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Identification and prevalence of *Anisakis pegreffii* and *A. pegreffii* × *A. Simplex* (s.s.) hybrid genotype larvae in Atlantic horse Mackerel (*Trachurus trachurus*) from some North African Mediterranean coasts

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ABSTRACT

Atlantic horse mackerel is one of the most popular fish for North African consumers. Mackerel constitutes a common host for the *Anisakis* species of infective larvae (L3). *A. pegreffii* and *A. pegreffii* × *A. simplex* (s.s.) hybrid genotype were identified using PCR-RFLP and entire ITS-DNA sequencing protocol. This research investigated the prevalence and mean intensity of *A. pegreffii* and its hybrid form in randomly collected mackerel samples throughout spring to summer seasons from the Libyan western coast. Briefly, 55 out of 240 (22.9%) fish samples were confirmed to be infected and the prevalence of *A. pegreffii* reached 22.08% with mean intensity of infection 29.13 ± 2.43 parasite/fish, while the prevalence of *A. pegreffii* × *A. simplex* (s.s.) hybrid genotype was 0.8% with mean intensity of infection 22 ± 0.85 parasite/fish. The prevalence of infection noticeably elevated during the summer season to reach 30.8%. Additionally, female fish samples were heavily infected as the mean intensity of infection was 34.75 ± 1.27 parasite/fish. Furthermore, results indicated that the highest intensity of infection was recorded in older and female mackerel. To sum up, the current study has provided substantial information about the impacts, diversity and epidemiology of *A. pegreffii* and its hybrid form in North African waters.
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Introduction

Anisakids (Nematoda: Anisakidae) are potential human seafood borne pathogens/ allergens, that pose a serious impact on the health and economy of the fisheries' industry. Fishermen and handlers are also at risk of contracting anisakiasis as well as developing occupational asthma caused by the inhalation of antigens from *A. simplex* (Daschner et al., 2000; Purello-D'Ambrosio et al., 2000; Serracca et al., 2014).

Adult *Anisakis* species reside in marine mammals, and the female worm lays eggs in their feces, which then become embryonated in sea water to develop first-stage larvae (L1). The larvae molt turn into second-stage larvae (L2) and then hatch to become free-swimming. The hatched L2 will then be ingested by crustaceans to grow into third-stage larvae (L3) which are infective to fish and squid. Third stage larvae (L3) migrate from fish intestines to their tissues where they grow to reach 3 cm in length. If ingested by humans through eating raw or undercooked infected marine fish, L3 will molt twice and develop into adult worms that penetrate the gastric and intestinal mucosa, causing the symptoms of anisakiasis (Kagei, 1968; Klimpel et al., 2004; Nagasawa, 1990).

Anisakiasis is a serious human disease caused by accidental ingestion of L3upon consuming raw or lightly preserved infected fish (Baptista-Fernandes et al., 2017). The disease triggers allergic host defenses as the ingestion of infested seafood can lead to an acute gastrointestinal disease associated with abdominal pain,

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nausea and diarrhea. Massive eosinophilic infiltration and granulomas in the gastrointestinal tract can occur if the larvae were not removed. Re-infection causes severe anaphylactic reactions and extra-gastrointestinal or ectopic forms with symptoms varying from gastrointestinal pathologies to deadly anaphylactic shock (Daschner and Pascual, 2005; Nieuwenhuizen, 2016; Nieuwenhuizen and Lopata, 2014).

The genus *Anisakis* contains nine species and mainly two species of the *A. simplex* complex: *A. simplex* sensu-stricto (s.s.) and *A. pegreffii* have been associated with infections in humans (Audicana and Kennedy, 2008; Mattiucci and Nascetti, 2008). *A. pegreffii* is similar to *A. simplex* (s.s.) in its pathogenic potential to trigger human anisakiasis when engulfed with contaminated food(Jeon and Kim, 2015). The first human anisakiasis with *A. simplex* was reported in the 1960s, and during the 1990s it was well established that even the consumption of dead larvae in seafood can cause severe hypersensitivity reactions. This may also occur upon exposure to allergens from dead worms through airborne or skin contact routes. These facts make *Anisakis* spp dangerous both dead and alive (Audicana et al., 2002; Audicana and Kennedy, 2008).

Anisakids parasitize a wide range of commercial marine fishes such as Sea Hake (*Merluccius merluccius*); Anchovy (*Engraulis encrasicholus*); Tuna (*Sardasarda*); Sardine (*Sardina pilchardus*), Atlantic horse mackerel (*Trachurus trachurus*) and blue jack mackerel (*T. picturatus*), and are globally reported in the North-eastern Atlantic and Northern waters of Europe, Portugal, Mediterranean Sea, Adriatic Sea and the Pacific, Atlantic waters of North America and Namdae River, South Korea (Buselic et al., 2017; Costa et al., 2016, 2003; Mattiucci et al., 2013; Mladineo et al., 2017; Setyobudi et al., 2010).

Several reports demonstrated that Mediterranean Sea has a prevailing distribution of *A. pegreffii* and a minimal presence of *A. simplex* (s.s.) in hybrid form with *A. pegreffii* in Atlantic horse mackerel (Costa et al., 2016; Mattiucci et al., 2008; Vincenzo et al., 2015). Significant positive correlations were found between Atlantic horse mackerel's host length and *A. simplex* occurrence, as well as its abundance in the Atlantic coast of Morocco (Shawket et al., 2017).

There were limited studies related to *Anisakis* spp larvae infection in North African Libyan waters (Abusdel, 2016; Farjallah et al., 2008b; Kassem and Bowashi, 2015). Therefore, the objectives of the current research are to accurately identify the potentially infective larvae *of Anisakis* spp. and explore their prevalence in Atlantic horse mackerel collected from North African Libyan waters.

Materials and methods

Fish sample collection, processing and morphological identification of anisakid nematodes

Atlantic horse mackerel (*T. trachurus*) samples were captured during spring and summer seasons of the year 2014. A total number of 240 fish were collected equally on a monthly basis by the Lampara fishing method from the area that extended from Tajura (east of Tripoli city) to Janzur (west of Tripoli city) at a rate of 20 fish/each fishing time. After collection, samples were transported to the Poultry and Fish Diseases Laboratory (PFDL), Faculty of Veterinary Medicine, University of Tripoli under refrigeration (4 °C) and were examined within 24 h.

Each fish sample was flushed with saline, and then the total length (TL) and body weight were determined. Approximate age of Atlantic horse mackerel samples was estimated by measuring TL and examining the whole otoliths with a light microscope, and then approximate fish age was estimated by identifying and counting annuli (Waldron and Kerstan, 2001). Furthermore, host samples were examined for the parasites as soon as morphometric measures were done. Briefly, peritoneal cavity and digestive tract of fish samples were examined after making an incision along the ventral line from the anus to the mouth opening. Fish meat (muscles) was removed from the spine and immersed in warm water for several minutes to facilitate the migration of the parasite outside the fish (Eissa, 2016; Stoskopf, 1993).

The collected *Anisakis* larvae were washed in saline solution for thirty minutes, then the larvae were relaxed in distilled water for ten minutes and killed in 80 °C hot glycerin alcohol (1 part glycerin: 3 parts 95% ethanol) according to the method adopted from (Oguz and Oktener, 2007). The retrieved larvae were cleared in lactophenol and examined under stereo-microscope.

Larvae were morphologically identified using the standard morphological criteria described by Quiazon et al. (2009) and Soewarlan et al. (2014). Briefly, the measured morphological parameters were the body width, esophagus length, ventriculus length/width and mucron length. The morphometric assessment keys were conducted as previously described (Cannon, 1977; Mattiucci et al., 2014; Shamsi et al., 2009a, b). Preliminary identified anisakid nematodes were preserved in 70% ethanol for further molecular identification.

Molecular identification of A. pegreffii and A. pegreffii × A. simplex (s.s.) hybrid genotype members using PCR-RFLP and rDNA sequencing protocol

Identification of retrieved anisakid nematode larvae at the species level was done by Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR-RFLP)using the standard protocol (http://www.iss.it/binary/crlp/cont/MI_04_website_EN.pdf) approved by the European Union Reference Laboratory for Parasites –*Instituto Superiore di Sanità* (ISS) at the department of infectious, parasitic and immuno-mediated diseases, unit of gastro-enteric and tissue parasitic diseases (Rome, Italy). Briefly, the procedures combined the standard PCR for amplification of Internal Transcribed Spacer (locus ITS "ITS1, 5.8S and ITS2" of ribosomal DNA) using the oligonucleotides primers (**NC5** 5'-GTAGGTGAACCTGCGGAAGGATCATT-3' and **NC2** 5'-TTAGT TTCTTTTCCTCCGCT-3') with RFLP protocol using *Hin*fI and/or *Hha*I restriction enzymes (D'Amelio et al., 2000) to allow the unambiguous authentication of all epidemiologically relevant anisakidae species. Accordingly, the restriction of digestion by the ITS fragments with the *Hin*fI enzyme allows the distinguishing of *A. pegreffi*, *A. shupakovi*, *A. ziphidarium*, *A. typical*, *A. physeteris* from *A. simplex* (s.s.), *A. simplex* C and *Pseudoterranova*. Also, using ITS sequence restriction digestion with *Hha*I enzyme, *A. simplex*, s.s can be distinguished from *A. simplex* C (La Rosa et al., 2006).

For molecular authentication of the hybrid genotype, the ITS PCR amplicons were excised from the gel, and the DNA was extracted from the gel using GF-1 AmbiClean kit (Vivantis, Malaysia) and sequenced using cycle sequencing PCR reaction with BigDye® Terminator v3.1 Kit (AB-Applied Bioscience), then sequenced in four-capillary ABI PRISM® 3100-Avant Genetic Analyzer. The chromatogram files were displayed and manually edited using ChromasPro version 2.1.6 software (Technelysium, Australia). Subsequently, BLAST search on NCBI (http://www.ncbi.nlm.nih.gov/pubmed) and Clustal W multiple sequence alignment was applied for the examined consensus sequences to compare with the previously published sequences.

Assessment of A. pegreffii and A. pegreffii × A. simplex (s.s.) hybrid larvae prevalence and mean intensity of infection

Prevalence of *Anisakis* spp. L3 infection in Atlantic horse mackerel fish samples was calculated by dividing the number of infected

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