	HinfI	*HhaI*
A. pegreffii	34, 67, 235, 284, 331	419, 532
A. simplex ss	34, 67, 235, 615	419, 532
A. simplex/ pegreffii	34, 67, 235, 284, 331, 615	419 ,532

Table I. Expected PCR/RFLP fragments size (in bp) for each Anisakidae species.

Results and Discussion

The primary objective of this work was to contribute to the molecular epidemiology and precise identification of *Anisakis* spp. in fish from the Albanian coast. Despite previous reports of the presence of anisakid nematodes in Albanian fish, no molecular identification has been conducted so far. A total of 158 fish samples were collected from Vlora and screened for the presence of Anisakis larvae. Of these, 10 fish (6.3%) tested positive, while the remaining 148 samples (93.7%) were negative. All positive cases were found exclusively in Trachurus trachurus, with 10 out of 14 individuals of this species infected. All other species, including Sparus aurata (68 samples), Pagellus erythrinus (27 samples), Solea vulgaris (6 samples), Dicentrarchus labrax (6 samples), Alburnus arborella (13 samples), and Merluccius merluccius (7 samples), tested negative. These results indicate that, among the fish species analysed in Vlora, Trachurus trachurus was the only species found to be positive with the presence of Anisakis larvae. The morphological analysis classified L3 larvae collected from *T. trachurus* from the Vlora district as *Anisakis* type I. The larvae were then subjected to species identification by molecular analysis using PCR/RFLP. Four larvae were identified as *A. pegreffii* and one larva as *A. pegreffi*/*A. simplex* hybrid (Figure 5).

The RFLP patterns were confirmed by sequencing, and the hybrid pattern showed the presence of two overlapping C/T peaks at the diagnostic nucleotide positions (i.e., positions 275 and 292 of the ITS1 region of rDNA) (Figure 6).

While earlier studies have suggested the presence of anisakid nematodes in Albanian fish, this study is the first to provide molecular identification of *A. pegreffii* and the hybrid genotype *A. simplex/A. pegreffii* at species level in *T. trachurus* in the Vlora region. This result could be expected since *Anisakis pegreffii* is widely prevalent in fish from the

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Mediterranean and Adriatic Seas and often reported as a dominant species across various fish hosts, including*T. trachurus* (Cipriani et al., 2018; Debenedetti et al., 2019; Mladineo et al., 2014; Molina-Fernández et al., 2018). Several studies have shown that the ITS region of the ribosomal DNA (ITS1–5.8S–ITS2) provides genetic markers for the accurate identification of Anisakis nematodes including the detection of recombinant genotypes or putative hybrids, such as between *A. simplex* and *A. pegreffii* (Costa et al., 2016; D'Amelio et al., 2000; Nadler et al., 2005; Zhu et al., 1998). The sequences obtained in our study matched the sequences previously deposited in GenBank for *A. pegreffii* and for hybrid genotype *A. simplex/A. pegreffii* (Costa et al., 2016). This hybridization can occur through interspecific crossing between closely related species, such as *A. simplex s.s*. and *A. pegreffii*, which can be found in coinfestation in different fish species and marine mammals (Abollo et al., 2011). These hybrid forms have been reported along the coasts of the Iberian Peninsula, including the Gibraltar Strait (Umehara et al., 2006), Japanese waters (Umehara et al., 2006), and the Mediterranean Sea (Cavallero et al., 2012; Chaligiannis et al., 2011; Farjallah et al. 2008; Meloni et al., 2011)

Over the past few years, anisakiasis has emerged as a significant zoonotic infection in countries such as Italy (Mattiucci et al., 2013), Korea (Lim et al., 2015), and Croatia (Brogli & Kapel, 2011), with *A. pegreffii* recognised as one of the leading etiological agents of human infections. Clinical case reports have highlighted that only *A. simplex* sensu stricto (s.s.) and *A. pegreffii* are responsible for causing invasive anisakiasis in humans (Zhu et al., 1998). Studies from Mediterranean countries such as Italy, Spain, and Croatia have reinforced the zoonotic significance of*A. pegreffii*, particularly in fish consumed raw or undercooked, such as in sushi dishes. As the popularity of raw fish dishes increases globally and locally in Albania, the health risks posed by *A. pegreffii* emphasize the need for continued surveillance programmes and stronger control measures to safeguard public health. In this perspective, our molecular confirmation of the species responsible for human infections marks an important milestone in understanding the epidemiological risks associated with seafood in Albania.

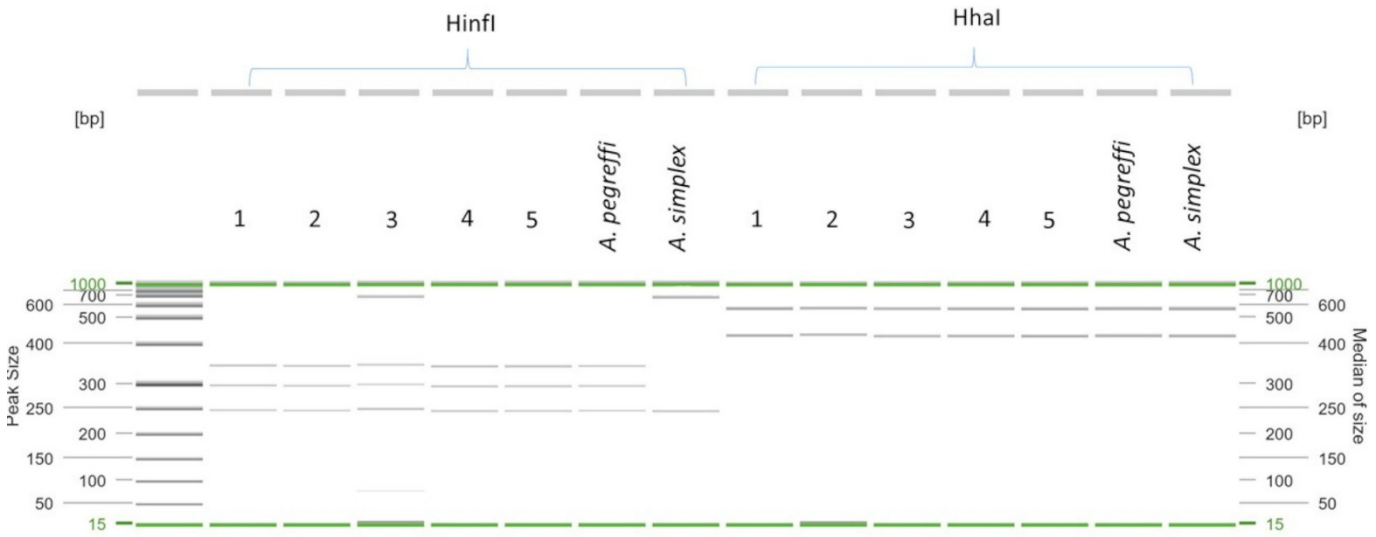

Figure 5. Restriction patterns with HinfI and HhaI. On the left, molecular markers 50–700 bp and alignment markers in green (1000 and 15 bp) are shown. Lanes 1–2 = Anisakis pegreffii; lane 3 = A. pegreffii × A. simplex s.s. hybrid; lanes 4–5 = A. pegreffii; ; lanes 4–5 = A. pegreffii; lane 6 = A. pegreffii (reference material); lane 7 = A. simplex (reference material). It should be noted that 34 bp and 67 bp fragments are not always detectable.

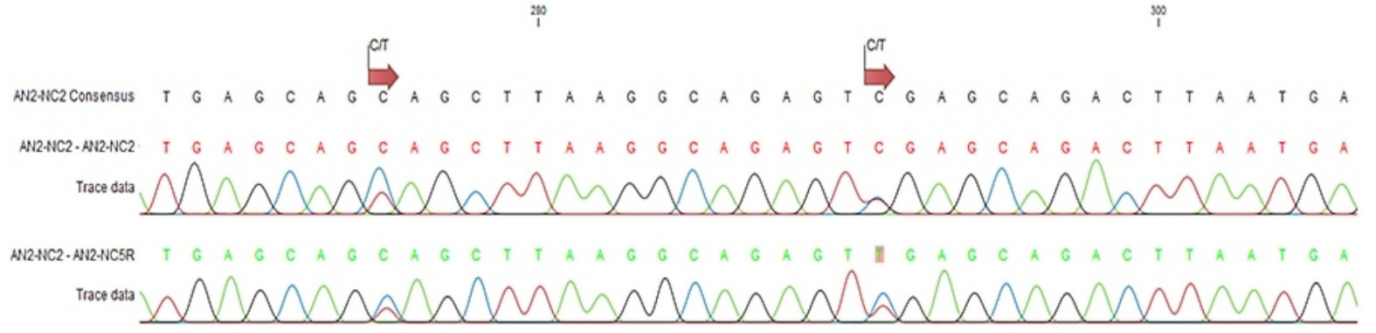

Figure 6. The RFLP pattern of the hybrid genotype A. simplex/A. pegreffii was confirmed by sequencing, showing overlapping C/T peaks at the diagnostic nucleotide positions 275 and 292 of the ITS1 rDNA region.

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Conclusion

This study provides the first molecular confirmation of A. pegreffii in T. trachurus from Albanian waters and reveals the presence of hybrid genotypes. The detection of both *A. pegreffii* and hybrid genotypes has important implications for seafood safety and public health. Continued surveillance, expanded species coverage, and public awareness initiatives are essential to mitigate the risk of anisakiasis in Albania. Regular monitoring of parasitic infections, coupled with public education on proper seafood handling and preparation, will be essential for mitigating the risks of anisakiasis and ensuring seafood safety in Albania.

Acknowledgments

The molecular identification of Anisakis species was carried out at the European Union Reference Laboratory for Parasites (ISS, Rome). Sincere thanks are due to Marco Lalle, Federica Santolamazza, and the staff of the Unit of Foodborne and Neglected Parasitic Diseases for their invaluable expertise and support in the molecular analyses.

Ethical approval

No ethical approval was required for this study, as it did not involve the collection of data from experimental animals or human subjects

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Author Contributions

Conceptualization: Ani Vodica, Federica Santolamazza, Marco Lalle; Methodology: Marco Lalle, Federica Santolamazza, Ani Vodica; Formal analysis: Marco Lalle, Federica Santolamazza, Ani Vodica; Writing original draft preparation: Ani Vodica, Federica Santolamazza, Marco Lalle; Writing, review and editing: Ani Vodica, Federica Santolamazza, Marco Lalle; Supervision: Marco Lalle; Project administration: Marco Lalle; Funding acquisition: Marco Lalle. All authors have read and agreed to the published version of the manuscript.

Data availability

Istituto Superiore di Sanità Rome, St. Regina Elena, Roma, Italy

Food safety and Veterinary Institute, St. Aleksander Moisiu, No.82, Tirana Albania.

Fundings

European Commission SMP-FOOD-2025-EURL-EURC-PJG-IBA under the grant agreement no. 101200161: “Work programme 2025-2027 of the European Union Reference Laboratory for the Parasites (EURL-P)”.

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