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Detection, distribution, diversity and phylogeny of the crayfish plague pathogen *Aphanomyces astaci* (Oomycetes)

Detekce, rozšíření, diverzita a fylogeneze původce račího moru *Aphanomyces astaci* (Oomycetes)

Ph.D. THESIS

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I declare that this thesis has not been submitted for the purpose of obtaining of the same or another academic degree earlier or at another institution. My involvement in the research presented in this thesis is expressed through the authorship order of the included publications and manuscripts. All literature sources I used when writing this thesis have been properly cited.

Eva Kozubíková

Prague, 15 July 2011

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Preface and acknowledgements

This thesis is a result of my already eight-year research focused on one of the most devastating diseases of invertebrates, crayfish plague. When I became interested in a molecular diagnostics of this disease as a topic offered for my MSc. thesis in 2003, I did not guess at all that I would spend on this research so long time and that it would develop to the present extent.

However, I did not work alone all the time... Until 2007, when I defended my MSc. thesis entitled “Molecular detection of the crayfish plague pathogen and its distribution in the Czech Republic” (already containing two publications not included to this thesis), only myself under the guidance of my supervisor worked at this topic at our department. During later years, when I continued the research as a Ph.D. student, our “crayfish plague team” was extended by three more students, Jiří Svoboda, Klára Matasová, and Lenka Filipová. It was a real pleasure to cooperate with them and I would like to thank them a lot for their work. Special thanks go to our supervisor Adam Petrusek for his invaluable advice and support during all the years and for language corrections of this thesis. I thank also Vojtěch Jarošík from our department who helped significantly with most of the statistical analyses presented in this thesis and Ondřej Koukol from the Department of Botany who provided facilities for our work with oomycete cultures.

A very nice cooperation during all the years was also with our colleagues from other Czech institutions, especially the Research Institute of Fish Culture and Hydrobiology in Vodňany (Pavel Kozák and his team), Department of Biology and Ecology of the University of Ostrava (Zdeněk Ďuriš and Ivona Horká) and the Agency for Nature Conservation and Landscape Protection of the Czech Republic (Monika Štambergová and others).

However, research of crayfish plague reacts to the whole-European need to protect indigenous crayfish species, and it is not possible to work on this topic within borders of a single country. Therefore, we established contacts with important European centres of crayfish plague research, especially the Royal Botanical Garden in Madrid, Spain (Javier Diéguez-Uribeondo, María Paz Martín), the Centre for Environment, Fisheries & Aquaculture Science (Cefas) in Weymouth, England (Birgit Oidtmann), the Norwegian Veterinary Institute in Oslo, Norway (Trude Vrålstad) and the Finnish Food Safety Authority (Evira) in Kuopio, Finland (Satu Viljamaa-Dirks, Sirpa Heinikainen). I would like to thank all the mentioned colleagues for their cooperation.

To complete acknowledgements, I am especially grateful to my family for their interest in my work and general support. In fact, they were those who supported my interest in anything alive when I was a child and thus they participated strongly in my achievements. Finally, my partner, friends, schoolmates and other colleagues, too many to list them, were with me in joyful as well as stressful situations during my studies for which they deserve many thanks. One special thank goes to Eva Hamrová with whom we took care of the molecular laboratory of the Department of Ecology and could divide this technical work to have enough time for our research projects and theses writing. Without all these people somehow involved, it would be much more difficult to finish this thesis. Once again, thank you all...

Abstrakt (in Czech)

Moje dizertační práce se zabývá různými aspekty výzkumu původce račího moru, *Aphanomyces astaci* (Oomycetes). Račí mor hubí celé populace původních evropských raků a je tak jedním ze zásadních problémů ochrany těchto živočichů, ohrožených i řadou dalších faktorů. Infekce je přenášena invazními druhy raků pocházejícími ze Severní Ameriky, kteří jsou vůči onemocnění mnohem odolnější a jsou zřejmě původními hostiteli *A. astaci*.

Těžiště této práce spočívá v šesti studiích, z nichž čtyři byly již publikovány a u dvou probíhá recenzní řízení pro publikaci. Pojítkem kapitol uvedených v mé dizertační práci není jen samotný původce račího moru, ale také využití, vývoj a ověřování spolehlivosti molekulárních metod detekce *A. astaci* a jeho různých genotypů.

Jádro práce je rozděleno do dvou částí. První z nich obsahuje čtyři studie, ve kterých se zabývám rozšířením *A. astaci* v populacích severoamerických raků v České republice (**kapitola 1**) a v Maďarsku (**kapitola 2**) a také faktory, které rozšíření parazita ovlivňují. Poznání rezervoárů nákazy totiž může přispět ke zlepšení ochrany původních raků. V uvedených zemích se vyskytují ve volné přírodě dva druhy severoamerických raků, rak pruhovaný (*Orconectes limosus*) a rak signální (*Pacifastacus leniusculus*). První dvě kapitoly ukazují, že míra nákazy u populací těchto raků může být značně rozdílná a může souviset s hustotou a historií populace a typem habitatu. Zdá se, že jako přenašeč *A. astaci* je ve zkoumané oblasti problematičtější spíše rak pruhovaný. První část práce dále ukazuje, že nakaženost populací raků pruhovaných nebo alespoň detekovatelnost nákazy molekulárními metodami může meziročně i mezisezónně kolísat (**kapitola 3**). Zároveň jsem zjistila, že výsledky detekce *A. astaci* mohou být částečně ovlivněny i použitou metodikou (**kapitola 4**), proto je potřeba výsledky používaných molekulárních detekčních postupů interpretovat opatrně a ověřovat je pokud možno více přístupy.

Druhá část práce je věnována studiu vnitrodruhové diverzity a fylogeneze *A. astaci*. **Kapitola 5** dokládá, že rak pruhovaný je přenašečem dříve neznámého genotypu patogenu. Studium těchto genotypů a jejich srovnávání s *A. astaci* z hynoucích raků může přispět ke zjišťování zdrojů nákazy. **Kapitola 6** přináší detailní fylogenetickou studii rodu *Aphanomyces* provedenou na základě genu pro ITS (internal transcribed spacers) v ribozomální jaderné DNA. Studie ukazuje tři jasně oddělené vývojové linie odpovídající životním strategiím oomycetů (paraziti rostlin, paraziti živočichů a saprobionti). Zároveň shrnuje většinu dostupných sekvencí ITS druhů tohoto rodu a vytváří tak rámec pro další vývoj molekulárních metod detekce parazitických druhů.

V příloze dizertační práce uvádím další tři studie související s výzkumem račího moru, které vznikly v průběhu mého studia. **Kapitola 7** se zabývá úhyny raků v souvislosti s račím morem v České republice. **Kapitola 8** se věnuje dlohodobému přežívání *A. astaci* v Turecku v populaci původního raka bahenního (*Astacus leptodactylus*) a v **kapitole 9** se zaměřuji na poznání druhů oomycetů, které se vyskytují na racích a mohou komplikovat izolaci *A. astaci*.

Abstract

My Ph.D. thesis deals with various aspects of research of the crayfish plague pathogen, *Aphanomyces astaci* (Oomycetes). Crayfish plague decimates whole populations of European crayfish; therefore, it is one of the main problems for conservation of these species endangered from other reasons as well. The infection is transmitted by invasive North American crayfish, which are much less susceptible to the disease and are apparently original hosts of *A. astaci*.

The core of this thesis consists of six studies. Four of them have been already published, the remaining two are manuscripts under review. The main linking motive among the presented studies is not only the crayfish plague pathogen itself, but also the use, development and verification of the reliability of molecular detection methods of *A. astaci* and its genotypes.

This core of the thesis is further divided into two parts. The first one contains four studies that deal with the distribution of *A. astaci* in North American crayfish populations in the Czech Republic (**Chapter 1**) and Hungary (**Chapter 2**), and factors influencing this distribution. Two North American crayfish species can be found in the wild in these countries, the spiny-cheek crayfish (*Orconectes limosus*) and the signal crayfish (*Pacifastacus leniusculus*). The first two chapters show that the prevalence of infection in North American crayfish populations varies and may be associated with population density and history, and habitat type. It seems that the spiny-cheek crayfish is more problematic as a carrier of *A. astaci* in the studied regions. Knowledge on the infection reservoirs is needed for efficient planning of conservational efforts. The first part of the thesis also shows that the prevalence of the parasite or at least its detectability by molecular methods may vary not only in space but also in time (**Chapter 3**). Finally, the results of the pathogen detection may be partly influenced by the used detection method as documented in **Chapter 4**. Therefore, it is necessary to interpret the results of the used molecular detection methods with caution and verify them using more approaches.

The second part of the thesis is dedicated to study of intraspecific diversity and phylogeny of *A. astaci*. **Chapter 5** documents that the spiny-cheek crayfish carries a novel *A. astaci* genotype. Comparison of genotypes from American crayfish and dying indigenous crayfish may assist uncovering sources of infection. **Chapter 6** brings a detailed phylogenetic study of the genus *Aphanomyces* based on the gene for ITS (internal transcribed spacers) of the ribosomal nuclear DNA. The study shows three clearly distinct lineages reflecting life strategies of oomycetes (plant parasites, animal parasites, and saprobionts). Furthermore, this study summarises most of the known ITS sequences of *Aphanomyces* spp. and provides a framework for further development of molecular detection methods for parasitic species.

As appendices to my thesis, I present additional three studies related to crayfish plague research, which were conducted during my study years. **Chapter 7** focuses on the recent crayfish plague outbreaks in Czechia. **Chapter 8** deals with the long-term coexistence of *A. astaci* and indigenous narrow-clawed crayfish (*Astacus leptodactylus*) in Turkey. And finally, in **chapter 9**, I describe diversity of oomycetes that can be found on crayfish and complicate isolation of *A. astaci*.

Introduction

Crayfish plague is the most damaging disease of crayfish. It has been devastating indigenous crayfish stocks in Europe already for about 150 years. Its causative agent is even listed among the 100 of the world's worst invasive species (Lowe et al. 2004). Unfortunately, we are still far from effective protection of susceptible crayfish from crayfish plague outbreaks, and further research is needed to look for new solutions. However, before I start to deal with crayfish plague itself and my research results, I would like to introduce generally also crayfish, as they were the primary reason for me to study the disease. By this research, I wanted to contribute to the knowledge potentially useful to conservation of endangered crayfish species.

1. Freshwater crayfish

1.1. Natural distribution and diversity

Freshwater crayfish (Astacida) is a highly diversified group of crustacean order Decapoda. There are about 600 crayfish species known at present (Sinclair et al. 2004) in three families. Astacidae and Cambaridae occur originally only in the northern hemisphere and Parastacidae inhabit the southern hemisphere (Souty-Grosset et al. 2006). Hot spots of crayfish diversity are in North America and Australia. On the other hand, large areas of Asia and South America and the whole African continent have no indigenous crayfish (Souty-Grosset et al. 2006) and low diversity of native crayfish can be found in Europe (section 1.4.).

1.2. Ecology

Crayfish are considered key species of many continental waters, being the largest benthic freshwater invertebrates occurring often in high densities. As omnivorous organisms, crayfish may substantially influence populations of other benthic animals as well as plants (Nyström et al. 1996). Together with consumption of decaying organic material, crayfish may accelerate recycling of nutrients in their environment (Usio et al. 2006).

Populations of crayfish are naturally regulated by their competitors and predators, and by various parasites (e.g., viruses, bacteria, protists, fungi or metazoans; for a detailed review on crayfish parasites see Longshaw 2011). Crayfish and these organisms underwent a long co-evolution, often resulting in well-balanced relationships. Disturbing these relationships, for example, by a loss of species or by introduction of exotic ones, may lead to alterations in the whole ecosystem.

In many regions, crayfish are also a traditional part of human diet and culture, and a socioeconomic value of their stocks is high (Holdich 1993, Gherardi 2011).

1.3. Human impact and invasive species

Natural distribution of crayfish has been remarkably changed by human activities during the last centuries. Species potentially attractive for consumption have been introduced for aquaculture

purposes to areas outside their original range. In addition, pet trade flourishing at present contributes to moving crayfish around the world (Faulkes 2010). Intentional as well as accidental releases of non-indigenous crayfish to open waters often lead to establishment of their viable populations (Souty-Grosset et al. 2006). Consequently, several species, especially of North American cambarids, became very successful invaders in new areas of North America (Lodge et al. 2000), Europe (section 1.4.) and some other parts of the world (e.g., Kawai et al. 2009).

Typical characteristics of a successful crayfish invader are fast reproduction, low sensitivity to altered environmental conditions, aggressiveness and good migratory abilities (Lindqvist and Huner 1999). Invasive crayfish may cause economic losses in agri- and aquaculture and have negative influence on freshwater communities (Holdich 1999). Further, they may outcompete indigenous crayfish and permanently occupy their habitats (Schulz et al. 2006). Moreover, together with these crayfish also their parasites and generally any microbiota potentially dangerous for local species may be spread (**chapter 9**). After all, this thesis is devoted to an extreme example of such situation, the dispersal and impact of crayfish plague.

On the contrary, many crayfish species are on severe decline at present; about 20 % of all crayfish species have critically endangered or endangered status according to the International Union for Conservation of Nature (www.iucnredlist.org). The main reasons for this situation are degradation of crayfish habitats (e.g., by regulation of watercourses), water abstraction and pollution, intensive agriculture and aquaculture activities, overfishing of crayfish, and spread of invasive crayfish and exotic parasites as mentioned above.

1.4. Crayfish in Europe

The taxonomy of European indigenous crayfish is still not completely resolved. Souty-Grosset et al. (2006) mention six species within two genera: noble crayfish (*Astacus astacus*), narrow-clawed crayfish (*Astacus leptodactylus*), thick-clawed crayfish (*Astacus pachypus*), stone crayfish (*Austropotamobius torrentuim*), and white-clawed crayfish separated to two species, *Austropotamobius pallipes* and *A. italicus*, based on genetic data. However, it seems that some of the mentioned species are rather species complexes (e.g., Trontelj et al. 2005). Moreover, there are also authors who suggest distinguishing even more crayfish genera in Europe (Starobogatov 1995, Smietana et al. 2006).

Crayfish fauna of European open waters has been “enriched” by at least eight species originating from North America up to now (Filipová et al. 2011). There are three so called “old non-indigenous crayfish species”, introduced before 1980 mainly for aquaculture purposes and widespread in many European countries at present (Holdich et al. 2009): the spiny-cheek crayfish (*Orconectes limosus*), the signal crayfish (*Pacifastacus leniusculus*) and the red swamp crayfish (*Procambarus clarkii*). These species are still invading new areas rapidly (e.g., Barbaresi and Gherardi 2000, Puky and Schád 2006, Skov et al. 2011) and eradication of their populations is almost impossible (Gherardi et al. 2011).

In addition to those, “new non-indigenous crayfish species” (several species of the genera *Orconectes* and *Procambarus*) appeared in the wild in Europe during the last two decades

(Holdich et al. 2009, Filipová et al. 2011). Apparently, these species originate from aquarium cultures and their distribution is still limited. However, they may become widespread in Europe in the future.

2. Crayfish plague

Crayfish plague is one of the most intensively studied diseases of invertebrates and much knowledge has been gathered during more than a century of its research. However, one must realise that to find out what we know now and what we consider as a general knowledge, is a result of a complicated process full of failed attempts and dead ends. Obtaining the present knowledge needed brilliant brains and dedication of many years of intensive work. In this section, I summarise the basic information on the disease.

2.1. Causative agent of crayfish plague and its life cycle

The crayfish plague pathogen *Aphanomyces astaci* (Schikora, 1903) belongs among saprolegnious oomycetes, the main life form of which is a fungal-like mycelium growing in a substrate (Cejp 1959). These organisms produce asexually biflagellate zoospores in order to colonize new sources of nutrients. The other way of reproduction in oomycetes is a sexual life cycle including female oogonia and male antheridia (Johnson et al. 2002). Oospores resulting from the sexual process are (unlike sensitive short-lived zoospores) durable propagules, which can withstand drying (Cejp 1959, Oidtmann et al. 2002b).

As far as it is known, *A. astaci* undergoes only an asexual life cycle (Unestam 1969a; Figure 1) similarly as some other saprolegnious oomycetes parasitic in aquatic animals (**chapter 6**). Hyphae growing outside the crayfish tissues produce sporangia, which release primary spores (Olson et al. 1984). They encyst directly after the release from a sporangium and stay attached together, forming so called “spore balls” of primary cysts. These spore balls are typical for the whole genus *Aphanomyces* (Johnson et al. 2002). Secondary spores (zoospores) released from primary cysts are mobile and able to infect a new host. After attachment to the crayfish cuticle, the zoospore encysts again, germinates, and a new hypha grows inside the host (Cerenius et al. 1988). Germination of the cysts seems to be activated by a specific chemical signal present in crayfish (Söderhäll and Cerenius 1999). If the cyst attaches on an unsuitable substrate, it can repeatedly transform back to the zoospore and continue in searching for a suitable host (this process is called “repeated zoospore emergence”; Cerenius and Söderhäll 1984).

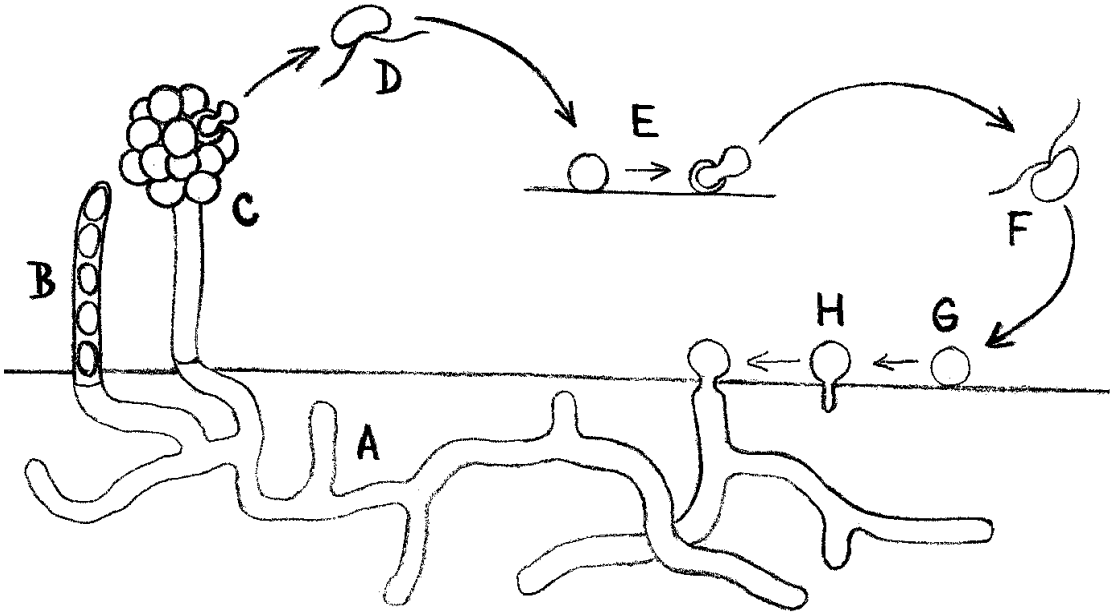

Figure 1. Life cycle of the crayfish plague pathogen *Aphanomyces astaci*. Mycelium growing in the crayfish cuticle (A), sporangium protruding to water (B), "spore ball" of primary cysts (C), zoospore of the first generation (D), encystment on a wrong surface and "repeated zoospore emergence" (E), zoospore of the second generation (F), a cyst attached on crayfish cuticle (G), germinating cyst (H). Original hand-drawn picture inspired by Cerenius et al. (1988) and already published in Kozubíková and Petrusek (2009).

2.2. Hosts of the crayfish plague pathogen

Up to the present knowledge, *A. astaci* seems to be a specific parasite of crayfish (Unestam 1972), although other freshwater decapods might possibly be infected as well (Benisch 1940).

Crayfish species originating from North America (hereafter "American") are well adapted to the coexistence with the parasite (Söderhäll and Cerenius 1999) and die from the infection only if their immune response is strongly weakened, typically by stress (Cerenius et al. 1988), or when infection doses are very high (Diéguez-Uribeondo and Söderhäll 1993). This evidence suggests that *A. astaci* originates from North America and coevolved with local crayfish hosts. American crayfish are capable of strong immune reaction against *A. astaci*, which first consists in quick production of enzymes synthesising melanin and other substances, which inhibit growth of the parasite in the cuticle (Söderhäll and Ajaxon 1982; more details on the crayfish immunity in context of *A. astaci* gives Cerenius et al. 2003). However, *A. astaci* usually remains alive in the host and is able to produce zoospores, which leads to transmission of the infection to other crayfish individuals. Thus, American crayfish may act as chronic carriers of *A. astaci* (Persson and Söderhäll 1983, Vey et al. 1983, Diéguez-Uribeondo and Söderhäll 1993). The infection may be sometimes visible as melanised spots in the cuticle but such depositions do not seem to be *A. astaci*-specific (**chapter 1**), as melanisation is a general immune reaction to various parasites in invertebrates (Söderhäll and Cerenius 1999).

On the contrary, immune reaction to *A. astaci* is much slower in crayfish from other parts of the world (Cerenius et al. 2003), so they usually succumb to an unlimited growth of the pathogen in their body (Unestam 1969b, 1975). Although crayfish plague outbreaks mostly lead to quick losses of the entire susceptible crayfish populations (Reynolds 1988, Alderman 1993, **chapter 7**), there are important exceptions from this rule leading to "chronic infections" of waterbodies (e.g. Fürst 1995, Viljamaa-Dirks 2008ab, **chapter 8**).

2.3. Transmission of crayfish plague

As far as we know, *A. astaci* does not survive long without the suitable host (Söderhäll and Cerenius 1999). Thus, the primary sources of infection are infected crayfish. The only infective units, zoospores, survive no more than a few weeks in wet conditions (Alderman et al. 1987, CEFAS 2000), usually substantially shorter (Diéguez-Uribeondo, pers. comm.). Infected crayfish may move to the vicinity of the healthy crayfish populations themselves, or be translocated by people or crayfish predators (Oidtmann et al. 2002b). Further, living zoospores may be transmitted with water or any wet item during human activities such as fishing (Alderman et al. 1987, Reynolds 1988), or possibly with animal movements in the wild. However, the sources of infection and transmission ways of the pathogen of particular crayfish plague outbreaks often remain unclear (Cammà et al. 2010, **chapter 7**), which complicates the disease management.

2.4. History and presence of crayfish plague in Europe and Middle East

The first crayfish mass mortalities presumably caused by *A. astaci* appeared in Italy in the late 1850s (Alderman 1996). During the following decades, the disease spread throughout the continental Europe, leading to massive decimation of indigenous crayfish populations (as reviewed in detail by Alderman 1996).

In some countries such as Finland or Sweden, *A. astaci* seems to have persisted in residual indigenous crayfish populations in complex lake systems from the first wave of crayfish plague outbreaks up to present (Wennerström et al. 1998) preventing restoration of crayfish stocks (Fürst 1995). In most other areas, the pathogen may have disappeared together with the susceptible crayfish that it infected (Matthews and Reynolds 1990, Alderman 1993). However, *A. astaci* started to spread again in later decades of the 20th century as a result of intentional introductions of American crayfish (Alderman 1996), which should have compensated for the lost indigenous crayfish (Bohman et al. 2006).

Losses of indigenous crayfish populations continue in many areas at present (e.g. Bohman et al. 2006, Hochwimmer et al. 2009, Cammà et al. 2010), endangering survival of these species (Holdich et al. 2009) and reducing economic profit from crayfish stocks (Viljamaa-Dirks 2008a, Paaver and Hurt 2009). However, experience with the disease history may differ among various regions. Two different cases are described in appendices of this thesis:

Chapter 7 describes the situation in the Czech Republic, where crayfish plague is primarily a conservational issue, as indigenous crayfish are endangered species not exploited commercially. Crayfish were decimated at this territory by crayfish plague at the turn of the 19th and 20th

centuries (Krupauer 1968). Very limited records have been available about this disease from later periods of the 20th century (Kozubíková et al. 2006). However, we confirmed *A. astaci* as a cause of nine crayfish mass mortalities and reported on a few additional suspected cases from a short period of 2004 to 2009 (**chapter 7**, Kozubíková and Petrusek 2009, and unpublished data), which suggests that this problem was either underestimated before or crayfish plague is on increase at present. *A. astaci* seems to have disappeared spontaneously after the outbreaks from localities where American crayfish were not found. Thus, reintroduction of indigenous crayfish might be possible. However, transmission pathways of the infection are often unknown, which leaves such localities under the permanent risk of further crayfish plague outbreaks. Similar situation as in Czechia may be also in other Central and Eastern European countries, in which crayfish plague is still understudied.

A completely different experience with crayfish plague has been in Turkey, as discussed in the **chapter 8**. Indigenous narrow-clawed crayfish populations inhabiting large Turkish lakes represent economically important resources. The country was affected by the disease outbreaks no earlier than in the mid-1980s. However, it seems that the *A. astaci* genotype, which affected Turkish populations, was the one that had caused the first wave of the crayfish plague outbreaks in Europe (Huang et al. 1994). Crayfish stocks collapsed in many Turkish lakes, leading to economic losses but some populations partly recovered after the disease outbreaks, exceptionally even to exploitable densities. American crayfish species have never been found in Turkey (Harlioğlu and Harlioğlu 2006) but *A. astaci* seems to be still present in some crayfish populations, as we confirmed by DNA analyses in the studied Lake Eğirdir. This finding suggests a coexistence of the local crayfish and *A. astaci* for more than two decades. Reasons for this coexistence are not clear. However, this situation resembling crayfish plague history in large Scandinavian lakes creates an exciting field for further research.

3. Diagnostic methods of the crayfish plague pathogen

3.1. Methods assisting *Aphanomyces astaci* determination

There are no reliable species determination characteristics on hyphae and sporangia of *A. astaci* (Royo et al. 2004). Therefore, microscopic observation of mycelium fulfilling the parameters for *A. astaci* (summarised in Cerenius et al. 1988) in crayfish cuticle or laboratory cultures may have only a supporting value for the species determination (Cerenius et al. 1988, Edgerton et al. 2004), and records of the species presence from various substrates based on morphological characters (Czeczuga 2002, 2004) are doubtful.

In case of crayfish mass mortalities, circumstances of the condition should be assessed. If there is no effect of the mortality on other organisms than crayfish, the mortality is spreading upstream and crayfish in different health state (alive, dying as well as dead) are found at the same time, a mortality from a crayfish-specific disease is highly suspected (**chapter 7**).

3.2. Cultivation of *Aphanomyces astaci*

First successful isolation of *A. astaci* from infected crayfish was performed by Nybelin in the 1930s. An experimental infection of susceptible crayfish with zoospores produced by the obtained cultures confirmed that *A. astaci* was the causative agent of crayfish plague (Söderhäll and Cerenius 1999). This happened about 70 years after the presumably first occurrence of the disease in Europe, which demonstrates the difficulties to recognize and isolate the crayfish plague pathogen.

For the rest of the 20th century, a cultivation followed by infection experiments remained the only reliable way of the parasite diagnosis. Although cultivation methods have been gradually improving (Unestam 1965, Alderman and Polglase 1986, Cerenius et al. 1988, Oidtmann et al. 1999, Viljamaa-Dirks and Heinikainen 2006), isolation and cultivation of the pathogen is still complicated and requires experienced personnel. Obtaining laboratory cultures of *A. astaci* is particularly difficult from American crayfish, in which the parasite growth is limited by melanisation (Cerenius et al. 1988, **chapter 5**).

The main source of problems in *A. astaci* isolation is its slow growth in artificial media (**chapter 6**). Therefore, this species is highly sensitive to overgrowing with bacteria and other faster-growing oomycetes, which are commonly present on crayfish. A contribution to the knowledge of diversity of oomycetes that may use crayfish as hosts and complicate *A. astaci* isolation is provided in the **chapter 9**. In that study, I attempted to determine oomycete cultures obtained during my efforts to isolate *A. astaci* from presumably infected Czech crayfish by both morphological and molecular approaches (the determination part of this work was performed under the guidance of a specialist on oomycetes Javier Diéguez-Uribeondo during my research stay in the Royal Botanical Garden in Madrid in 2008).

Although more reliable molecular methods are available for a routine parasite diagnostics nowadays (section 3.3.), cultivation of *A. astaci* remains very important. Molecular detection methods are based on comparison with the standard DNA obtained from pure laboratory cultures of the parasite (Oidtmann et al. 2006, Vrålstad et al. 2009) and cultivation of *A. astaci* is still necessary for determination of parasite genotypes (**chapter 5**). Further research of *A. astaci* (e.g., studies of its physiology, host specificity and transmission) and development of molecular detection methods require clean laboratory cultures. Finally, cultivation of the pathogen, if successful, may corroborate the molecular detection results and it is desirable at least in some cases.

3.3. Molecular detection of *Aphanomyces astaci*

During the last decade, methods based on the analysis of DNA became available for diagnostics of the crayfish plague pathogen with the aim to avoid infection experiments and cultivation. The first such method employed the restriction fragment length polymorphism (RFLP) analyses, and was suitable only for determination of *A. astaci* in pure cultures (Oidtmann et al. 2002a). Later, methods were developed based on presumably species-specific polymerase

chain reactions (PCR), which allowed detection of *A. astaci* DNA directly in DNA extracts from crayfish tissues.

The internal transcribed spacers (ITS) in ribosomal nuclear DNA has been a marker of choice for development of the *A. astaci*-specific assays, similarly as in the detection methods for other pathogenic fungal-like microorganisms (e.g. Boyle et al. 2004, Vandersea et al. 2006). There are several reasons for choosing ITS: 1) a suitable level of variability enabling to distinguish species of these organisms (Oidtmann et al. 2004), 2) its presence in multiple copies per genome (Álvarez and Wendel 2003), which increases the sensitivity of detection, and 3) availability of general primers for amplification of this DNA region for fungal-like organisms (White et al. 1990). Conventional (Oidtmann et al. 2004, 2006) as well as quantitative real-time PCR (Vrålstad et al. 2009) ITS-based protocols for *A. astaci* detection are available at present. The second mentioned approach allows quantification of the target DNA in samples and thus, a rough estimation of the parasite amount in crayfish individuals.

The only attempt to use an alternative marker for *A. astaci* detection is the method developed by Hochwimmer et al. (2009). As *A. astaci* in contrast to other oomycetes permanently expresses chitinase (Andersson and Cerenius 2002), a gene for this enzyme has been chosen to develop specific PCR assays. The comparison of various methods by Tuffs and Oidtmann (2011) suggests that chitinase-based detection is specific to *A. astaci* but substantially less sensitive than ITS-based methods. However, the chitinase- and ITS-based PCR methods showed partly inconsistent results when applied to the same set of DNA extracts from crayfish cuticles (Kozubíková, unpublished data). Thus, further research is desired to elucidate the source of these discrepancies.

History of development of the above mentioned methods, and their advantages and disadvantages are described in **chapter 4** and in Tuffs and Oidtmann (2011).

Molecular detection methods of *A. astaci* allowed faster and more reliable diagnostics of crayfish mass mortalities, which is crucial for appropriate management of such cases. These methods have also facilitated further research of *A. astaci* (for examples, see part 1 of this thesis and Strand et al. 2011). However, development of species-specific detection methods applicable to DNA extracts from crayfish tissue is a continuous process largely depending on the level of knowledge on other organisms potentially occurring on crayfish, especially oomycetes mostly related to *A. astaci*. Cross-reaction with DNA of such organisms may lead to obtaining false positive results (**chapters 1** and **4**). Another source of false detections may be laboratory contamination, especially in the highly sensitive real-time PCR approaches. On the contrary, false negative results may stem from PCR inhibition (**chapters 4** and **8**) in case of low-quality DNA extracts, or from the use of less sensitive methods (**chapter 4)** and reagents. Therefore, molecular detection methods should be used and their results interpreted with care, and combined with other approaches to confirm correct pathogen detection when appropriate.

Outline of publications and manuscripts

The core of my Ph.D. thesis consists of six studies (four published papers, and two manuscripts at different stages of the review process), three more studies relevant to the topic are included as appendices. In my research, I have been especially interested in distribution of the crayfish plague pathogen in populations of American crayfish, potential reservoirs of infection (**chapters 1** to **4**). A draft version of the **chapter 1** has already been included in my MSc. thesis but it has been partly extended and significantly edited during my Ph.D. studies. Furthermore, I participated in the research of the diversity and phylogeny of *A. astaci* (**chapters 5** and **6**). Therefore, I divided the core of the thesis to two parts, which, however, are interconnected. The main linking motive of my whole study is not only the crayfish plague pathogen itself, but also molecular detection methods of *A. astaci* and its genotypes, practical applications of these methods, and improving knowledge allowing their further development.

The three additional chapters included to the thesis as appendices demonstrate other research topics I participated in during my Ph.D. studies. **Chapter 7** dealing with crayfish mass mortalities in the Czech Republic stems from my MSc. thesis, in which it has been included as an unpublished manuscript. Later, it has been slightly extended and published. The two remaining studies, on the detection of *A. astaci* in a Turkish lake (**chapter 8**) and on the diversity of oomycetes cultivated from crayfish (**chapter 9**), are attached in the form of unpublished manuscripts and can be considered as "by-products" of our research activities. **Chapters 7** and **8** are discussed in the section 2.4., and the **chapter 9** is mentioned in the section 3.2.

Part 1: Distribution of *Aphanomyces astaci* in American crayfish populations and factors that influence the distribution

As American crayfish are the predominant sources of the crayfish plague pathogen in Europe (sections 2.2., 2.4.), data on the distribution and prevalence of *A. astaci* in their populations may assist management of the disease. However, detection of the parasite in American crayfish had been extremely difficult before molecular detection methods for *A. astaci* appeared and, therefore, the knowledge on this topic was very limited (Cerenius et al. 1988, Nylund and Westman 2000).

In **chapter 1**, we brought the first large-scale screening of the prevalence of *A. astaci* in populations of invasive American crayfish based on molecular detection methods. We showed here that American crayfish populations may substantially vary in the prevalence of the pathogen and, thus, probably also in the potential of the infection transmission to the indigenous crayfish populations. The prevalence seems to be related much more to the crayfish species and locality type connected to the population history than to the size, sex, or the presence of melanised spots in the cuticle of the crayfish individuals. In Czechia, the signal crayfish appeared to be much less infected than the spiny-cheek crayfish, and the populations of the latter species from running waters were evaluated as the most problematic reservoirs of infection. In the paper, we suggested possibilities of using such data in indigenous crayfish conservation.

From this keystone study emerged questions for further research. We were interested 1) if a similar pattern in the pathogen prevalence occurs also in other regions with the presence of the same American crayfish species, 2) whether there are temporal changes in the prevalence (detectability) of the pathogen and, 3) if the results would be substantially changed by the use of alternative molecular detection methods. We attempted to answer these questions in the subsequent studies.

Chapter 2 first confirmed the presence of infected American crayfish in Hungary, where the same two invasive species as in Czechia are present and spreading. The data obtained from Hungary were too limited to compare fully the situation in both countries. However, *A. astaci* was detected in both, signal crayfish as well as spiny-cheek crayfish, and the prevalence of the parasite markedly varied, roughly resembling some aspects observed in Czechia. What is, however, most important in this paper, is the finding that infected invasive crayfish are present in very close vicinity of populations of endangered stone crayfish. This highlights the need of this kind of studies for indigenous crayfish conservation.

In the **chapter 3**, we showed that the prevalence of the parasite in the populations of American crayfish (or at least its detectability) may vary not only spatially but also temporally. In one of three investigated Czech spiny-cheek crayfish populations, we observed a decrease of individuals that tested positive for *A. astaci* during an eight-year study. Moreover, less infected crayfish were usually detected in autumn (after the main period of crayfish moulting) than in spring. These findings suggested that: 1) the results of the two previous studies might have been partly influenced by the crayfish sampling performed in different years and seasons, 2) crayfish should be collected preferably in spring for future similar studies, 3) if the population density plays a role in the decrease of the parasite in American crayfish populations as we suggested in the study, management leading to the crayfish population density decrease (e.g., stocking of predatory fish) could result in at least partial suppression of the parasite. However, if there are any factors which may cause a decrease of the parasite prevalence, other factors resulting in its increase cannot be excluded as well. Finding out the determinants of the pathogen prevalence in American crayfish should be thus an important line of further research.

The last study of this part of the thesis focused on reliability of the molecular detection method used in the previous studies based on the semi-nested PCR after Oidtmann et al. (2006). In the course of my master studies, I learnt this procedure at my study stay at the Institute of Zoology, Fishery Biology and Fish Diseases of the Ludwig-Maximilians University in Munich. I established it in the laboratory of the Department of Ecology and applied on crayfish samples from Czechia and Hungary (**chapters 1** and **2)**. In the study on temporal changes of the pathogen prevalence (**chapter 3)** we also used the same method to obtain data comparable to the previous results from **chapter 1**.

However, the authors of the semi-nested PCR assay already found that it may exceptionally suffer from false positive results caused by the cross-reactions with the DNA of oomycetes closely related to *A. astaci* (Oidtmann et al. 2006). Therefore, I re-analysed most of the DNA extracts still available from the studies in **chapters 1** and **2** with a later-developed advanced

molecular detection method based on quantitative TaqMan MGB real-time PCR, which promises higher specificity and sensitivity of detection (Vrålstad et al. 2009). I had a chance to do so directly in the laboratory where the method has been first established, at the Norwegian Veterinary Institute in Oslo.

Results from this stay are presented in the **chapter 4**. We found that the previous results were not significantly influenced by the false positive results, which shows that conventional PCR methods are robust enough for the research presented in the previous chapters. On the contrary, our previous results appeared to be underestimated. The number of crayfish found to be infected increased by about 10 % when using the real-time PCR, supporting its claimed higher sensitivity. Although the increase in the parasite detections seems to be striking, only very low amounts of the target DNA were found in all newly detected parasite carriers. Thus, the general conclusions from **chapters 1** and **2** remained valid. Further interesting result of this study was a positive correlation between the prevalence of infection carriers in American crayfish populations and the average amounts of *A. astaci* DNA detected in local crayfish. This may be related to the increased numbers of zoospores in water at the localities, where crayfish populations with high parasite prevalence are present.

Part 2: Diversity and phylogeny of *Aphanomyces astaci*

Studies of intraspecific variation of *A. astaci* as well as diversity of closely related congeneric lineages are necessary for development and continuous improvement of molecular diagnostic methods for this parasite (section 3.3.) and distinguishing its genotypes. However, the genus *Aphanomyces* that includes species with various life strategies (parasites, saprobionts) is also a suitable model for studies of oomycete evolution.

A. astaci strains may vary in physiological characters and virulence (Viljamaa-Dirks and Torssonen 2008) as well as genetically. The whole-genome analysis of various isolates based on Random Amplification of Polymorphic DNA (RAPD-PCR) employed by Huang et al. (1994) and Diéguez-Uribeondo (1995) showed four distinct groups of genotypes associated with different crayfish species present in Europe. As *A. astaci* is not known to produce sexual structures, a clonal propagation is supposed to be the case in this species. These clones might have evolved in parallel with crayfish speciation in North America and, thus, the overall clonal diversity of *A. astaci* may be very high.

An association of *A. astaci* genotypes with different host species may have a practical use. Comparison of the strains from crayfish dying from crayfish plague with the already defined strains may point to the source of infection for the mass mortality (e.g. Vennerström et al. 1998, Diéguez-Uribeondo and Söderhäll 1999). However, as no method for distinguishing the genotypes of *A. astaci* other than RAPD-PCR analysis of pure genomic DNA is available at present (Makkonen et al. 2011), obtaining laboratory cultures of the parasite is necessary to determine its genotype group. As already mentioned in the section 3.2., cultivation of *A. astaci* is difficult and, thus, the diversity of its strains is poorly known.

In Czechia, the sources of infection for the crayfish plague outbreaks often remain unknown (**chapter 7**). To learn more about these sources, I made many attempts to isolate *A. astaci* from dying indigenous crayfish as well as from the spiny-cheek crayfish (the most common invasive crayfish in Czechia), the isolates from which had not been available for comparison. As I did not succeed in this work on my own, I asked researchers from Finnish Food Safety Authority (Evira) for help. The study summarised in the **chapter 5** is the result of this cooperation. We described a successful isolation of *A. astaci* from the spiny-cheek crayfish and confirmed that this species carries a novel genetically distinct genotype of the parasite. Obtaining these isolates is a very important step for future searching for sources of pathogen in crayfish plague outbreaks in European countries where the spiny-cheek crayfish is present together with other invasive crayfish species. This is also the first confirmation of *A. astaci* presence in the studied area by cultivation techniques.

Besides *A. astaci*, there are about 40 other species in the genus *Aphanomyces* known at present. However, only minority of these species are available in culture collections because their cultivation and maintenance is difficult (**chapter 6**). Most of them were defined based on morphological characters on their sexual structures (Johnson et al. 2002). However, definition of species in which sexual reproduction is unknown was based only on physiology or host specificity (**chapter 6**). For example, *A. astaci* has been traditionally defined as a species of the genus *Aphanomyces* pathogenic to crayfish, with a specific combination of physiological characteristics (Cerenius et al. 1988). Later, molecular methods brought a further tool useful in species definition. In oomycetes, the ITS of rDNA is used as a marker for this purpose up to now.

During my study stay in the Royal Botanical Garden in Madrid in 2006, I had an opportunity to participate in the research of the phylogenetic relationships among *Aphanomyces* species. We collected all available ITS sequences of this genus from GenBank and obtained sequences from laboratory cultures of all species accessible at that time. The resulting study (**chapter 6**) suggested that the genus is monophyletic and uncovered three distinct evolutionary lineages inside the genus: animal parasitic, plant parasitic and saprobiotic. More related species tended to have more similar physiology. Moreover, this study contributed to better definition of some *Aphanomyces* species and provided an important framework for future development and improvement of molecular detection methods for the parasitic species.

However, three new *Aphanomyces* species have been described since 2009, one of them very closely related to *A. astaci* (O'Rourke et al. 2010, Takuma et al. 2010, Takuma et al. 2011), and intraspecific variability of the ITS sequences of *A. astaci* has been uncovered (Makkonen et al. 2011). This further highlights that diversity inside the genus is still poorly known (but its research is progressing). Although presently recognized *Aphanomyces* species seem to be distinguishable using ITS sequencing, we cannot exclude that taxa with overlapping characteristics (e.g., not pathogenic to crayfish but with identical ITS sequences as *A. astaci*) will be found in the future, and the species definitions will have to be further discussed and detection methods adjusted.

Conclusions and outlook for further research

Emerging diseases of wildlife are of increasing concern of scientists, conservationists as well as farmers at present (Dobson and Foufopoulos 2001). Partly, these problems might seem to be on increase because they are more intensively studied. However, the real increase of risk for wildlife seems to be the case because of the higher propagule pressure (caused by rapidly increasing human-mediated dispersal of organisms) in combination with anthropogenic environmental change (Daszak et al. 2000, 2001).

Crayfish plague is one such case. Import of its causative agent to Europe was facilitated by intercontinental transport (Alderman 1996, Gherardi et al. 1999), and spread of the pathogen has been further supported by trade with indigenous European crayfish, and later by introductions of American crayfish species. Unlike in its original hosts of the North American origin, *A. astaci* became an aggressive pathogen in naive crayfish species and crayfish plague is one of the main factors negatively influencing future prospects of indigenous crayfish in Europe (Holdich et al. 2009). Management of such disease is not possible without 1) fast and reliable detection methods, 2) detailed knowledge of infection reservoirs, and 3) understanding the transmission pathways of the pathogen.

The research of the pathogen distribution compiled in this thesis is based on the molecular detection methods, which have been applied for the first time on such large crayfish sample collection (more than 800 crayfish individuals tested for *A. astaci*). This allowed better insight into benefits and limitations of these relatively new detection approaches. We showed that combination of alternative molecular assays is very important to uncover possible erroneous results. In particular, the negative results should be considered with care as sensitivity of detection depends on the PCR technology, the amount of crayfish tissues analysed, presence of potential inhibitors as well as other factors. In my thesis, I also contributed to the first comprehensive study of phylogeny of the genus *Aphanomyces*, which summarises most of the known ITS sequences of the genus and may serve as a basis for further parasite detection methods development.

My thesis brought new data on the distribution of *A. astaci* in its original host populations in Czechia and Hungary, where crayfish plague had been understudied. In this region, the spiny-cheek crayfish seems to be the main source of infection. We performed the first extensive studies of this invasive species in connection with crayfish plague pathogen. Traditionally, the signal crayfish was more often studied in context of *A. astaci* infections (Souty-Grosset et al. 2006), although the spiny-cheek crayfish is more common in some Central European countries including Czechia. We showed that the prevalence of *A. astaci* in American crayfish populations substantially varies. However, it must be stressed that it is nearly impossible to declare any American crayfish population to be “plague-free” and this term should be used very carefully (if at all). Thus, all American crayfish should be considered as potential sources of infection, e.g., for legislative purposes or in education of public.

My studies of the spiny-cheek crayfish in context of *A. astaci* have been suitably supplemented by the first isolation of the parasite from this crayfish species. Finding that the

spiny-cheek crayfish carries a new genotype of *A. astaci*, and including the obtained isolates to the publicly available culture collections, are important steps for potential more detailed tracing of infection sources in crayfish plague outbreaks in the future.

In hope to cope better with crayfish plague, further complex research of its pathogen is still required. I see a particular need of the further studies in the following areas (some of them will be objectives of a Ph.D. project of Jiří Svoboda):

1) Continuous improvement and verification of detection methods for *A. astaci*

This aim is particularly connected to studies of the diversity of aquatic oomycetes, especially of the genus *Aphanomyces* potentially present in the same habitats as crayfish, and research of the intraspecific variability in the diagnostic molecular markers (such as in Makkonen et al. 2011).

2) Study of the dynamics of the pathogen in invasive crayfish populations

Although this thesis suggested that prevalence or at least detectability of *A. astaci* in the spiny-cheek crayfish populations may vary in time, the factors causing the variation remain unclear and further research should uncover them.

3) Development of a method for distinguishing of *A. astaci* genotypes in crayfish tissues

Availability of appropriate molecular tool would facilitate routine searching for infection sources of crayfish plague outbreaks without the need of the difficult pathogen cultivation. Further extension of our knowledge on the diversity of *A. astaci* genotypes is also needed, particularly in the "new non-indigenous crayfish species" occurring in Europe. No less important is also the further research of physiological characteristics and pathogenicity of various *A. astaci* genotypes.

4) Study of the transmission pathways of the infection

It is not clear which vectors are the most important in indirect transmission of crayfish plague (if American crayfish are not found at the localities of outbreaks), and these should be better investigated. We also do not know enough about the host specificity of *A. astaci*. Potential for infection in other freshwater crustaceans (especially decapods) and fish was only marginally studied. Additionally, a new exciting area for research has been opened by the possibility of tracing the zoospores in the environment using molecular methods (Strand et al. 2011).

5) Study of mechanisms of the long-term coexistence of *A. astaci* and European crayfish

Further research should be conducted on the rare populations of European crayfish that seem to survive with the infection longer than expected. Results of such research might be potentially useful in the disease management.

Last but not least, although it is a very serious disease with a devastating history, it would be a mistake to consider crayfish plague as the only important disease of crayfish. There are disease outbreaks in crayfish populations clearly not related to crayfish plague (Edgerton et al. 2004) and, for example, some viruses of crustaceans may cause nearly 100% mortality of crayfish (Baumgartner et al. 2009). Thus, other crayfish parasites and diseases, which are understudied in comparison to *A. astaci*, should be investigated thoroughly as well, to provide a complete picture on crayfish pathology necessary for effective crayfish conservation.

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