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Detection of Aphanomyces astaci in spiny-cheek crayfish from selected water reservoirs of north-western Poland

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Summary

Aphanomyces astaci is a fungus-like oomycete agent responsible for an illness called crayfish plague, reaching 100% mortality in infected animals. Therefore, the aim of the work was to detect and estimate the rate of infection of spiny-cheek crayfish (Orconectes limosus) by A. astaci in selected water reservoirs of north-western Poland, as this crayfish is described as a main cause of crayfish plague. The material for the study were 54 spiny-cheek crayfish individuals from 3 sites in Poland: Trzebiocha River, Lake Sominko and Lake Dąbie. A total of 162 samples (muscle samples were taken from abdomen, legs and carapace) were taken and used for DNA extraction followed by PCR and bidirectional sequencing of 5.8S ribosomal RNA gene. The electrophoretic separation of the PCR products confirmed the presence of A. astaci in 17 samples (Trzebiocha River and Lake Dąbie). Lake Sominko proved to be a zone free of the investigated pathogen. The collected information on the presence or absence of A. astaci in the investigated reservoirs might be used for restocking purposes.

Keywords: A. astaci, crayfish plague, restitution program, 5.8S rRNA

Aphanomyces astaci is an oomycete also known as water mould. Oomycetes are a group of filamentous, unicellular heterokonts, which are fungus-like in their growth form and adsorption and formation of spores, but also relatively closely related to photosynthetic algae (11). *A. astaci* may occur in three forms: mycelium, cysts and swimming zoospores (3, 12). Depending on crayfish species the pathogen can cause the lethal state called crayfish plague that can lead to 100% mortality in infected animals. The clinical signs in infected crayfish are non-specific and difficult to identify, particularly since the characteristic symptoms of infection are visible only in the end-stage of crayfish plague disease. A characteristic sign of crayfish plague includes the appearance of brown-red spots on the muscles under the cuticula, while the main infection sites are located within tissues of the abdomen, limb joints, gills, telson and eyes. At the final stages of the infection, signs of neurotoxicity (behavioural changes) are also observed, mainly as an increase in the diurnal activity or leaving water reservoirs and entering land, physiological changes such as loss of movement coordination, increasing paralysis and even autotomy (dropping limbs). The external sign of infection are the visible hyphae of the thallus emerging from areas of soft epidermis such as joints or eyestalks (2). Transmission of *A. astaci* can occur via different routes (13). It can also spread through direct contact with highly infected or asymptomatic crayfish individuals, or via ingestion of infected food, such as fish feeding on infected crayfish (12). The possibility of transmission of crayfish plague via direct contact with infected fish was observed based on the presence of *A. astaci* on scales (4), but also on equipment that had been in contact with contaminated water (1).

In Europe, there are five indigenous crayfish species: the noble crayfish (*Astacus astacus*), the narrow-clawed crayfish (*Astacus leptodactylus*), the Caspian crayfish (*Astacus pachypus*), the white-clawed crayfish (*Austropotamobius pallipes*) and the stone crayfish (*Austropotamobius torrentium*) (19). The noble crayfish (*Astacus astacus*) and the Danube crayfish (*Astacus leptodactylus*) represent the indigenous fauna of Poland

(8). All the above listed species are susceptible to severe infection with water mould. It cannot be clearly stated what the vector of the first wave of crayfish plague in Europe was (1, 6, 9). Despite the various information on introducing crayfish plague to Europe, spiny-cheek crayfish (*Orconectes limosus*) and signal crayfish (*Pacifastacus leniusculus*) mostly play an unquestionable role as a vector of this disease. Observations of the native populations of crayfish in the European waters indicate a significant reduction in the size of their population in relation to the historical data from 150 years ago (1, 19, 20). The defence against the development of *A. astaci* is based on prophenoloxidase (proPO-system) (19). In this type of response, the pathogen is closed within semigranular blood cells, and a layer of granular cells associates to form capsules, which causes an activation of the proPO-system via the degranulation of the granular cells (22). As a result of the proPO-system activation, the pathogen is coated by sticky melanin. Despite the fact that the growth and spread of the pathogen become strongly limited, it remains viable (17). This defence system enables American crayfish species to carry the disease which poses a lethal threat to the indigenous European crayfish species that are highly susceptible to infections and do not exhibit immunological responses (18, 21, 23).

Noble crayfish is one of the indigenous invertebrate species of Poland. Once very abundant, currently, however, it is considered by the International Union for Conservation of Nature and Natural Resources as vulnerable (VU) in Poland, for some regions (Pomerania – NW Poland) its status is estimated as critically endangered (CR) (20). The rate of decline in the recent decades indicates a real possibility of the loss of Noble crayfish as a part of the aquatic biocenoses in next 10 years (20). This species has to be protected from extinction through interdisciplinary restitution programmes applied to carefully selected water reservoirs where no presence of *A. astaci* has yet been confirmed. In spite of the fact that there are some scientific papers on *A. astaci* in north-western Poland (15, 16), the scientific knowledge regarding detection of *A. astaci* in freshwater crayfish seems to be still insufficient. Therefore, the aim of this study was to detect and estimate the rate of infection of spiny-cheek crayfish by *A. astaci* in selected water reservoirs of north-western Poland in order to protect Noble crayfish which are valuable not only for breeders, but also from a biodiversity perspective.

Material and methods

The material for the study were spiny-cheek crayfish individuals from 3 sites in Poland: Trzebiocha River (N:54°3'59.85" E:17°53'43.9", Pomeranian Voivodeship), Lake Sominko (N:54°4'52.97" E:17°52'43.27", Pomeranian Voivodeship) and Lake Dąbie (N:53°24'45.32" E:14°39'40.42", West Pomeranian Voivodeship). The material was harvested in the last quarter of 2015. A total of 54 individuals were obtained: 30 from the Trzebiocha River, 8 from Lake Sominko and 16 from Lake Dąbie. The individuals were collected by free diving or from the shore, using a landing net. Once the crayfish were transported to the laboratory, muscle samples were taken from their abdomen, legs and carapace. A total of 162 samples were taken and used for DNA extraction using the High Pure PCR Template Preparation Kit (Roche, Germany). Qualitative and quantitative assessment of the extracted DNA was conducted by measuring absorbance using the NanoDrop 2000 UV-VIS spectrophotometer (ThermoScientific). Subsequently, the DNA isolates were separated electrophoretically on 1.5% agarose gel. A fragment of the *A. astaci* 5.8S rRNA sequence was detected via PCR using the Aast58R and Aast58F primers, in accordance with the methodology by Holdich (5) (Tab. 1). The results of each PCR were assessed by separating the PCR products on 1.5% agarose gel followed by bi-directional Sanger sequencing of each PCR product. The sequencing was ordered from Genomed (Poland).

Tab. 1. Primer pair used in the study to detect the *Aphanomyces astaci* genome

Primer name	Primer sequence	Reference
Aast58R	ATTCTGCAATTCGCATTACG	Hochwimmer et al. 2009
Aast58F	ATACAACTTTCAACAGTGGATGTCT	

Explanation: the results of sequencing were analysed using the BLAST, MEGA5 and BioEdit software

Results and discussion

The qualitative and quantitative analysis of the obtained DNA isolates demonstrated that the method employing spin columns determines a high degree of purity of the obtained DNA samples (A_{260}/A_{280} = 1.8-2.0). The electrophoretic separation of the PCR products revealed the presence of *A. astaci* in 17 samples. Positive results were obtained for 4 individuals from the Trzebiocha River. The following tissues were infected: leg muscles, carapace and abdominal muscles. In the case of Lake Dąbie, a positive result was obtained for 5 individuals, with all investigated tissue types infected. Lake Sominko proved to be a zone free of the investigated pathogen, which is rather important from the epidemiological point of view. Table 2 lists the number of *O. limosus* individuals against the number of infected individuals and the type of tropism to each tissue and organ.

Bidirectional sequencing confirmed that the isolated fragment of genome corresponded to *A. astaci*. Negative results may indicate the absence of the pathogen or the presence of an amount of *A. astaci* DNA too small to exceed the detection threshold of the reaction.

Infections in crayfish are difficult to diagnose and poorly known due to the crayfish life-style and biology, as well as the small size of their natural populations. The

Tab. 2. List of positive results of the detection of the *A. astaci* DNA

Collection site/Sample type	Trzebiocha River 6/30	Lake Sominko 0/8	Lake Dąbie 11/16
Leg muscles	++	–	+++
Carapace	++	–	++++
Abdominal muscles	++	–	++++

Explanation: + number of positive samples

nature of the infections caused by the pathogens from the Phylum Oomycota, also known as water moulds, is also poorly understood (14). This concerns mainly the time of occurrence of the first symptoms counted from the moment of infection of the host organism. The lack of earlier signs indicating an early stage of infection with *A. astaci* prevents any control and elimination of infected crayfish from the reservoirs or selection of candidates for artificial reproduction. Probably even in the case of monitored crayfish reintroduction, it is not certain that the introduced material is free of *A. astaci*. As reported by Jussila et al. (7), *A. astacus* individuals with no clinical signs of infection demonstrated positive results of *A. astaci* detection using PCR. The analysed population was used commercially. It is hypothesized that crayfish can spread the infection via latent individuals, which further increases the risk of contamination of clean reservoirs with *A. astaci* by crayfish reintroduction. In Slovenia (10), the first infection in a wild crayfish population was reported for stone crayfish, in which clinical manifestations of the disease were observed. The most appropriate conclusion considered was that the pathogen was transmitted to Slovenia along with the first introduction of this crayfish species. The problem of transmission poses an extremely serious threat for the management of crayfish populations in Poland and Europe, but also contributes to the unintentional contamination of water reservoirs considered as free of the disease. Our results indicate the need for caution on all attempts to obtain crayfish from the entire estuary of the Oder River. Due to the physical link between Lake Dąbie and the Regalica River, as well as the Płonia and Chełszcząca Rivers, it is necessary to take special care during selection and restitution activities. The positive results of detection obtained in the crayfish of the Trzebiocha River are worrying, as the river is part of the Wda-Trzebiocha natural reserve. The river also runs into Lake Sudomie, part of the Graniczna–Trzebiocha waterway. Due to the diversity of ichthyofauna, the presence of the pathogen in the Trzebiocha River constitutes a risk of transmitting the disease via the hydrological system. It is believed that there is a possibility of transmitting *A. astaci* spores through water and fish as vectors of infection. However, monitoring the sources of infected crayfish or latent carriers such as fish is problematic. This is due to the fact that several weeks to even several months can pass between infection and the first diagnosis of crayfish mortalities. Introducing juvenile crayfish into water reservoirs is also poses a risk, as no resistant populations have been found to date (14). No vaccines or immunostimulants are available commercially to effectively reduce morbidity by means of the crayfish immune system. The individuals from Lake Sominko, which proved to be free of *A. astaci*, offer the possibility to expand the studies aiming to estimate the genetic similarity of this population to other crayfish populations. It is necessary to characterize populations that are more resistant to infection and use these populations as the basis for selection. The individuals from Lake Sominko constitute potential material for such studies.

Crayfish populations are extremely important for freshwater ecosystems in Poland and around the world. Therefore, identification of "healthy" populations is important for breeding programmes aimed at re-establishing crayfish in water bodies in which crayfish plaque has not been detected. The presented studies clearly indicate that individuals from Lake Sominko, which proved to be free of *A. astaci*, offer the possibility to expand studies aimed at estimating the genetic similarity of this population to other crayfish populations. However, it is necessary to characterize populations that are more resistant to infection and use these populations to develop a stock for selection. The individuals from Lake Sominko constitute potential material for such studies.

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