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Presence of *Aphanomyces astaci* in *Pontastacus (Astacus) leptodactylus*, detected in Polyfytou lake

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ABSTRACT: A case of the oomycete *Aphanomyces astaci* infection in crayfish *Pontastacus* (*Astacus*) *leptodactylus*, is described in the present study. During June 2023, in lake Polyfytou, 60 individuals of crayfish were collected from five different sites of it and transferred to the Faculty of Veterinary Medicine, at University of Thessaly. According to macroscopic and microscopic examination, 32 crayfish that exhibited obvious signs of infection, such as carapace erosion, melanized body patches and spots on their body, underparts, tail, pleiopods and walking legs joints, or individuals with amputation of the walking legs and visible inking at the amputation site, were separated for further analysis. Molecular analysis showed that 32/60 samples from lake Polyfytou were positive to *A. astaci*. Further research is required to investigate the presence of the pathogen in freshwater crayfish species, sites and temperature ranges.

Keyword: *Aphanomyces astaci*; *Pontastacus* (*Astacus*) *leptodactylus*; molecular analysis; lake Polyfytou; crayfish plague

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INTRODUCTION

The oomycete *Aphanomyces astaci* is the causative agent of crayfish plague, a disease that has severely affected populations of European native crayfish species for over a century (Schikora, 1903; Alderman and Polglase, 1987; Edgerton et al., 2002). It is a sapromycete (mushroom-like) and grows on the crayfish epidermis while infecting other healthy crayfish individuals by producing zoospores. The zoospores swim for a few days in search of a host crayfish and when found, attach to its surface, they hook and germinate to begin a new cycle of growth. At the point of invasion by zoospores there may be small pin prick melanised areas (Alderman and Polglase, 1987; Edgerton et al., 2002). Furthermore, histological lesions are also limited, in part due to the rapid onset of death following initial infection. The presence of hyphae in the cuticle at the site of infection is an important diagnostic feature of the disease. The unlimited growth of *A. astaci* leads to the death of the infected animal within only a few weeks of infection (Alderman and Polglase, 1988). Signs of infection, include carapace erosion, melanized body patches and spots on their body, underparts, tail, pleiopods and walking legs joints and amputation of the walking legs along with visible inking at the amputation site (Unestam and Weiss, 1970; Alderman and Polglase, 1987; Alvanou et al., 2024).

The introduction of "invasive" crayfish of North American origin (such as species *Pacifastacus leniusculus* & *Procambarus clarkii*) and their widespread distribution have greatly contributed to the spread of crayfish plague among native populations (Unestam and Weiss, 1970; Vey et al., 1983; Diéguez-Uribeondo et al., 1995). North American crayfish coevolved with this oomycete succumb to the disease only under specific conditions (Diéguez-Uribeondo and Söderhäll, 1993) but mainly act as vectors of the infection (Persson and Söderhäll, 1983; Diéguez-Uribeondo and Söderhäll, 1993; Jussila et al., 2015; OIE 2019).

Molecular typing from random amplified polymorphic DNA (RAPD) of *A. astaci* (Huang et al., 1994) allowed the identification of five distinct genotype groups, labeled alphabetically from A to E (Rezinciuc et al., 2015). The majority of crayfish plague cases in Europe analyzed so far have been linked to one of the four genotypes defined by the RAPD groups or isolated from one of the widespread crayfish invaders, such as group B, (Huang et al., 1994; Diéguez-Uribeondo et al., 1995; Kozubíková et al., 2011b) or related to historical mortality i.e. group A (Huang et al., 1994). Group B, was originally isolated from the crayfish *P. leniusculus* (Huang et al., 1994) while group D, isolated from the red crayfish *P. clarkii* (Diéguez-Uribeondo et al., 1995), was identified in southwestern Europe, where this invasive species is particularly widespread (Caprioli et al., 2018). Recently, group D strains were found to be associated with mortality in the Czech Republic (Mojžišová et al., 2020). Group E was originally isolated from the crayfish *Faxonius limosus* (Kozubíková et al., 2011a). This host is widespread in France, throughout Central Europe and the Baltic countries (Kouba et al., 2014) and corresponds to areas with mortality associated with group E. (Kaldre et al., 2017). Interestingly, *A. astaci* distribution and genotype data from Eastern Europe including Turkey have recently described in chronic infections of the crayfish *Pontastacus leptodactylus*, a native species in these areas (Ungureanu et al., 2020) In this species, at least three different strains have been documented, including those of genotype groups A and B (Kokko et al., 2018) as well as the enigmatic genotype "SSR-Up" (Panteleit et al., 2018). The aim of the present study was to ascertain whether the crayfish plague was present in the examined lake, or whether other environmental factors accounted for the observed absence of infection.

MATERIALS AND METHODS

Samples

During June 2023 in Polyfytou lake, sixty individuals of crayfish *Pontastacus leptodactylus* were caught randomly at a depth of 10 m, from five different sites (Figure 1), near Velventos town, and transferred to the laboratory of Aquaculture and Fish Diseases, at the Faculty of Veterinary Science, University of Thessaly, Greece.

All samples were collected live using classic cylindrical crayfish traps (76 × 23 cm) with a funnel at each end (Holdich, 2002). According to macroscopic and microscopic examination, thirty two crayfish were separated into suspected individuals with obvious signs of infection by blackening of the prophenoloxidase reaction on their body, underparts, tail, pleiopods, telso and walking legs joints or individuals with amputation of the walking legs and visible blackening at the amputation site (Figure 2a & 2b).

Crayfish were euthanized according Protocol of European Commission, Euthanasia of experimental animals, Part 2, (1997) Directive 86/609/EEC.

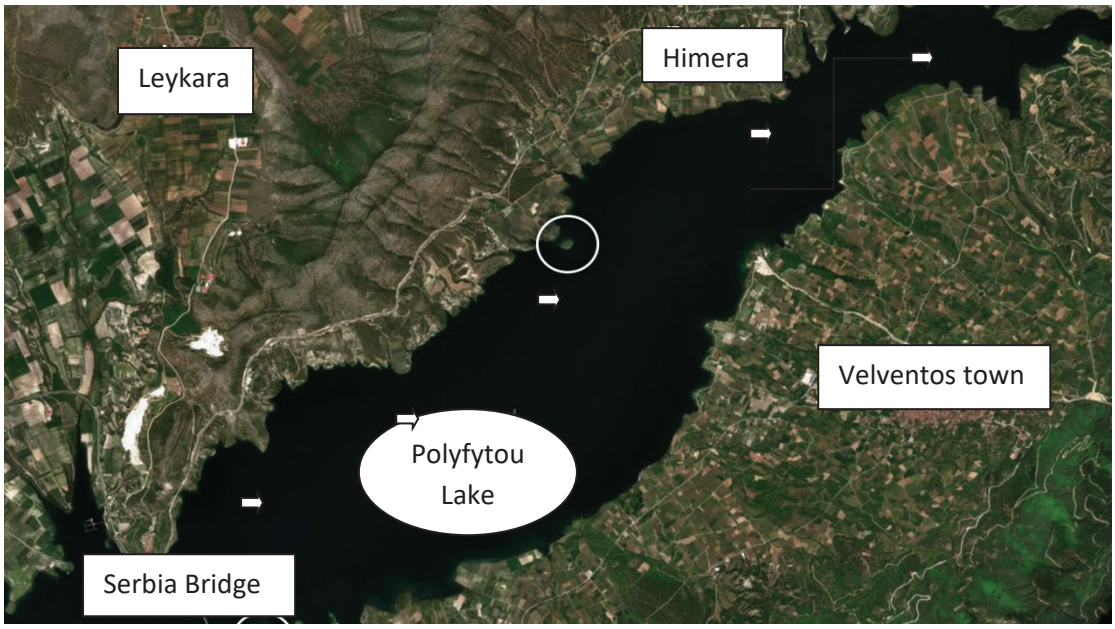

Figure 1. Crayfish fishing spots in lake Polyfytou, June 2023.

Eugenol injection at a concentration of 75 ppm per individual was inserted into the synovial membrane that creates the joint between the abdominal cavity and the cephalothoraxes from the upper part of the body reaching the pericardial sac, according to Waterstrat and Pinkham, (2005). These individuals were then frozen and kept at temperature -40°C for further DNA analysis (Kušar et al., 2013).

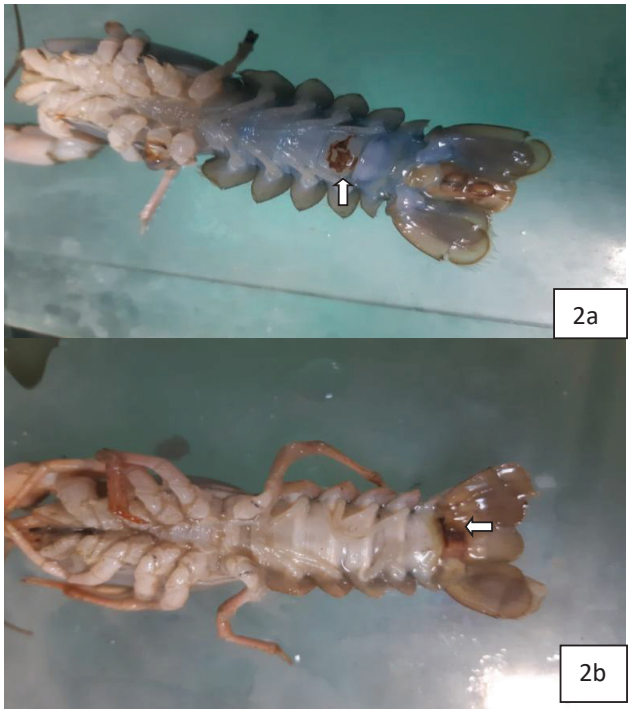

Figure 2a & 2b. Suspicious indication points of the infection from *A. astaci*, by blackening of the prophenoloxidase reaction.

Molecular analysis

For the DNA extraction, the cuticle was wiped with cotton swabs and sterile water to remove any external contamination. The samples were collected mainly from the abdomen and the pleopods, the uropods and the abdominal skin. DNA was extracted using the DNeasy tissue kit (Qiagen, Germany) following the manufacturer's instructions (Peiró et al, 2016). A pestle was used for each sample for mechanical tissue disruption, after the addition of Proteinase K. The extracted DNA of analyzed crayfish samples, were compared with two different extracted DNA samples of genotype A and B of the crayfish plague mycelium from Finland (Finnish Food Authority Kuopio WOAH reference laboratory for crayfish plague, Kuopio, Finland), that were used as positive controls.

The semi-nested PCR assay was chosen using the species-specific primer sites located in the ITS1 and ITS2 regions (Oidtmann et al., 2006). Extracted DNA was subjected to amplification, previously designed from Oidtmann et al (2004), using the primer pair: Forward primer (BO 42) 5'-GCT-TGT-GCT-GAG-GAT-GTT-CT-3' and Reverse primer (BO 640) 5'-CTA-TCC-GAC-TCC-GCA-TTC-TG-3'(598 bp). PCR amplifications were carried out in a 50 µl re-

action volume containing Reddy Mix PCR Mastermix (ABgene AB-0575, with 1.5 mM MgCl2) and Primers 42 and 640 at a final concentration of 0.5 μM each. The mixture was denatured at 96°C for 5 min, followed by 40 cycles of 1 min at 96°C, 1 min at 59°C, 1 min at 72°C, followed by a final extension step of 7 min at 72°C. PCR products were run on a 2% agarose for 50 min at 100 V. After purification, the PCR products (obtained from primer pairs 42/640) were bidirectionally sequenced (Tuffs & Oidtmann, 2011). Amplicons with an expected length of 569 bp were recovered (Figure 3). Standard Nucleotide BLAST (BLASTN) service at NCBI was used to compare each isolate sequence to the J6S rRNA sequences data base by using default settings.

RESULTS

From the sixty individuals of crayfish that were randomly collected and examined from Polyfytou lake, thirty two were positive (53%) in the presence of *A. astaci*, with obvious macroscopically signs, such as melanized body patches and spots on walking legs joints. Molecular analysis, using Primers 42 and 640, an amplicon of the expected length of 569 bp was obtained with all *A. astaci* strains included in this study. (Fig. 3)

DISCUSSION

The present study is the first case of *A.astaci* detection in *P. leptodactylous* from Polyfytou lake. Seventeen species of freshwater fish have been recorded in the waters of the lake Polyfytou. The area is an important habitat for birds of prey, it is also used by migratory species as a winter refuge. The lake is surrounded by urban and rural areas and receives urban, industrial and agricultural wastes which deteriorate water quality and affect condition and survival of its aquatic organisms (Shields, 2012). Therefore, it was considered of crucial importance to investigate the occurrence of *A. astaci* in local crayfish populations.

European crayfish, as in the case of the crayfish *Pontastacus leptodactylus* of Lake Polyfytou, have been characterized as susceptible species to the plague fungus by many studies (Alderman and Polglase, 1988; Holdich et al., 2009) but also as a species tolerant to the fungus (Unestam and Weiss, 1970) in genotype A but also recently in B (Panteleit et al., 2018). According to a recent study on the spread of the species in the Greek area, (Perdikaris et al., 2017) in this particular lake, these crayfish may coexist with the American *Pacifastacus leniusculus* which act as carriers of the disease. Also, the same author mentions the existence of *A. leptodactylus* in the Evros River, as well as in many lakes in Turkey (Harlioglu et al., 2004), where the fungus has been detected since 2000, with the massive collapse of the populations there. The existence of the fungus has been documented in Moldova, Romania (Alvanou et al., 2024), Ukraine (Ungureanu et al., 2020) as well as in Croatia (Pavic et al., 2021).

The growth rate of the pathogen is significantly dependent on temperature. Lower temperature delays the development of mortality in experimental infections (Alderman and Polglase, 1987). Reduced growth rate could be a survival strategy of the pathogen *A. astaci* in highly susceptible European indigenous crayfish species (ICS). Defensive melanization in these new host animals appears to be activated slowly compared to more resistant North American

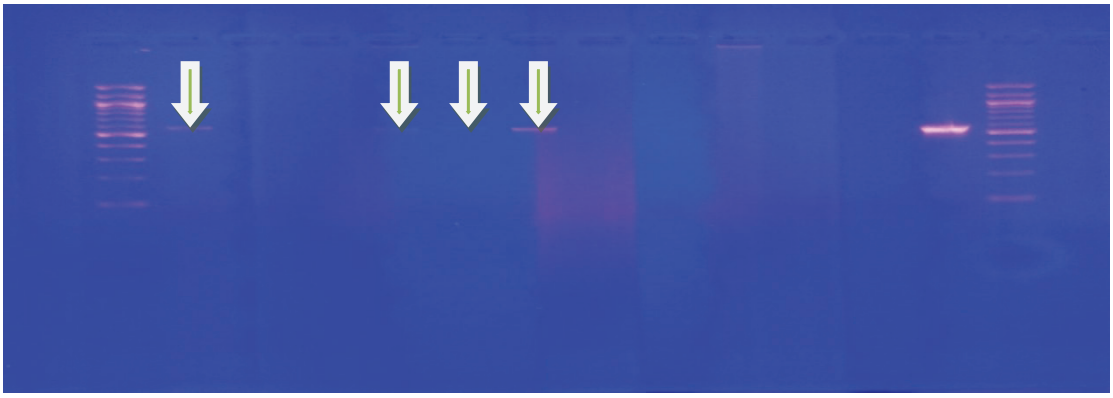

Figure 3. Results for 10 samples with Genotype A, positive control. In the photo, a 100bp ladder was placed in the first and last position. One position before the last ladder that lights up strongly is the positive control and you see the lines that light up in positions (1, 4, 5, and 6).

crayfish species (NACS). In European crayfish species, enzymatic activation of the prophenoloxidase reaction is often too inefficient and slow to successfully control the disease (Cerenius et al., 2003). Therefore, in European crayfish infection usually leads to death within a few days or weeks, depending on the pathogen strain and virulence (Makkonen et al., 2014). The limited availability of weak populations and generally low water temperatures in deeper areas where crayfish are often fished in this particular lake may create favorable conditions for the maintenance of low virulent strains of *A. astaci*. However, the existing risk of anoxic conditions at times, of minimal food resources at these depths due to eutrophication, the enhancement of sporiosis due to the water physico-chemical characteristics, (Cerenius et al., 1984; Pavic et al., 2022) as well as, the low biodiversity in Lake Polyfytou in zooplankton species (Stamou et al., 2022) contribute to the loss of populations in summer months during the development of young individuals and in the sensitive phase of hatching in older individuals.

Furthermore, their tolerance to a wide range of conditions including pollution, organic enrichment (Souty-Grosset et al., 2006) and eutrophication, resulting from the spatial planning of the specific lake near the dam area (Tsioptsias et al., 2019) has been noted as a contributing factor to their persistence. The overfishing of crabs, the spread of the fungus not only through non-native crayfish, but also through fish (Oidtmann et al., 2002) and other aquatic species, the transport of *A. astaci* zoospores through fishing gear and nets (Kozubíková et al., 2008), or even via birds and mammals (Anastácio et al., 2013) are additional potential threats for the ecological balance of the lake. Some aquatic pathogens like *Saprolegnia* spp. existing as *S. parasitica* (Spt) (Söderhäll et al., 1991) and *Fusarium* spp as an opportunistic factor causing disease (Edgerton et al., 2002), are elements that have troubled the past years regarding the future of the lake's biodiversity. However, the increase in temperature and especially this year the drought that Greece is facing and at the local level in Polyfytou lake, with the dramatic receding of the waters to unprecedented levels, has made a nightmare scenario come true in terms of the collapse of the population according to local fishermen, despite the fishing ban.

Finally, it is also necessary to determine the genotype of any mortality event caused by crayfish plague, where the probability of complete population eradication is much higher with infection with a strain of genotype Ps1 (genotype B) (Viljamaa-Dirks et al., 2011) than with genotype As (genotype A) which appears to be more variable in virulence (Makkonen et al., 2012b, 2014) and paradoxically, makes the successful reintroduction of native crayfish more likely and clearly without the coexistence of non-native species that the fungus could to exist in latent form (Fürst, 1995).

CONCLUSIONS

This study verified that the crayfish plague exists in Lake Polyfytou, Greece. Macroscopic symptoms were confirmed through molecular analysis. Further research is required to investigate the presence of *A. astaci*, to specify the prevailing genotypes in various populations along the lake. This will enable local authorities, to repopulate local water reservoirs with more indigenous freshwater crayfish species with no clinical signs of *A. astaci*. And clearly without the coexistence of non-native species that the fungus could exist in latent form.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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