Collection of Pathogen-free *Astacus* sp. Crayfish and Acclimation in Laboratory Aquaria: The First Step Towards Freshwater Crayfish Aquaculture Development

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Abstract

Aquaculture constitutes a sector of high importance for Greece, however, so far concerns only fish and bivalves. On the other hand, freshwater crayfish represent a product of particular interest, encompassing a greatly export oriented character. Except from their economic value and great protein source, freshwater crayfish species comprise important benthic invertebrates and are considered as appropriate model organisms. Nevertheless, *Aphanomyces astaci*, a fungus-like eukaryotic microorganism pathogen spread in Europe, which has been transmitted also in Greece from North American crayfish species, devastated native populations causing freshwater crayfish plague. Recently, crayfish populations from lake Vegoritida and Polyphytos declined, leading local authorities to take measurements for preventing further crayfish population reductions. Thus, the development of an aquaculture protocol establishment is of urgently high importance not only for commercial activities of local fishermen, but for restocking purposes and biodiversity conservation as well. Towards this direction, detection of the aforementioned pathogen in order to be aware of a possible disease outbreak, represents a core issue. The present study represents a small-scale monitoring for the presence of *A. astaci* in freshwater crayfish populations from in North Greek wetlands, and an initial effort for acclimation of pathogen free *Astacus leptodactylus* individuals collected from lake Vegoritida as the first step for an aquaculture protocol establishment.

Keywords

freshwater crayfish, aquaculture, *Astacus leptodactylus, Aphanomyces astaci*, crayfish plague

1. Introduction

Among the various aquatic organisms that are cultivated or farmed worldwide, the vast majority belong to fish, followed by bivalves, whereas the crustaceans and decapods are placed in the third position [1]. Within this last category, mainly on account of the major species produced in worldwide aquaculture, red swamp crayfish *Procambarus clarkii* come in the second place in terms of quantity, after the whiteleg shrimp *Penaeus vannamei* [1]. Particularly in Greece, aquaculture concerns only fish

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and bivalves, but no crustacean species so far, with the only exception of few recently licensed marine shrimp units which, however, have not initiated yet.

Freshwater crustaceans have been known to human since prehistoric times, when they were used as a primary source of animal protein [2]. The interest in these aquatic animals is also scientific, due to the peculiarities in the evolutionary history of this taxon and in its biology and ecology. They constitute excellent biological models for anatomists and physiologists and in recent years they have been also studied by molecular biologists [3].

In Greece, there are three indigenous species of freshwater crayfish, namely the *Astacus astacus* (noble crayfish), the *Astacus leptodactylus* (narrow clawed crayfish) and the *Austropotamobius torrentium* and one invasive species from North America, the *Pacifastacus leniusculus* [4]. Among the aforementioned, *A. leptodactylus* individuals are caught in significant quantities in the Lake Polyphytos, wherefrom there is also the largest available information regarding their production and trade, mostly performed through small scaled exporting companies. It should be noted, that based on their great export potential, both species (*A. astacus* and *A.leptodactylus*) are valuable for local fishing communities, and are thus considered of high conservation priority.

Similarly to other European countries, freshwater crayfish overfishing practices resulted in conservation issues, with sustainable fishing remaining one of the biggest question marks for inland wetlands. Keeping this in mind, the establishment of an aquaculture protocol for freshwater crayfish is of high importance, in order to maintain biodiversity as well as for restocking purposes [5].

The fungus-like oomycete *Aphanomyces astaci*, introduced in Europe by invasive North American crayfish, is responsible for the crayfish plague that devastated many European native crayfish populations. Worth noting that *A. astaci* has been listed among the 100 worst invasive species [6]. Hence, monitoring and early detection of this pathogen contributes in population screening for pathogen carries, setting legislation in order to prevent a possible disease outbreak [7,8] The scope of the present study was the investigation of *A. astaci* presence in freshwater crayfish from North Greece, the collection of pathogen free populations and their subsequent acclimation in laboratory aquaria.

2. Materials and Methods

2.1. Sample Collection

Astacus sp. crayfish specimens were collected from three different sampling sites, namely lake Vegoritida, lake Polyphytos and wetlands Agras-Nisi-Vrita. Collections were carried out with the use of specific net-traps, located approximately 8-10 meters from the coast, where they remained for 8 days (Figure 1). The 8th day the traps were removed, and the crayfish specimens were collected. Identification of collected freshwater crayfish individuals was performed morphologically using specific keys [9]. All crayfish individuals were initially examined morphologically for the *A. astaci* presence before transportation to the laboratory [10].

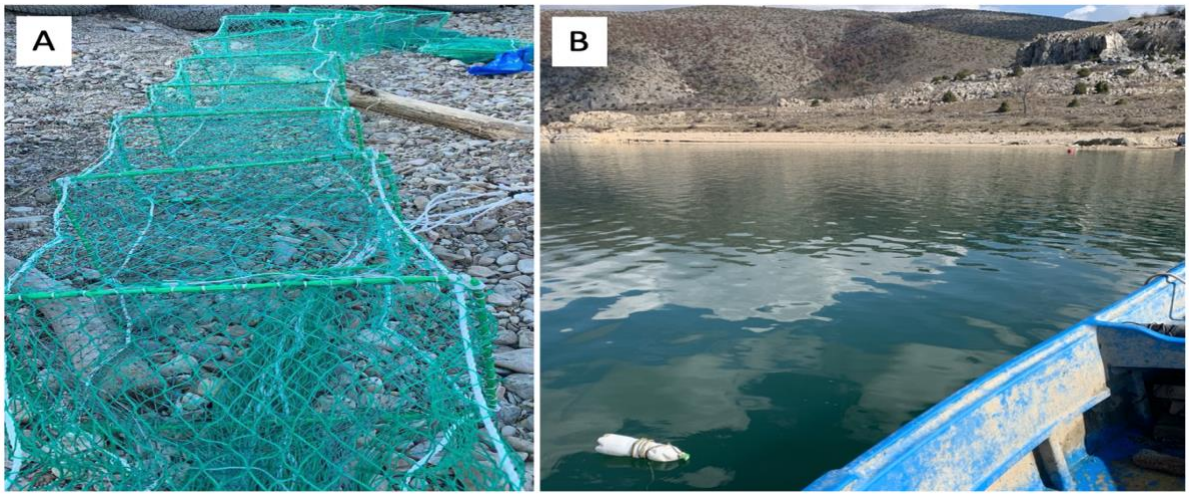

Figure 1: Net-traps (A) used for freshwater crayfish collections, placed in lake Vegoritida (B)

2.2. Molecular Assay for the Pathogen *Aphanomyces Astaci* Detection

From each sample composed by approximately 50 individuals, half of them were transferred to the lab aquaria while the remaining ones were examined for the presence of *A. astaci*. A partial tissue of the uropod, soft abdominal cuticle, eye stalk and walking leg joint of each individual were crashed and used for DNA isolation using the NucleoSpin DNA extraction kit (Macherey Nagel, Germany) and sterile instruments. A 569-bp fragmant of the *A. astaci* ITS nrDNA was targeted using the PCR as previously described [10]. Considering that the absence of an amplified PCR product may be observed due to insufficient amounts of DNA in the PCR reaction, control PCR reactions were run with primers used for all Oomycetes and other microorganisms, i.e. ITS1 and ITS4 as described in Oidtmann et al. [11].

2.3. Transfer to Laboratory and Acclimation in Aquaria

All collected freshwater crayfish samples belonging to *Astacus leptodactylus* species were placed in aquaria. Each aquarium was filled with approximately 50 L of water and was equipped with air pump (Figure 2). After alive crayfish specimens transfer in the lab, each male individual was placed with two females in each aquarium and were fed with commercial growth diet for *Sparus aurata* as basal diet once every week. The water was changed the day after feeding. The temperature was kept stable at ±15 ^{o}C and the conductivity was calculated at ±600 μS/cm. Also, since freshwater crayfish are more active during darkness, all tanks were provided with PVC shelters (Figure 2).

Figure 2: Aquaria where the collected crayfish were placed (left), equipped with a PVC shelter (right)

3. Results

In total, 150 individuals of freshwater crayfish belonging to two species, namely *A. leptodactylus* and *A. astacus*, were collected, half of which were examined for the pathogen *A. astaci* presence, whereas the remaining were placed in lab aquaria. Using the primers 42 and 640 [10], an amplicon of length 569 bp would be expected if the pathogen were present. PCR products corresponding to *A. astac*i were not amplified in any specimen examined (Figure 3). DNA from these individuals was successfully amplified with primers ITS1 and ITS4 (Figure 3) that serve as internal control, corresponding to other organism oomycetes as reported in Oidtman et al. [11]. Thus, all collected crayfish specimens were scored as pathogen free.

Post *A. astaci* detection investigation, pathogen free individuals were placed in the lab aquaria. During their acclimation process many different behaviours were observed. Firstly, some female crayfish produced eggs as shown in Figure 4A. This specific behaviour may be observed owing to stress response of acclimation process. In addition, mating behaviour between individuals was observed (Figure 4B). More specifically, the male on the top observed to hold the female firmly with the chelipeds and the ischial hooks. Similar mating behaviour has been described in Vogt et al. [12] in marbled freshwater crayfish *(Procambarus fallax)*. These results indicate the successful acclimation in the lab aquaria, where freshwater crayfish were kept alive for more than two months.

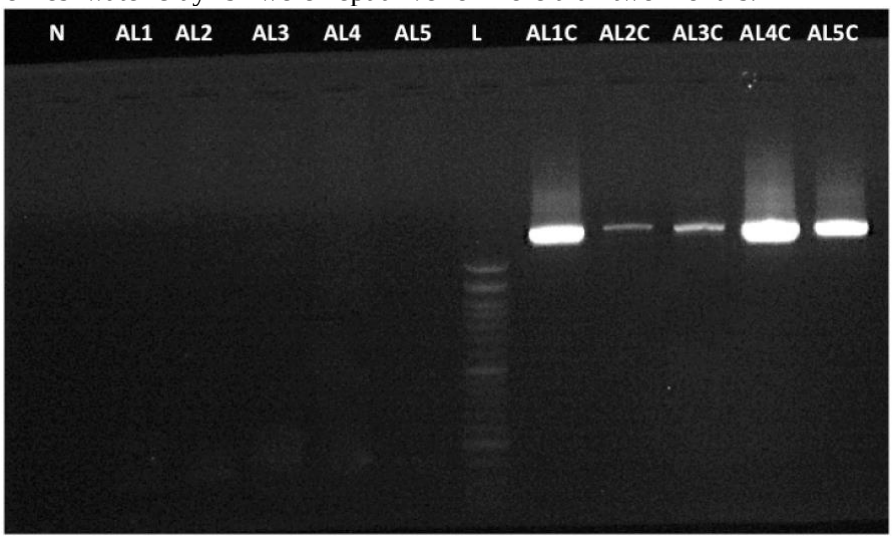

Figure 3: Agarose gel electrophoresis banding patterns of the PCR performed for the investigation of the *A. astaci* presence in tissues of *Astacus* sp. Lane N: negative control; lanes AL1-AL5: *Astacus leptodactylus* tissue samples; Lane L: DNA Mass ladder (Nippon Genetics); AL1C-AL5C: internal controls.

Figure 4: *A. leptodactylus* individual with eggs (A) and two individuals *A. leptodactylus* exhibiting mating behaviour (B)

4. Discussion

Native freshwater crayfish species are vulnerable not only to the pathogen causing the crayfish plague but to overfishing practices as well. Based on the recently observed population reductions, local authorities have forbidden crayfish fishing for commercial purposes in lakes Vegoritida and Polyphytos for one year from September 2021 to September 2022. However, it should be noted that it remains obscure, if this measurement is followed by all local fishermen (personal communication).

Despite its great economic value, freshwater crayfish aquaculture remains underdeveloped in Greece so far. According to economic prediction models, the post-lignite era is expected to lead to increased rates of unemployment in Western Macedonia. These problems could be at some extent mitigated with the development of freshwater crayfish aquaculture. Additionally, aquaculture field development may provide crayfish stock for restocking purposes in an attempt to support biodiversity balance.

All *Astacus* individuals examined were found negative to the pathogen *A. astaci*. Their transfer and acclimation to aquaria was completed successfully and some of them exhibited physiological behaviours such as molting, mating and egg production. However, it should be emphasized that these behaviours may occasionally be observed due to extensive stress [13]. The above indications during acclimation were encouraging for next experimental set ups towards the development of a freshwater crayfish aquaculture protocol in Greece.

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