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**Reservoirs and diversity of the crayfish plague pathogen: from
Central Europe to its native range**

**Rezervoáry a diverzita patogenu račího moru: od střední Evropy po
jeho původní areál**

Ph.D. Thesis

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DECLARATION OF ORIGINALITY

I declare that this thesis has not been submitted for the purpose of obtaining 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. All literature sources I used when writing this thesis have been properly cited.

Prague, December 10, 2024

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ABSTRAKT

Patogen račího moru *Aphanomyces astaci* způsobuje úbytek původních populací raků v Evropě i jinde v Palearktu a představuje vážnou hrozbu i pro akvakultury raků, kteří jsou vůči račímu moru citliví. Pochopení vztahu mezi hostiteli a *A. astaci*, způsobů jeho zavlékání a epidemiologie je nezbytné pro přijetí vhodných strategií ochrany raků. Má doktorská práce se zaměřuje na rozšíření, prevalenci a diverzitu *A. astaci* v populacích původních evropských raků i v severoamerických druhů, a to jak v Evropě, tak i v původním areálu výskytu patogenu v USA. Klíčové výsledky mého výzkumu jsou shrnuty ve čtyřech hlavních kapitolách, které doplňují tři další spoluautorské publikace. Studie využívají řadu molekulárních metod ke zkoumání genetické variability *A. astaci* a vztahují výskyt nalezených genotypů patogenu s hostitelskými druhy a zeměpisnými oblastmi. Klíčové výsledky disertační práce zahrnují identifikaci neočekávaných kmenů *A. astaci*, které se podílely na nedávných úhynech raků na račí mor v Česku, charakterizaci nového genotypu *A. astaci* v populacích původně akvarijních druhů raků v Maďarsku, vyhodnocení relativní stability prevalence *A. astaci* v populacích jejich hostitelů v čase nebo potvrzení, že charakter míry infekce *A. astaci* je obdobný v populacích invazních severoamerických raků v Evropě i v původním areálu výskytu patogenu. Výsledky také naznačují možnou roli obchodu s okrasnými raky při zavlékání a šíření genotypů *A. astaci*. Můj výzkum odhalil případy horizontálního přenosu mezi různými asymptomatickými druhy hostitelských raků nebo výměnu dominantních genotypů *A. astaci* ve studované populaci v relativně krátkém časovém období. Výsledky však také potvrzují, že identifikace genotypů *A. astaci* je užitečným nástrojem pro sledování zdrojů infekce v invadovaných oblastech, kde kontakt mezi různými hostitelskými druhy patogenu není častý. Kromě studií zapracovávajících evropské vzorky jsme zkoumali variabilitu *A. astaci* také v Pensylvánii, což přispělo k znalostem o rozšíření a genotypové diverzitě patogenu v jeho původním severoamerickém areálu. Podílela jsem se také na dvou metodicky zaměřených studiích (na vývoji a ověření genotypovací metody založené na kvantitativní PCR a na pilotní studii zaměřené na simultánní monitoring hostitelských raků i *A. astaci* z environmentální DNA) a na objevu chronických infekcí v populacích původního evropského druhu, raka bahenního *Pontastacus leptodactylus*, na Ukrajině.

ABSTRACT

The crayfish plague pathogen *Aphanomyces astaci* is causing declines in native crayfish populations across the Palaearctic and poses a serious threat to aquaculture of susceptible crayfish species. Understanding the host-pathogen relationship of *A. astaci*, its introduction pathways and epidemiology is essential for the adoption of appropriate conservation strategies for *A. astaci*-susceptible crayfish worldwide. My PhD thesis focuses on the distribution, prevalence and diversity of *A. astaci* in native European crayfish populations, as well as in North American crayfish populations both in Europe and in the pathogen's native range in the USA. The key results of my research are provided in four core chapters, which are supplemented by three additional co-authored publications. The studies use multiple molecular tools to explore the genetic variability of *A. astaci*, linking genotype patterns to host species and geographical regions. The key findings included the identification of unexpected *A. astaci* strains involved in crayfish plague outbreaks in Czechia and novel *A. astaci* genotype in populations of non-native aquarium crayfish species in Hungary, evaluation of relative stability in *A. astaci* infection rates in populations of their hosts over time, and revealing comparable patterns of *A. astaci* prevalence between populations of North American crayfish in Europe and in the native range of this pathogen. My research revealed cases of horizontal transmission between different asymptomatic host crayfish species upon contact and temporal replacement of dominant *A. astaci* genotypes in a population studied over time, and highlights the potential role of the ornamental crayfish trade in the introduction and spread of *A. astaci* genotypes. However, our results also confirm that the identification of *A. astaci* genotypes is a useful tool for tracing the source of infection in the invaded regions where contact between different reservoir host species is not frequent. In addition to studies analysing European samples, we investigated the variability of *A. astaci* in Pennsylvania, shedding light on the broader distribution and genotype diversity of the pathogen its native range in North America. I also contributed to two methodological studies (on the development of real-time PCR genotyping assays for *A. astaci*, and a simultaneous screening of environmental DNA for crayfish and *A. astaci*) and to the discovery of chronic infections in populations of the native European crayfish *Pontastacus leptodactylus* in Ukraine.

AUTHOR CONTRIBUTION TO PUBLICATIONS AND MANUSCRIPT INCLUDED IN THE THESIS

This PhD thesis consists of six published articles and a submitted manuscript. My personal contribution to each of them is listed below.

CHAPTER I

Mojžišová M, Mrugała A, Kozubíková-Balcarová E, Vlach P, Svobodová J, Kouba A, Petrusek A (2020) Crayfish plague in Czechia: Outbreaks from novel sources and testing for chronic infections. *Journal of Invertebrate Pathology* 173: 107390. <https://doi.org/10.1016/j.jip.2020.107390>

I partially participated in the field work. Then I processed and molecularly analysed all the samples and evaluated the data. I wrote the first draft and contributed to its revisions.

CHAPTER II

Mojžišová M, Svobodová J, Kozubíková-Balcarová E, Štruncová E, Stift R, Bílý M, Kouba A, Petrusek A (2022) Long-term changes in the prevalence of the crayfish plague pathogen and its genotyping in invasive crayfish species in Czechia. *NeoBiota*. 74: 105–127. <https://doi.org/10.3897/neobiota.74.79087>

I partially participated in the field work and isolated DNA from about half of the samples collected. I analysed all the DNA isolates and evaluated the data. I wrote the first draft and contributed to its revisions. Furthermore, I methodologically guided a visiting student (Robin Stift) who contributed to sample processing.

CHAPTER III

Mojžišová M, Weiperth A, Gebauer R, Laffitte M, Patoka J, Grandjean F, Kouba A, Petrusek A (2024) Diversity and distribution of *Aphanomyces astaci* in a European hotspot of ornamental crayfish introductions. *Journal of Invertebrate Pathology* 202: 108040. <https://doi.org/10.1016/j.jip.2023.108040>.

I processed and analysed all the samples and evaluated the data. I wrote the first draft (with a substantial contribution of my supervisor) and contributed to its revisions.

CHAPTER IV

Petrusek A, **Mojžišová M**, Mikešová A, Piálková R, Lieb DA: Prevalence and diversity of *Aphanomyces astaci* in cambarid crayfish of Pennsylvania: Where native and introduced hosts meet. *Submitted manuscript*.

I isolated DNA from about one third of the samples. I then analysed all the isolates and evaluated the data. I participated in the writing of the draft with comments and suggestions.

APPENDIX A

Ungureanu E, **Mojžišová M**, Tangerman M, Ion MC, Pârvulescu L, Petrusek A (2020) The spatial distribution of *Aphanomyces astaci* genotypes across Europe: Introducing the first data from Ukraine. *Freshwater Crayfish* 25: 77–87. <https://doi.org/10.5869/fc.2020.v25-1.077>

I methodologically guided a MSc student Elena Ungureanu who isolated DNA from all the samples she collected in Ukraine. Then I analysed the isolates and evaluated the molecular data. I participated in the writing of the draft with comments and suggestions, as well as in the planning of the review part of the paper.

APPENDIX B

Di Domenico M, Curini V, Caprioli R, Giansante C, Mrugała A, **Mojžišová M**, Cammà C, Petrusek A (2021) Real-time PCR assays for rapid identification of *Aphanomyces astaci* genotypes. *Frontiers in Ecology and Evolution* 9: 597585. <https://doi.org/10.3389/fevo.2021.597585>

I provided A. astaci DNA isolates from crayfish plague outbreaks in Czechia, which I had originally isolated and analysed for the pathogen presence and diversity. I participated in the manuscript writing process with comments and suggestions.

APPENDIX C

Rusch JC, **Mojžišová M**, Strand DA, Svobodová J, Vrålstad T, Petrusek A (2020) Simultaneous detection of native and invasive crayfish and *Aphanomyces astaci* from environmental DNA samples in a wide range of habitats in Central Europe. *NeoBiota* 58: 1–32. <https://doi.org/10.3897/neobiota.58.49358>

I contributed to the field work. I provided DNA isolates from tissues of native crayfish species present in Czechia and data on the presence of A. astaci in selected non-native crayfish populations in this region, which were matched to eDNA data where possible. I participated in eDNA analyses performed at Charles University and contributed to manuscript writing and its revisions.



Supervisor declaration

As the senior author of all papers, and corresponding author of the unpublished manuscript included in the thesis of Michaela Mojžišová, I confirm that her contributions described above are correct.

prof. Adam Petrusek

PREFACE & ACKNOWLEDGEMENTS

Even as a child I was fascinated by the animal kingdom, and thanks to my parents who supported me, I fell in love with the aquatic world. My journey with crayfish and the crayfish plague pathogen began in the first year of my undergraduate degree when Adam Petrusek convinced me that crayfish were actually great, and he was right. I gradually became so interested in the subject that I also fell in love with the world of parasites and their evolution and stayed with it throughout my MSc. and PhD. Every time I got a result, it was like opening a present under the Christmas tree. I had the opportunity to travel across Europe and collaborate with different interesting researchers and institutions. Even though it wasn't always easy during my studies, e.g. the COVID-19 situation or becoming a parent, I never gave up. I see the end of my PhD as the closure of a door that will open further into the academic world, which I am already looking forward to.

Without my supervisor, Adam Petrusek, I would never be where I am today, so I owe him a huge debt of gratitude. He has always been a source of support and motivation for me, and after so many years of working together, he has not only been a great supervisor but has become like a second father to me. Thank you, Adam!

I would also like to thank Eva Kozubíková-Balcarová and especially Agata Mrugała, who both guided me methodically and were always there for me when I needed help in the molecular lab or in analysing the results.

My deep gratitude also belongs to my good friend and colleague Nataša Katanić who took care of my daughter Amélia during the last months, and especially during the last days, when I needed her help. I literally could not have written the last pages of this thesis without her support.

My national and international collaborations, which have been very fruitful, have been essential to the existence of this thesis and therefore my profound gratitude go to Jitka Svobodová (T.G. Masaryk Water Research Institute, Czechia); Antonín Kouba and Radka Piálková (University of South Bohemia in České Budějovice, Czechia); Pavel Vlach (University of West Bohemia, Czechia); Jiří Patoka (Czech University of Life Sciences Prague, Czechia); Johannes C. Rusch, David Strand and Trude Vrålstad (Norwegian Veterinary Institute, Norway); David A. Lieb and his team (Pennsylvania Fish and Boat Commission, PA, USA); Marco Di Domenico and his colleagues (Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G. Caporale, Italy); András Weiperth (Hungarian University of Agriculture and Life Sciences, Hungary); Lucian Pârvulescu and his team (West University of Timisoara, Romania). Thank you! Děkuji! Takk! Grazie! Köszönöm! Multumesc!

Over the years, I have also methodologically supervised a number of students. They have not only contributed a large part of the data for my doctoral thesis but have also become my friends. I would like to express my immense gratitude to Elena Ungureanu-Tangerman, Michiel Tangerman, Robin Stift, Adéla Mikešová and Jiří Hladovec.

I would like to extend my special thanks to my colleagues in Poitiers, France, where I spent several months during my Erasmus-funded internship. Namely, Frédéric Grandjean, for his very motivating guidance and help, as well as Maude Laffitte, who not only helped me professionally and to integrate into the team, but

she also helped me to feel warm and welcome. I would also like to thank Charlotte Noizat, Cécilia Mondet, Baptiste Lheraud, Victor Desclos le Peley, Hélène Paulac, Benoit Monnerau for their warm hospitality and funny talks during our lunches and hangouts. Merci Beaucoup!

I am also grateful to my colleagues in the Ecology Department, namely Barbora Augstenová, Pavel Karel Bystřický, Tomáš Pšenička, Eleni Anna Charmpila, Lucija Andjel and Jakub Hradečný. Thank you all for supporting me over the years, for constructive discussions, hangouts, lunches, parties. It was fun!

Lastly, I would like to express my deep gratitude to my family, who supported me during my studies and urged me to finally complete my PhD thesis. My sister Martina Alakšová painted beautiful crayfish and lobster pictures, which are included in this thesis. Ďakujem Vám!



INTRODUCTION

Emerging infectious diseases have been characterised as those that are newly recognised or newly appeared in the population and have recently increased in incidence or geographical range (Daszak et al. 2000; Petersen et al. 2018). Their appearance and subsequent spread are often associated with the introduction and spread of non-native species, which often serve as reservoirs of infection (Daszak et al. 2000; Dunn et al. 2009; Lymbery et al. 2014; Blackburn & Ewen 2017; Zhang et al. 2022). In addition, introduced disease agents can spread to new areas through host-switching, when they spread in populations of naive hosts after spilling over from the original ones (Daszak et al. 2000; Peeler et al. 2011). Such unwanted invaders often have negative economic, ecological, and conservation impacts (Daszak et al. 2000; Murray & Peeler 2005; Holdich et al. 2009), and can ultimately be devastating to native species (Lymbery et al. 2014).

Some of these emerging disease agents are so successful that they have been included in the list of the 100 worst invasive species in the world (Lowe et al. 2000). This list also includes several fungal and fungal-like pathogens that have been on the rise in recent decades, causing major population losses or even extinctions of various species (Fisher et al. 2012). One such pathogen is the chytridiomycete *Batrachochytrium dendrobatidis*, which has devastated amphibian populations around the world (Scheele et al. 2019). Another example is the oomycete *Phytophthora cinnamomi*, which infects nearly 5000 plant species, making it one of the most destructive plant pathogens in the world (Cahill et al. 2008; Jung et al. 2013).

The oomycete *Aphanomyces astaci*, the causative agent of crayfish plague, which is the subject of my PhD thesis, was also included in the above-mentioned list of the worst world's 100 invasive species. This pathogen was introduced to Europe in the late 19th century and has caused massive mortalities of native European crayfish populations across the continent since its introduction (Alderman 1996). The spread of the disease accelerated with the subsequent repeated introductions of North American crayfish in the 20th century (Alderman 1996; Holdich et al. 2009). These are assumed to be the original hosts of *A. astaci*, with which they have co-evolved in a symbiotic relationship and often act as asymptomatic carriers of this pathogen (Söderhäll & Cerenius 1999; Boštjančić et al. 2022). With the continuous introductions of North American crayfish, either for aquaculture or pet trade, *A. astaci* is currently causing mortalities of susceptible crayfish not only in Europe but also in Asia (Jussila et al. 2021a; Hsieh et al. 2016; Martín-Torrijos et al. 2018). Moreover, its presence has recently been confirmed in many other regions of the world, mainly in farmed populations of the red swamp crayfish *Procambarus clarkii* (Putra et al. 2018; Martín-Torrijos et al. 2021a), in pet shops (Putra et al. 2018), but also in feral populations of the marbled crayfish *Procambarus virginalis* and *P. clarkii* (Andriantsoa et al. 2019; Peiró et al. 2016). There is also preliminary evidence of the presence of *A. astaci* in representatives from local endemic crayfish in Brasil (Peiró et al. 2016), meaning that local crayfish populations might be under threat of crayfish plague outbreaks. Recently, some studies on the distribution or diversity of *A. astaci* in its native range have also been conducted (Makkonen et al. 2019; Panteleit et al. 2019; Butler et al. 2020; Martín-Torrijos et al. 2021b, 2023).

The lifecycle of *A. astaci*

The crayfish plague pathogen, *Aphanomyces astaci*, is a saprolegnial oomycete. This fungal-like organism consists of three main biological stages: mycelium, biflagellate zoospores, and cysts (Söderhäll & Cerenius 1999; Diéguez-Urbeondo et al. 2006).

In general, the genus *Aphanomyces* includes saprophytic species with the ability to become opportunistic pathogens as well as highly specialised parasites of plants or animals (Diéguez-Urbeondo et al. 2009). For the parasitic *Aphanomyces* spp. that infect plants, sexual reproduction is critical for their survival, whereas it is rare or absent in those that infect animals (Diéguez-Urbeondo et al. 2009). This also appears to be the case for *A. astaci*, although there were some reports of a sexual apparatus of *A. astaci* in the past (Rennerfelt 1936; Schäperclaus 1935; Johnson et al. 2002) and some speculation about its potential for sexual reproduction (Johnson et al. 2002; Rezinciuc et al. 2015). However, to date its existence has never been conclusively documented (Kozubíková-Balcarová et al. 2013; Martínez-Ríos et al. 2023). Further investigations of biological characteristics and interactions of *A. astaci* axenic cultures obtained from its native area may provide a better insight into this topic.

The asexual stage of *A. astaci* is characterised by the formation of the only infectious unit, the biflagellate zoospores. Their only goal is to quickly find the suitable substrate (the crayfish host or other decapods) and start germination (Cerenius & Söderhäll 1984a, 1985; Svoboda et al. 2017). However, *A. astaci* zoospores are non-specifically chemotactically attracted to most nutrients (Cerenius & Söderhäll 1984b), so it can happen that zoospores land on an unsuitable surface and initiate encystment triggered by non-specific stimuli (Cerenius & Söderhäll 1984b, 1985). For such cases, *A. astaci* has an adaptation: repeated zoospore emergence (Cerenius & Söderhäll 1984b). During this process, the encysted zoospore releases another motile zoospore instead of germinating. This gives *A. astaci* up to three attempts to find a suitable host and establish infection before its energy reserves are exhausted. Once the biflagellate zoospores encyst and begin germination, *A. astaci* produces a mycelium of non-segmented hyphae inside an infected host. When the hyphae growing in the host tissue reach the surface of the body and get into contact with the surrounding water, primary spore production is initiated. Specialised structures called sporangia develop at the tips of the hyphae, and inside them, a row of primary spores is formed. The mature primary spores are released from the sporangia. They encyst, forming a protective wall around themselves, and can clump together in clusters called 'spore balls'. Each primary cyst transforms into a motile secondary biflagellate zoospore which may infect a suitable host, completing the life cycle (Cerenius & Söderhäll 1984a, 1985). For more details on the biology of *A. astaci* see, e.g., Cerenius et al. (1988), Söderhäll & Cerenius (1999), Diéguez-Urbeondo et al. (2006), Rezinciuc et al. (2015), Svoboda et al. (2017), or Martínez-Ríos et al. (2023).

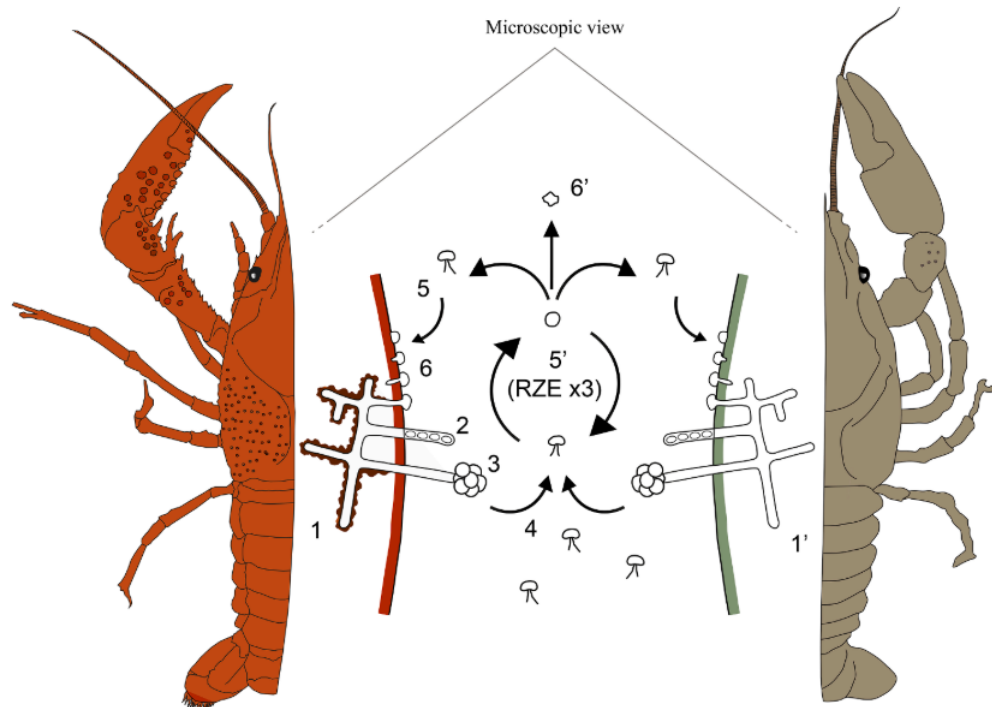


Figure 1: The life cycle of *Aphanomyces astaci* (based on Martínez-Ríos et al. 2023): The melanized hyphae in the cuticle of North American crayfish species (1), infected susceptible crayfish species without defensive melanisation (1'), the formation of a sporangium protruding to water (2), “spore ball” of primary cysts (3), the secondary biflagellate zoospores (4), encystment on a wrong surface and repeated zoospore emergence (RZE) (5'), unviable zoospore (6'), localization of the host by chemotaxis (5), the formation of secondary cysts and their germination (6).

Transmission of *A. astaci* and its potential reservoirs

The biflagellate zoospores (infective stages) of *A. astaci* have a short lifespan, usually days to weeks (Unestam et al. 1966; Alderman & Polglase 1986; Cefas 2000; Svoboda et al. 2013), so the pathogen does not survive in the environment long without a suitable host (Oidtmann et al. 2002; Svoboda et al. 2017). *A. astaci* is predominantly freshwater and was thought to be sensitive to elevated salinities (Unestam 1969a), but recently its presence has been recorded also in a brackish-water population of *P. clarkii* (Martín-Torrijos et al. 2021c).

Its zoospores are extremely sensitive to desiccation (Alderman & Polglase 1986; Smith & Söderhäll 1986), but when in a moist environment, they ensure efficient spread of the pathogen between hosts. Apart from horizontal transmission, *A. astaci* may also get transmitted trans-generationally in populations of chronically infected hosts. Direct vertical transmission of *A. astaci* from mother to eggs to offspring has not been confirmed, but it has been experimentally demonstrated that juveniles under natural conditions may acquire infection already in the early stages of their ontogeny, when they are still attached to an infected mother (Kozák et al. 2023).

The dominant mode of spread of *A. astaci* is undoubtedly horizontal. The crayfish plague pathogen was most likely introduced into Europe through transoceanic vessel (possibly with yet unknown host crayfish) and spread across the continent with infected native crayfish, causing mass mortalities of native European crayfish populations (Alderman 1996). In addition, the firm establishment and further expansion of *A. astaci* was amplified by subsequent deliberate introductions of North American crayfish species into European freshwaters (Alderman 1996; Holdich et al. 2009). Crayfish of North American origin are assumed to be the original hosts and primary reservoirs of *A. astaci* (Svoboda et al. 2017); this is also supported by higher diversity of *A. astaci* in that region (Martín-Torrijos et al. 2021b, Chapter IV). Due to a long co-evolutionary history with this pathogen, immune response of North American crayfish to infection is much more effective than that of other crayfish species (Cerenius et al. 1988, 2003). However, in the last decade, latent infections have also been detected in some populations of native European crayfish (Svoboda et al. 2017; Pavić et al. 2021; Satmari et al. 2023; Akhan et al. 2024; Alvanou et al. 2024; Brady et al. 2024). Apart from crayfish, the latent asymptomatic *A. astaci* infections have also been confirmed in other decapods, namely in wild populations of the Chinese mitten crab *Eriocheir sinensis* in Europe (Svoboda et al. 2014a; Schrimpf et al. 2014; Tilmans et al. 2014), in the Levantine freshwater crab *Potamon potamios* in Turkey (Svoboda et al. 2014a) as well as in the Paddy field crab *Parathelphusa convexa* in Java (Putra et al. 2018). Horizontal transmission between *E. sinensis* and crayfish in both directions has also been demonstrated experimentally (Schrimpf et al. 2014). The presence of *A. astaci* has also been reported in wild populations of the Riceland shrimp *Macrobrachium lancesteri* (Putra et al. 2018) and the Mississippi grass shrimp *Palaemon kadiakensis* (Martín-Torrijos et al. 2021b). Presumed growth of *A. astaci*, at least over short term, has also been observed in experimental studies of the Chocolate shrimp *Macrobrachium dayanum* (Svoboda et al. 2014b), the Bamboo shrimp *Atyopsis moluccensis* and in the African fan shrimp *Atya gabonensis* (Mrugała et al. 2019). This evidence suggests that *A. astaci* may also develop on other decapod groups but their mass mortalities have not been reported yet.

Apart from infected hosts, the crayfish plague pathogen can also be transmitted through wet objects (such as fishing gear, waders, or nets) or with spore-contaminated water (Svoboda et al. 2017). In addition, animals that feed on infected crayfish can transmit *A. astaci* to uninfected crayfish populations, for example through the transport of infected crayfish carcasses by birds or otters, or through fish faeces (Oidtmann et al. 2002). Fortunately, passage through the digestive tract of warm-blooded predators makes transmission of *A. astaci* unlikely (Svoboda et al. 2020). For more details, an overview of the transmission of *A. astaci* is provided by Oidtmann et al. (2002) and Svoboda et al. (2017).

The DNA isolation & molecular detection of *A. astaci* and its crayfish hosts

In the past, the oomycete *A. astaci* was mainly diagnosed by isolation on axenic cultures followed by microscopic diagnosis (Alderman & Polglase 1986; Cerenius et al. 1988). However, reliable identification of the presence of *A. astaci* in crayfish individuals using this method was often laborious and time-consuming (Martínez-Ríos et al. 2023). Currently, cultivation is generally used to maintain and expand biological knowledge of known and novel *A. astaci* strains (Francesconi et al. 2021, 2024; Martín-Torrijos et al. 2021b,

2023) and to discover new closely related species, some of which might even interfere with molecular detection methods (Viljamaa-Dirks & Heinikainen 2019; Strand et al. 2023), rather than for diagnosis itself. Nowadays, the diagnosis of *A. astaci* is usually performed by isolating DNA from crayfish tissue, which was originally developed to detect this pathogen from crayfish mass mortalities (Oidtmann et al. 2004). Later, it was optimised to effectively identify *A. astaci* even in clinically inapparent carriers, i.e. crayfish of North American origin (Oidtmann et al. 2006). However, analysis of tissue samples requires crayfish specimens, obtaining of which may entail substantial effort in either manual searching or trapping (Abrahamsson 1983; Reynolds & Matthews 1993). *A. astaci* DNA is usually extracted from the soft abdominal cuticle, telson and melanised spots, where this pathogen is most frequently found (Oidtmann et al. 2006; Vrålstad et al. 2011). However, this type of isolation usually requires killing of the animal. The WOA only describes cuticle sampling from recently dead or euthanised animals (OIE 2021).

In the case of endangered native crayfish species, this method is suitable in the event of mass crayfish mortality. However, special permits are required to sample protected crayfish populations without evidence of mortality, as such research may involve euthanasia of crayfish (Kušar et al. 2013; Maguire et al. 2016) or cutting off specific parts that may regenerate (Schrimpf et al. 2012; Mojžišová et al. 2020), which is still destructive and stressful to them. Recently, as latent infections in native crayfish species have been more studied, non-destructive methods such as analysis of cuticle swabs have been applied (Pavić et al. 2020; Basso et al. 2023). Specifically, Pavić et al. (2020) showed that *A. astaci* DNA is also present on the cuticle of infected crayfish, including non-symptomatic carriers, and can be collected by swabbing the surface with brushes. This method has recently been used to monitor *A. astaci* in latently infected native crayfish populations in Croatia (Pavić et al. 2021). Basso et al. (2023) optimised a different swabbing protocol using disposable swabs, as this appeared to be more practical in the field and less prone to cross-contamination. This study compared the success of *A. astaci* detection between traditional tissue-based and non-destructive isolation from cuticle swabs, and tested the performance of non-destructive methods, including the environmental DNA (eDNA) method, in the field pilot distribution study. Both non-destructive methods were suitable for detection of *A. astaci* and Basso et al. (2023) suggested that they could be used simultaneously for monitoring or reintroduction programmes.

The term environmental DNA is generally described as "DNA obtained directly from environmental samples (soil, sediment, or water) without evidence of biological material" (Thomsen & Willerslev 2015); eDNA can also be obtained from snow tracks (Franklin et al. 2019; De Barba et al. 2024), plant surfaces (Valentin et al. 2018; Lee-Rodriguez et al. 2024), airborne material (Johnson & Barnes 2024) or faecal samples (Ruppert et al. 2019), as well as from dead or decomposing organisms (Kamoroff & Goldberg; Curtis & Larson 2020).

The eDNA methodology does not seem particularly applicable for biomass quantification and population density estimation, at least when crayfish are concerned (Johnsen et al. 2020; Rusch et al. 2022). However, an increasing number of studies show that eDNA screening is suitable for providing information on the presence/absence of target species (Hempel et al. 2020; Mason et al. 2021; Satmari et al. 2023), although 'absence' data should be interpreted with caution (Rusch et al. 2020). Specifically in aquatic environments, eDNA is suitable for detecting both common and rare species (Cardás et al. 2020; Mizumoto et al. 2020), as

well as aquatic invaders (Larson et al. 2020), including “door knocker” species (Ekrem et al. 2023), pathogens and parasites (Ruppert et al. 2019; Bass et al. 2023).

To reliably detect *A. astaci* and its hosts using eDNA, it is necessary to know their biological characteristics and the environmental conditions that may limit their successful detection. Previous studies indicate that the zoospores of *A. astaci* (free-swimming propagules) are easily detectable from eDNA (Strand et al. 2011, 2012, 2014; Makkonen et al. 2013). In contrast, due to the hard exoskeleton, crayfish shed much smaller amounts of DNA compared to other aquatic organisms such as fish or amphibians, making their detection more challenging (Forsström & Vasemägi 2016; Johnsen et al. 2020). Crayfish DNA seems more detectable during their reproduction, moulting (Rusch et al. 2022) and mortality (Strand et al. 2019), but, interestingly, the decomposition of their carcasses does not necessarily produce more DNA to the environment (Curtis & Larson 2020). The reproduction phase of *A. astaci* also increases its detectability, especially during crayfish plague outbreaks when mass sporulation is present (Strand et al. 2019). In contrast, the production of *A. astaci* DNA is more or less continuous in waters with chronically infected crayfish populations (Strand et al. 2012; Svoboda et al. 2013). Cold (about 10°C) and clear water (i.e. in the absence of food supply) increases the detectability of DNA of crayfish as well as *A. astaci* (Rusch et al. 2022). To increase the detectability of *A. astaci* and its hosts, it is important to consider these aspects and additionally collect large volumes of water and multiple field replicates (Ruppert et al. 2019; Basso et al. 2023). Aquatic eDNA can be collected by precipitation, but for efficient eDNA collection from larger volumes, filtration in situ or from water collected in the field and transported to the laboratory is more appropriate (Ruppert et al. 2019; Peixoto et al. 2021). Currently, many protocols have been developed to extract eDNA from filters; those used in crayfish research were summarised in Baudry et al. (2024). In the study by Rusch et al. (2020), which is included in this thesis as Appendix C, we used the CTAB protocol for eDNA extraction according to Vrålstad et al. (2009) and modified by Strand et al. (2014).

At present, there are three different approaches to analyse eDNA isolates: environmental metagenomics based on shotgun sequencing of all genetic material present in the eDNA isolate, particularly suitable for analyses of microbial communities (Thoendel et al. 2020; Fadiji & Babalola 2020; Zhang et al. 2021); metabarcoding based on amplicon sequencing of polymerase chain reaction (PCR) products with general primers, used for analysis of whole communities or taxonomic groups on a broader scale (Ruppert et al. 2019); and targeted screening for specific taxa based on conventional or quantitative PCR (qPCR) with taxon-specific primers (Lu et al. 2024). The last-mentioned approach has been used for the detection of *A. astaci* (Strand et al. 2019), and usually also for its crayfish hosts (Baudry et al. 2024; Rusch et al. 2020).

Screening for eDNA has proven to be a very effective tool for rapid detection of both above-mentioned target taxa and is gradually replacing or complementing traditional manual or trap-based sampling methods (Wittwer et al. 2018, 2019; Mirimin et al. 2022; Pisano et al. 2024). There has been a particular increase in efforts to develop or optimise eDNA assays that reliably detect the presence of target crayfish species worldwide (Rusch et al. 2020; reviewed in Baudry et al. 2024). These crayfish assays typically target the mitochondrial gene for cytochrome c oxidase subunit I (COI) and are subsequently tested by qPCR, end-point PCR or Droplet Digital PCR (Baudry et al. 2024). Screening for the associated conservationally relevant

pathogen *A. astaci* is more commonly performed as an extension to targeted eDNA biomonitoring for specific non-native and native crayfish species in Europe (Casabella-Herrero et al. 2023; Basso et al. 2023; Satmari et al. 2023; Oficialdegui et al. 2024; Baudry et al. 2024).

Several detection assays have been developed to detect *A. astaci*, applicable to mixed-genome DNA isolates, using conventional PCR (Oidtman et al. 2004, 2006) or qPCR (Vrålstad et al. 2009; Hochwimmer et al. 2009; Makkonen et al. 2012a). Those targeting the ribosomal internal transcribed spacer (ITS) region have been shown to be the most sensitive and specific for *A. astaci* diagnosis (Tuffs & Oidtmann 2011; Kozubíková et al. 2011a). The specificity of the diagnostic assay for *A. astaci* is essential as its geographical range overlaps with other *Aphanomyces* spp. (Makkonen et al. 2019; Butler et al. 2020; Becking et al. 2022), and the true diversity of this genus has not yet been fully explored. The method by Vrålstad et al. (2009) based on TaqMan qPCR, has been used extensively for monitoring crayfish plague (Maguire et al. 2016; Grandjean et al. 2017; Mojžišová et al. 2020, 2022). Shortly after its development, it was also shown to be suitable for eDNA water samples (Strand et al. 2011, 2012, 2014; Makkonen et al. 2013). However, Viljamaa-Dirks & Heinikainen (2019) showed that this method unfortunately cross-reacts with the new closely related species *Aphanomyces fennicus*. Therefore, the team of Strand et al. (2023) developed a new, more specific method to detect *A. astaci*, suitable for all types of mixed genome samples, which was quickly implemented in recent studies (Mojžišová et al. 2024; Pisano et al. 2024; Lipták et al. 2024; Laffitte et al. 2024; Chapter IV). However, it should be noted that false-positive records of *A. fennicus* have so far only been documented from Fennoscandia (Viljamaa-Dirks & Heinikainen 2019; Casabella-Herrero et al. 2021; Strand et al. 2023), and we did not detect *A. fennicus* in any of our studies from Central Europe or from the native range of *A. astaci* where Vrålstad et al. (2009) assay has been applied (Mojžišová et al. 2020, 2022, 2024; Chapter IV). The reliability of *A. astaci* detection in these studies was supported by subsequent sequencing using mtDNA markers (Makkonen et al. 2018), which can reliably diagnose *A. astaci* and distinguish some of its strains (Casabella-Herrero et al. 2021; Martínez-Ríos et al. 2023). However, in the qPCR-positive samples where mtDNA or other genotyping assays cannot be amplified due to low levels of the target DNA, the potential presence of *A. fennicus* or another cross-reacting taxon cannot be excluded. This is also the case for some of our study populations (e.g. in Mojžišová et al. 2022; Chapter IV).

Identification of *A. astaci* genetic variability

The genetic diversity of *A. astaci* was first studied by molecular methods at the end of the 20th century, when axenic laboratory cultures were characterised by randomly amplified polymorphic DNA (RAPD). This fingerprinting method initially identified three distinct *A. astaci* genotype groups (Huang et al. 1994). In the following years, two additional genotype groups also characterised by RAPD were recognised (Diéguez-Uribeondo et al. 1995; Kozubíková et al. 2011b). This grouping was then supported by the amplified fragment length polymorphism (AFLP) (Rezinciuc et al. 2014). However, both methods are only applicable to axenic cultures.

The first *A. astaci* genotyping method applicable to clinical samples was based on the nuclear chitinase gene (Makkonen et al. 2012a). Phylogenetic analyses using this method and AFLP indicated that all *A. astaci*

genotype groups based on RAPD split into two main lineages (Makkonen et al. 2012a; Rezinciuc et al. 2014; Makkonen et al. 2018). However, the chitinase variation was limited, unable to discriminate between some genotype groups (B and E) originally identified by RAPD (Makkonen et al. 2012a).

Shortly afterwards, a genotyping method based on nuclear microsatellite markers was published (Grandjean et al. 2014). It is applicable for DNA samples from pure cultures as well as from host tissues (Grandjean et al. 2014), including historical samples preserved from past crayfish mass mortalities (e.g. Kozubíková-Balcarová et al. 2014; Kaldre et al. 2017; Caprioli et al. 2018). These markers can also be applied to eDNA samples if the concentration of target taxa DNA is sufficient (own unpublished results).

As a higher DNA concentration is required, this method is more suitable for identifying *A. astaci* genotypes from crayfish plague outbreaks (e.g. Mojžišová et al. 2020) than from chronically infected carriers. However, reliable results can also be obtained from immunocompromised individuals which are latent *A. astaci* carriers (Mojžišová et al. 2022, 2024; Laffitte et al. 2024; Chapter IV). This method has the highest resolution to date and distinguishes not only all *known A. astaci* genotype groups identified by RAPD, but also reveals variability within these groups as well as novel *A. astaci* genotypes or multiple strain co-infections (Grandjean et al. 2014; Maguire et al. 2016; James et al. 2017; Mojžišová et al. 2024; Chapter IV). However, in case of mixed patterns or identification of novel genotypes, the results should be interpreted with caution (Grandjean et al. 2014), as some of the obtained fragment lengths may belong to other Oomycetes species commonly found in crayfish (Kozubíková-Balcarová et al. 2013). Furthermore, the results may be complicated by the presence of null or partially null alleles due to primer mismatches or preferential amplification of some alleles over others (Dakin & Avise 2004; Jarne & Lagoda 1996). Therefore, especially in cases when microsatellite multilocus genotypes do not match known *A. astaci* strains, it is suggested to supplement microsatellite markers with other complementary genotyping methods described below (Makkonen et al. 2018; Di Domenico et al. 2021).

Although these have a lower resolution in distinguishing *A. astaci* genetic diversity than microsatellite markers, the concentration of *A. astaci* DNA in the isolate required for successful genotyping is also lower (Grandjean et al. 2014; Makkonen et al. 2018; Di Domenico et al. 2021), allowing more reliable data to be obtained from chronically infected individuals (e.g. Mojžišová et al. 2022).

Another marker frequently used in recent studies is based on the sequencing of two mitochondrial small and large ribosomal subunits (rnnS and rnnL), amplified by conventional PCR (Makkonen et al. 2018). The sensitivity of this approach is higher than amplification and/or sequencing of single-copy nuclear markers due to the higher copy number of mitochondria present in cells. The mtDNA markers allow the differentiation of most known RAPD-defined genotype groups but one (group C), as well as the differentiation of *A. astaci* from other known related oomycete species (Makkonen et al. 2018; Viljamaa-Dirks & Heinikainen 2019; Casabella-Herrero et al. 2021). In addition, the method also revealed significant additional variation in *A. astaci*, especially from areas not previously studied (Makkonen et al. 2018; Martín-Torrijos et al. 2018, 2021b, 2023).

Whole genome analysis of strains representing all known *A. astaci* genotype groups originally identified by RAPD allowed Minardi et al. (2018) to identify genomic regions unique to each representative RAPD-based

genotype group and to develop specific primers for a conventional PCR-based nuclear genotyping assay, which focuses on the presence/absence of PCR product after amplification. However, an additional step was required to distinguish one of the known *A. astaci* genotype groups (Minardi et al. 2018). Shortly afterwards, an alternative method based on restriction fragment length polymorphism (RFLP) of mtDNA markers was published (Minardi et al. 2019). However, this method is not widely used and seems to be a dead end. If someone can afford the molecular diagnosis and genotyping of *A. astaci*, the sequencing can be ensured too. The most recently developed *A. astaci* genotyping method is based on the genomic regions previously described by Minardi et al. (2018), but instead of using conventional PCR, it was developed for qPCR, which increases both specificity and sensitivity (Di Domenico et al. 2021). This method has been applied to pure cultures (Di Domenico et al. 2021), clinical crayfish samples (Di Domenico et al. 2021; Mojžišová et al. 2022, 2024; Chapter IV) and eDNA samples (own unpublished data; Sword et al. 2022). It was originally developed to rapidly assign common *A. astaci* RAPD-based genotype groups causing crayfish plague outbreaks in Europe. However, it also seems that unusual or new *A. astaci* genotypes not originally identified by RAPD also yield positive for developed qPCR assays (Mojžišová et al. 2024; Chapter IV) and moreover assay "C" cross-reacts with *A. fennicus* (Di Domenico et al. 2021). Cross-reactivity with *A. fennicus* is resolved using the recent *A. astaci* diagnostic method by Strand et al. (2023), and for other uncertain cases, it is strongly recommended to use other complementary genotyping methods whenever possible (Grandjean et al. 2014; Makkonen et al. 2018). Overall, these three genotyping methods, when used, seem to provide reliable information on the genetic variability of *A. astaci* (Becking et al. 2022; Mojžišová et al. 2022, 2024; Chapter IV).

A. astaci in the Western Palearctic

The first introduction of *A. astaci* into Europe probably occurred in the 19th century, with the earliest record of mass mortalities dating back to 1859 from the Po valley in Italy (Alderman 1996). Thereafter, it presumably began to gradually spread through European crayfish populations, and by the end of the 19th century many European crayfish populations were eradicated or on the verge of extinction (Alderman 1996; Jussila et al. 2021a). The RAPD genotype group A, originally isolated from the European noble crayfish *Astacus astacus* and the narrow-clawed crayfish *Pontastacus leptodactylus*, was presumably involved in these historical crayfish mass mortalities (Huang et al. 1994).

Subsequent waves of crayfish plague outbreaks in European crayfish populations were caused by other *A. astaci* strains (originally defined by RAPD; Svoboda et al. 2017), which were introduced to Europe along with three North American crayfish species in the late 19th and 20th centuries to supplement stocks (Alderman 1996; Holdich et al. 2009): RAPD genotype group B was isolated to axenic cultures from the signal crayfish *Pacifastacus leniusculus* (Huang et al. 1994); group D from *P. clarkii* (Diéguez-Urbeondo et al. 1995) and group E from the spiny-cheek crayfish *F. limosus* (Kozubíková et al. 2011b). These crayfish plague outbreaks still occur throughout Europe (Martín-Torrijos et al. 2019; Mojžišová et al. 2020; Brady et al. 2024), in contrast to the RAPD genotype group C, which was originally and only once isolated to axenic cultures from Canadian *P. leniusculus*, but never again unambiguously identified in Europe (Ungureanu et al. 2020).

Most information on *A. astaci* genotypes in Europe comes from crayfish plague outbreaks or axenic cultures, but information on *A. astaci* in chronic carriers, both native crayfish and their original hosts, is relatively scarce (Ungureanu et al. 2020). The *A. astaci* RAPD genotype groups B, D and E have a very uneven distribution (Ungureanu et al. 2020) and to some extent reflect the distribution of their original North American crayfish host species across Europe (Kouba et al. 2014), with some exceptions (e.g. Ungureanu et al. 2020; Mojžišová et al. 2020; Brady et al. 2024). These data suggest an association between these *A. astaci* genotype groups and their native North American hosts, but this species specificity was not observed in the pathogen's native range (Butler et al. 2020; Martín-Torrijos et al. 2021b; Chapter IV). However, our results in recent studies (Mojžišová et al. 2022; Laffitte et al. 2024) support the existence of this association in Europe, at least in regions where several North American crayfish species are not in contact (Mojžišová et al. 2022, 2024). This particular association of *A. astaci* with its hosts, which is probably the result of the founder effect and genetic drift (Francesconi et al. 2024), may be a useful indicator for tracing the sources of the pathogen during outbreaks of crayfish plague in Central and Western European countries (Caprioli et al. 2018; Martín-Torrijos et al. 2019; Mojžišová et al. 2020).

The various *A. astaci* strains we encounter may differ in physiological abilities, i.e. tolerance of higher temperatures or salinities (Diéguez-Urbeondo et al. 1995; Rezinciuc et al. 2014; Martín-Torrijos et al. 2021c) or in virulence (e.g. Makkonen et al. 2012b, 2014; Viljaama-Dirks et al. 2013, 2016; Becking et al. 2015; Francesconi et al. 2021). Laboratory experiments have shown that strains from the RAPD genotype group A tend to be less virulent than RAPD genotype groups B, D or E (Makkonen et al. 2012b, 2014; Jussila et al. 2013; Viljamaa-Dirks et al. 2013, 2016; Becking et al. 2015). As the RAPD genotype group A has so far only been detected in native European crayfish populations and its original host remains unknown (Huang et al. 1994; Jussila et al. 2021a), this has prompted the hypothesis that this genotype group was the first one introduced into Europe (Huang et al. 1994) and during more than a century and half of coexistence, this *A. astaci* strain has coevolved with some populations of European crayfish species (Jussila et al. 2014; Francesconi et al. 2021; Jussila et al. 2021b). It has been frequently detected in chronically infected populations of native crayfish in Europe and occasionally in Turkey (reviewed in Svoboda et al. 2017; Ungureanu et al. 2020; Akhan et al. 2024), but mass mortalities caused by the RAPD genotype group A are still being reported from Europe (Kozubíková-Balcarová et al. 2014; Mojžišová et al. 2020; Ungureanu et al. 2020; Brady et al. 2024). It is thus likely that strains from this group vary in virulence (but difference in susceptibility of the host populations, or environmental factors that may influence the intensity of host immune response may also play a role).

It has been experimentally demonstrated that under certain circumstances, even *P. leniusculus* can succumb to infection caused by *A. astaci* RAPD genotype groups A and B (Aydin et al. 2014). Subsequently concurrent infection with *A. astaci* and *Fusarium* species complex may cause eroded swimmeret syndrome and the collapse of some wild *P. leniusculus* populations in Fennoscandia (Edsman et al. 2015; Jussila et al. 2021c). On the contrary, it has been shown experimentally that some native European crayfish populations have become more resistant to *A. astaci* (e.g. Makkonen et al. 2012b, 2014; Becking et al. 2015; Martín-Torrijos et al. 2017; Jussila et al. 2020; Martínez-Ríos et al. 2022). Furthermore, despite the presumed higher virulence

of RAPD genotype group B (Jussila et al. 2013, 2017), some wild native crayfish populations chronically infected with this strain have also been detected in Eastern Europe, Turkey and Armenia (Ungureanu et al. 2020; Svoboda et al. 2017; Akhan et al. 2024). The results of these experiments, together with observations in the wild, underscore that variable host resistance also plays an important role in *A. astaci* chronic infections (Jussila et al. 2014). Chronic *A. astaci* infections in European native crayfish seem to be more common in some European countries, such as Croatia (Maguire et al. 2016; Pavić et al. 2020, 2021), and the number of countries in Western Palearctic detecting such populations in recent years is increasing (reviewed in Ungureanu et al. 2020; Martín-Torrijos et al. 2017; Mirimin et al. 2022; Alvanou et al. 2024). In Czechia, however, we have not yet detected such populations (Mojžišová et al. 2020).

More recent *A. astaci* genotyping methods applicable to mixed genome samples, in particular nuclear microsatellite and mtDNA markers (Grandjean et al. 2014; Makkonen et al. 2018), have allowed the identification of additional variability among *A. astaci* genotype groups originally defined by RAPD involved in both mass mortality (Grandjean et al. 2014; Martín-Torrijos et al. 2018) and chronic carriers (Maguire et al. 2016; James et al. 2017; Mrugała et al. 2017; Panteleit et al. 2019; Martín-Torrijos et al. 2018, 2021b, 2023; Mojžišová et al. 2024; Chapter IV). *A. astaci* mtDNA markers distinguished so far four additional haplotypes *usa6*, *d1*, *d2*, *d3* within the *A. astaci* haplogroup 'D', the latter being so far only present in Japan (Makkonen et al. 2018; Martín-Torrijos et al. 2018, 2021b). However, mtDNA markers were unable to differentiate various strains (namely: RAPD genotype groups A and C; *rust1-2*; *Up*; Budapest; PA1-6) that all shared the same *A. astaci* haplogroup 'A' (Makkonen et al. 2018; Panteleit et al. 2019; Mojžišová et al. 2024; Chapter IV). Therefore, it is not surprising that different strains from haplogroup 'A' showed substantial differences in virulence (Francesconi et al. 2024).

An example of *A. astaci* genotype that highlights problems with reliance to any single genotyping markers is the genotype *Up*, identified originally from a crayfish mass mortality in Czechia (from Úpořský brook, hence the abbreviation *Up*) but never obtained to axenic culture. It is assigned to *A. astaci* haplogroup 'A' by mtDNA markers (Makkonen et al. 2018), yields a positive signal for assay "B" by qPCR genotyping (Di Domenico et al. 2021) but has a unique microsatellite multilocus genotype (Grandjean et al. 2014). It has been involved in mass mortalities in Czechia (Grandjean et al. 2014; Mojžišová et al. 2020) but also has been detected in a chronically infected population of *P. leptodactylus* in the Danube delta (Panteleit et al. 2018). Its original North American host remains unknown to date.

In recent decades, the popularity of ornamental crustaceans in the pet trade has increased in Europe (Chucholl 2013; Faulkes et al. 2015; Patoka et al. 2016a), resulting in the deliberate or accidental introductions of a wider range of invasive North American crayfish species into the wild (e.g. Weiperth et al. 2019, 2020; Bláha et al. 2022; Laffitte et al. 2023). The presence of *A. astaci* in ornamental crayfish and evidence of its indirect horizontal transmission between *Procambarus* spp. has already been reported from the aquarium trade (Keller et al. 2014; Mrugała et al. 2015; Panteleit et al. 2017).

In the study conducted by Mrugała et al. (2015), the *A. astaci* genotype group D was identified using the RAPD method. Similar findings were reported in other studies investigating aquarium crayfish, where sequencing of the chitinase gene of *A. astaci* also classified the pathogen in this genotype group (Keller et al.

2014; Panteleit et al. 2017). It is believed that this strain(s) of *A. astaci* may have been transmitted from *P. clarkii*, which is the typical carrier of the *A. astaci* genotype group D; however, this remains uncertain. It cannot be excluded that some species of the family *Cambaridae* are natural hosts of *this A. astaci* genotype group (Mrugała et al. 2015; Martín-Torrijos et al. 2021b). Therefore, when crayfish plague outbreaks caused by two different haplotypes d1 and d2 were recorded in Czechia, we assumed that the source of infection may have originated from the aquarium trade (Mojžišová et al. 2020). The hypothesis that ornamental crayfish populations spread *A. astaci* in open waters was indirectly supported by our subsequent research (Mojžišová et al. 2024), however we did not detect these *A. astaci* strain(s) in Budapest. The *A. astaci* variability in other North American crayfish species introduced to Europe is poorly documented so far. In addition to data on the genetic diversity of *A. astaci*, information on the distribution and prevalence of *A. astaci* in the populations of its chronic carriers is also important for effective management of native European crayfish. This is particularly important for the identification of regions where native susceptible crayfish populations are at risk. Recently, country-wide surveys of the spatial distribution and/or prevalence of *A. astaci* have been carried out in many more European countries (e.g. Martín-Torrijos et al. 2019; Sieber et al. 2023; Basso et al. 2023; Laffitte et al. 2024). Large-scale prevalence studies have been performed on populations of *P. leptodactylus* in Turkey (Akhan et al. 2024) and on populations of the most widespread North American crayfish species in Europe, *F. limosus*, *P. leniusculus* and *P. clarkii* (e.g. Grandjean et al. 2017; Mojžišová et al. 2022; Laffitte et al. 2024). For other North American host species present in Europe, prevalence was also frequently examined (e.g. Filipová et al. 2013; Tilmans et al. 2014; Lipták et al. 2017; Laffitte et al. 2023; Mojžišová et al. 2024). Most studies have shown that the prevalence of *A. astaci* can vary considerably between species/populations and regions, as well as within regions (e.g. Tilmans et al. 2014; Maguire et al. 2016; Grandjean et al. 2017; Akhan et al. 2024) from no detection (e.g. Schrimpf et al. 2013; Caprioli et al. 2018; Mojžišová et al. 2022; 2024) to 100% (e.g. Kozubíková et al. 2011a; Filipová et al. 2013), a similar pattern was also observed in the native range of *A. astaci* (Chapter IV).

A crayfish population where no *A. astaci* is detected, even if free of the pathogen at a given time, may still acquire the infection from some source later. However, once *A. astaci* is present in the population of its natural host, its prevalence does not tend to change significantly over time (Mojžišová et al. 2022). Although there were some temporal variations in the prevalence of *A. astaci*, these could be explained by determinants such as the density of the host population, or the level of stress to which the crayfish hosts are exposed (Matasová et al. 2011). However, it appears that the founder effect is a more important determinant of *A. astaci* prevalence in invasive crayfish populations (Kozubíková et al. 2011a; Tilmans et al. 2014).

A. astaci in other regions in the world

Apart from introductions of North American crayfish to Europe, some of those species, especially *P. clarkii* but also *P. leniusculus*, have been introduced for commercial purposes (aquaculture of fisheries) in other regions of the world (Hobbs et al. 1989). In addition, various crayfish species including *P. clarkii* have been rapidly gaining popularity as an ornamental species (Chucholl 2013; Faulkes et al. 2015; Souty-Grosset et al. 2016). Because *P. clarkii* is so extensively used for various purposes, it is currently the most widely

distributed crayfish in the world (Oficialdegui et al. 2020). Other ornamental crayfish species popular in the pet trade include the parthenogenetic crayfish *P. virginialis* (Faulkes 2015), which has also been recently recorded from different parts of the world including Europe, Israel, Madagascar or Japan (see <https://sites.google.com/view/marmorkrebs/>; Sánchez et al. 2024). The introduction of these crayfish species, which are known carriers of *A. astaci* (Svoboda et al. 2017), has contributed to the global spread of this pathogen (see: <https://map.crayfish.ro/en/map/crayfish/>).

Similar to Europe, *A. astaci* is frequently present in both invasive crayfish species widespread in Japan, *P. clarkii* and *P. leniusculus*, and its detected prevalence varied from no detection to 100% (Mrugała et al. 2017). It was proven to cause mass mortalities in endemic populations of the Japanese crayfish *Cambaroides japonicus* (Martín-Torrijos et al. 2018). *A. astaci* involved in crayfish plague outbreaks in *C. japonicus* was characterised by mtDNA sequencing (Martín-Torrijos et al. 2018), which revealed haplotypes b, d1 and d3. The last-mentioned one was revealed in a local populations of *P. clarkii* (Martín-Torrijos et al. 2018), and the two other haplotypes are those typically identified in the respective host crayfish species in Europe (Ungureanu et al. 2020; Mojžišová et al. 2022; Laffitte et al. 2024). Crayfish plague outbreaks have also been reported in cultured population of the redclaw *Cherax quadricarinatus* in Taiwan (Hsieh et al. 2016).

Other studies confirmed the presence of *A. astaci* as a subclinical infection of North American crayfish species in subtropical and several tropical regions, where no mass mortality of native crayfish species has been recorded to date (OIE 2014; Peiró et al. 2016; Putra et al. 2018; Andriantsoa et al. 2019; Martín-Torrijos et al. 2021a). The crayfish plague pathogen has been reported from a closed system of farmed population of the Everglades crayfish *Procambarus alleni* in Israel (OIE 2014) and in established populations of *P. virginialis* in Madagascar (Andriantsoa et al. 2019). Additional records of *A. astaci* presence were associated with *P. clarkii* in Java, both in the wild population and in the pet shop (Putra et al. 2018), in its established populations in Brazil (Peiró et al. 2016), and in a feral population in Costa Rica. In the latter, *A. astaci* haplogroup 'D' was identified based on one mitochondrial marker (Martín-Torrijos et al. 2021a). Some of these records (from Brazil, Indonesia and Madagascar) are from or close to regions where crayfish of the family Parastacidae are native (Peiró et al. 2016; Putra et al. 2018; Andriantsoa et al. 2019; Kawai & Crandall 2016; De Los Rios Escalante et al. 2022). It has been shown experimentally that species of this family occurring in Australasia are generally highly susceptible to the crayfish plague pathogen (reviewed in Svoboda et al. 2017). However, the level of resistance to *A. astaci* in crayfish species native to South America or Madagascar remains unknown. There is some evidence that a pathogen spillover may have occurred to their populations in Brazil (Peiró et al. 2016) and that native crayfish coexist with weakly infected marbled crayfish in Madagascar (Andriantsoa et al. 2019) without causing documented mass mortalities (at least not yet).

Data on the distribution and diversity of *A. astaci* in its native range, North America, remains scarce, with six studies conducted to date (Panteleit et al. 2019; Makkonen et al. 2019; Butler et al. 2020; Martín-Torrijos et al. 2021b, 2023; Chapter IV). Recent studies of *A. astaci* in its native range indicate that the eastern USA appears to be a center of *A. astaci* diversity and that, unlike in Europe, the association between crayfish host species and *A. astaci* genotypes or haplogroups is not significant (Panteleit et al. 2019; Butler et al. 2020;

Martín-Torrijos et al. 2021b, 2023; Chapter IV). However, as could be expected, the presence of matching *A. astaci* genotypes or haplogroups has been confirmed in populations of *P. clarkii* and *P. leniusculus* from source regions in the USA and from regions invaded by these species out of North America (Makkonen et al. 2019; Martín-Torrijos et al. 2021b). *A. astaci* was frequently recorded in the studied areas, and already no less than 29 North American crayfish species were found to carry *A. astaci*, and 11 haplotypes (namely a, b, d1, d2, mex1, USA1-6) were identified, seven of which were previously unknown from invaded regions (Panteleit et al. 2019; Makkonen et al. 2019; Butler et al. 2020; Martín-Torrijos et al. 2021b, 2023; Chapter IV). Infections in other North American crayfish species have also been reported in the pet trade (Mrugała et al. 2015; Panteleit et al. 2017). These infections may be the result of interspecies transmission or the potential introduction of the pathogen during the capture process. Although the exact source remains unclear, this observation suggests the ability of these species to host *A. astaci*.

The use of additional complementary genotyping methods (Di Domenico et al. 2021; Grandjean et al. 2014) revealed even greater *A. astaci* diversity, and within haplogroup 'A' the presence several other *A. astaci* strains not known from Europe was indicated (Panteleit et al. 2019; Chapter IV). However, none of the studies conducted in North America so far have recorded some of the *A. astaci* genotype groups frequently encountered or studied in Europe: A (known only from native European crayfish mass mortalities and chronic infections; Ungureanu et al. 2020); C (isolated to axenic culture from *P. leniusculus* of Canadian origin; Huang et al. 1994) and E (carried by *F. limosus* in Europe and frequently causing mass mortalities of native European crayfish; Ungureanu et al. 2020; Mojžišová et al. 2022).

AIMS OF THE THESIS

The crayfish plague pathogen is responsible for the decline of native crayfish populations across Eurasia and represents a significant threat to crayfish aquaculture production in various regions, particularly for farmed crayfish. Research on the variability of *A. astaci* contributes to a deeper understanding of the host-pathogen relationship and the pathways of its introduction.

Furthermore, knowledge of the epidemiology of this pathogen aids in identifying regions where native crayfish populations are at-risk, enabling conservation authorities to prioritise these areas for intervention. It also helps to identify 'crayfish plague-free' regions suitable for the reintroduction of native crayfish populations. In the context of aquaculture, it helps to implement effective disease management strategies such as biosecurity protocols for transport and stocking of crayfish.

The principal aim of my doctoral research was to investigate the genetic variability of *A. astaci* and its distribution and prevalence in different potential crayfish hosts, both native and non-native, in different regions, and to investigate interspecific transmission of different *A. astaci* genotypes.

The partial aims are given separately for each Chapter and appendix:

CHAPTERS:

- I. To provide up-to-date data on crayfish plague outbreaks in Czechia, characterize the genotypes of *A. astaci* responsible for these recent outbreaks, and assess the potential occurrence of chronic infections in native crayfish populations in this region.
- II. To examine long-term changes in *A. astaci* prevalence in selected populations of invasive crayfish re-sampled after more than ten years, and to update *A. astaci* distribution and prevalence in host populations in Czechia. Additionally, to examine links between specific *A. astaci* genotypes and their North American crayfish hosts.
- III. To investigate the potential role of ornamental crayfish taxa in the spread and interspecific horizontal transmission of *A. astaci* in open waters, by conducting a case study on the distribution, prevalence, and diversity of this pathogen in populations of (mostly) ornamental crayfish established in urban waters of Budapest.
- IV. To investigate the distribution, prevalence and diversity of *A. astaci* in its native range, specifically in Pennsylvania, a US state where populations of native and introduced North American crayfish frequently meet and which hosts native populations of *Faxonius limosus*, the original chronic carrier of *A. astaci* genotype group E.

APPENDICES

- A. To test for *A. astaci* chronic infections in selected populations of *P. leptodactylus* in the Dnieper River in Ukraine, and to summarise literature data on the distribution of *A. astaci* genotypes recorded across Europe.
- B. To develop and validate a qPCR method applicable to mixed genome samples (primarily from crayfish hosts) that would rapidly assign *A. astaci* to common RAPD-based genotype groups causing crayfish plague outbreaks in Europe.
- C. To evaluate the specificity of the qPCR-based assays for selected crayfish species present in Central Europe and to assess the presence of *A. astaci* and its different crayfish hosts using analysis of environmental DNA obtained from water samples from various habitats in Czechia (and nearby countries). In addition, to evaluate the applicability of a robust, easy-to-use and low-cost hardware set-up suitable for eDNA sampling.

OVERVIEW OF THE RESEARCH INCLUDED IN THE THESIS

(thesis Chapters are highlighted in bold)

The core of my PhD thesis consists of three already published first-author papers and one submitted co-authored paper (**Chapters I-IV**), which share the overall research questions and methodological approaches. My research focused on the distribution, prevalence and genetic diversity of *A. astaci* in 1) populations of European native crayfish, and 2) in populations of North American crayfish species in some invaded regions in Europe as well as in the native range of this pathogen. **Chapter I (Mojžišová et al. 2020)** is partially based on the results already obtained during my MSc studies but the data set has been substantially expanded afterwards. Moreover, three additional studies are included in my thesis as appendices (**Appendix A-C**). These co-authored publications are the outcomes of different collaborations related to *A. astaci* to which I contributed by laboratory work, for example by detection of *A. astaci* or its genotyping.

For more than a century and a half, native crayfish populations have been devastated by crayfish plague throughout the European continent (Alderman 1996). In the past, it was believed that when the crayfish plague strikes, the entire native crayfish populations become extinct within weeks (Alderman et al. 1987, Kozubíková et al. 2008). Nevertheless, already in the 20th century it was shown experimentally that some individuals of the native European crayfish *P. leptodactylus* survived exposure to crayfish plague infection (Unestam 1969b). However, it is only in recent years, with the development of new molecular-based assays, that crayfish populations of various native European species chronically infected with *A. astaci* have been discovered in different European countries, as were the populations of *P. leptodactylus* from Turkey or Armenia (reviewed in Svoboda et al. 2017; Pavić et al. 2021; Satmari et al. 2023; Akhan et al. 2024; Alvanou et al. 2024; Brady et al. 2024). We contributed to the knowledge of this phenomenon by studying the distribution and diversity of *A. astaci* in populations of *P. leptodactylus* in Ukraine (in the Dnieper River), which we also found to be chronically infected with this pathogen (**Appendix A: Ungureanu et al. 2020**). In **Chapter I**, we focused on screening for chronically infected native crayfish populations in Czechia. However, their presence has not been detected in this region. On the contrary, we could not but notice a continuing trend in native crayfish mass mortalities caused by *A. astaci*.

With the molecular methods developed in the last decade (Grandjean et al. 2014; Makkonen et al. 2018; **Appendix B: Di Domenico et al. 2021**), we can not only detect the presence of *A. astaci*, but also characterize the pathogen genotypes involved in mass mortalities and chronic infections of carrier crayfish (e.g. Grandjean et al. 2017; Martín-Torrijos et al. 2019). The genotyping of *A. astaci* is the main topic that links all core Chapters and most of the Appendices of my PhD thesis.

The outbreaks in Czechia studied in the last two decades were caused by a diverse range of *A. astaci* genotypes known from Europe (Grandjean et al. 2014; Kozubíková-Balcarová et al. 2014). Specifically, in **Chapter I**, with the use of nuclear and mitochondrial markers, we confirmed persisting presence of genotype groups A and E, and after more than 10 years, we recorded once more the unusual *A. astaci* genotype “Up”. However, these results were not as surprising as the discovery that two unrelated outbreaks were each caused by a different haplotype assigned to genotype group D.

This *A. astaci* genotype group, usually carried by *P. clarkii* (Dieguez-Uribeondo et al. 1995; Mrugała et al. 2015, 2017; Makkonen et al. 2018; Martín-Torrijos et al. 2018, 2021a,b; 2023), has also been documented in several other species of the family Cambaridae, including the Cajun dwarf crayfish *Cambarellus shufeldtii* and the Hatchie River crayfish *Procambarus ablusus* from the wild (Martín-Torrijos et al. 2021b), and *Procambarus llmasi*, *P. virginalis*, and *P. alleni* from aquaria (Keller et al. 2014; Mrugała et al. 2015; Panteleit et al. 2017).

However, neither *P. clarkii* nor any of the abovementioned species have been recorded in the wild in Czechia, apart from *P. virginalis*, in whose known populations we have not recorded the presence of *A. astaci*. (Patoka et al. 2016b; **Chapter II: Mojžišová et al. 2022**). However, we assume that the most likely introduction pathway of the genotype group D to Czechia was through the release of some cambarids originating from the ornamental trade, which has been recently the most common way of their spread in Central Europe (Chucholl 2013; Faulkes 2015).

The isolates from *A. astaci*-positive crayfish collected mostly during the abovementioned crayfish plague outbreaks from Czechia and from central Italy, together with DNA extracts from pure *A. astaci* cultures, were used to evaluate a new rapid genotyping assay. In **Appendix B**, we describe the development of a real-time PCR TaqMan assay aimed to distinguish the common *A. astaci* genotype groups known to cause crayfish mass mortalities in Europe. Although some of the markers are not completely specific for the target genotype group, the method itself turned out to be useful for genotyping both crayfish plague outbreaks and asymptomatic hosts with sufficiently high *A. astaci* infection load. In subsequent projects, I have successfully employed this genotyping method in combination with other available assays to study *A. astaci* genetic diversity (**Chapter II - IV**).

Due to substantial introduction bottlenecks, *A. astaci* genotypes in Europe seem to be associated with their original North American hosts, with which they were introduced to the continent. Hence, the genotype assignment may help us to trace the sources of infection. However, most of the data on *A. astaci* genotypes in Europe were obtained from mass mortalities of native crayfish (see **Chapter I**, and the review in **Appendix A**) or from laboratory cultures (reviewed in Svoboda et al. 2017), and data from North American carriers were less frequent. Therefore, one of the main objectives of the subsequent three Chapters (**Chapter II; Chapter III: Mojžišová et al. 2024; Chapter IV: Petrusek et al. submitted**) was to study the *A. astaci* variability in populations of pathogen's original hosts in different regions. In **Chapter II** we examined *A. astaci* variability in selected populations of two North American crayfish species established in Czechia. In our study, we detected the same genotype of *A. astaci* in representatives of both *P. leniusculus* and *F. limosus* at the only known locality where these species coexist in Czechia, suggesting horizontal transmission of *A. astaci* from the former to the latter. Nevertheless, the rest of data from this study supports the existence of a specific association between the *A. astaci* genotype and its original host. At least in invaded areas where there is no frequent contact between different North American crayfish species, tracing the source of *A. astaci* infection by genotype assignment appears to be a reliable strategy.

Besides the investigation of *A. astaci* variability in infected North American hosts, another goal of this study was to examine temporal changes in *A. astaci* prevalence over a period of more than ten years in selected

populations, known from previous screenings of the two alien crayfish species common in Czechia. Our data suggest that, despite some fluctuations, the prevalence of *A. astaci* in North American host populations generally does not tend to vary significantly over longer period. In addition, we updated the data on the distribution of *A. astaci* and its prevalence in selected populations of all non-native crayfish species present in Czechia. We discovered new hotspots of highly infected *P. leniusculus* populations but, fortunately, did not find the pathogen in the two known *P. virginalis* populations in Czechia.

In **Chapter III** we examined the variability, distribution and prevalence of *A. astaci* in selected populations of five North American crayfish species established in Budapest (plus the native *P. leptodactylus*). This capital city of Hungary has become the “epicenter” of multiple undesirable aquarium species, including crayfish, thanks to the locally abundant thermal waters (Weiperth et al. 2019; 2020). Aquatic habitats with elevated temperatures are particularly attractive to hobbyists who abandon their pets for various reasons (summarized in Bláha et al. 2022). Unfortunately, their stable temperatures facilitate the establishment of non-native species in the wild (Emde et al. 2016). A large proportion of the ornamental crayfish species sold in the pet trade originate from the native range of *A. astaci* (Chucholl 2013; Patoka et al. 2014; Chucholl & Wendler 2017). The sites in Budapest where these species come into contact provided us with a good opportunity to investigate their role in the spread of this pathogen in open waters. The results in **Chapter III** indirectly supported the hypothesis that *A. astaci* is spread with the ornamental crayfish and released into the wild with them. We detected infections in most of the North American alien crayfish species found in the vicinity of Budapest, three of which are certainly of aquarium origin. Our genotyping results revealed the presence of two *A. astaci* mtDNA haplogroups, 'B' and 'A', which have changed their dominance in the population of three coexisting alien crayfish species over a short period of time, implying at least two introductions of the pathogen into Budapest. Additional genotyping methods revealed that haplogroup 'A' was represented by a novel *A. astaci* genotype, likely introduced with the ornamental taxa. The results in **Chapter III** underscore the complexity of *A. astaci* dynamics in regions with multiple potential hosts and indirectly indicate horizontal transmission between them. The contact between multiple hosts and pathogen genotypes can lead to unpredictable infection patterns, preventing the source of the mass mortality from being reliably traced.

When we initiated our project presented in **Chapter IV**, virtually no data were available on the distribution and variation of *A. astaci* in North America, its native range. Meanwhile, five studies have been published (Panteleit et al. 2019; Makkonen et al. 2019; Butler et al. 2020; Martín-Torrijos et al. 2021b, 2023) but the amount of information still remains very limited. The study by Martín-Torrijos et al. (2021b), based on sequence variation of mitochondrial markers, is most extensive and suggests, as expected, a higher *A. astaci* variability in its native range than in the invaded regions. These data suggest only a weak, if any, link of *A. astaci* haplotypes to the specific host crayfish (Martín-Torrijos et al. 2021b). However, the studied markers have only a limited resolution, so the real diversity of *A. astaci* strains may be much higher, and their distribution among host species different from what mtDNA suggests.

To better understand the *A. astaci* strain-host relationships, we examined in **Chapter IV** variability, distribution and prevalence of *A. astaci* in populations of multiple native and non-native North American

crayfish species in this pathogen's native range. We focused specifically on Pennsylvania, an area where such host species get into contact (Lieb et al. 2011a, b), and from which we had a possibility to obtain material thanks to collaboration our co-author David A. Lieb from Pennsylvania Fish and Boat Commission. Furthermore, the Delaware River, which flows through this US state, was believed (Schikora 1916; Henttonen & Huner 1999) to be the original source of the only successful introduction to Europe of *F. limosus*, the sole so far known North American host of the *A. astaci* genotype group E (Kozubíková et al. 2011b; **Appendix A; Chapter II**). In this study, we detected the presence of *A. astaci* in most of the host taxa studied, at more than three quarters of the sampling sites, confirming a really broad distribution and common infections of this pathogen. Population-level prevalence and pathogen loads in cambarids were similar to those in the invaded range. Genotype group E was not detected in populations of *F. limosus* or any other cambarid tested, possibly due to its limited spatial distribution. We only identified two *A. astaci* mtDNA haplotypes, but the nuclear markers revealed that these comprised at least seven distinct strains of *A. astaci*, none of which are known to occur in invaded areas. These results emphasize that the combination of genotyping methods is essential when studying the diversity of (not only) *A. astaci*.

During the project focusing on distribution of invasive crayfish and their infection by *A. astaci* (presented in **Chapters II and III**), we also collected, in collaboration with colleagues from Norwegian Veterinary Institute in Oslo, environmental DNA (eDNA) from a wide range of localities in Czechia as well as from additional urban sites invaded by marbled crayfish in Berlin and Budapest. These eDNA samples were used to screen for the presence of three invasive and native crayfish, as well as for the crayfish plague pathogen (**Appendix C: Rusch et al. 2020**). The eDNA results were complemented, whenever possible, with screening for *A. astaci* in the local crayfish populations. Apart from evaluating the specificity of the qPCR-based assays for the target taxa, we presented in this study a robust, easy-to-use, and low-cost eDNA sampling device that remains in use in our research group. We have successfully implemented these methods in our laboratory and continue to use them in subsequent projects.

CONCLUSIONS

The studies compiled in this thesis have contributed to the knowledge of prevalence, distribution and genetic variability of *A. astaci* in both susceptible and original hosts of this pathogen in different regions, from Central and Eastern Europe to the USA.

The prevalence of *A. astaci* depends on many factors, including its introduction history, interactions with other species, or host population density. The prevalence of *A. astaci* in North American crayfish populations can vary significantly among invaded areas, a pattern that is analogous to the native range of this pathogen. However, we found that the prevalence of *A. astaci* in North American crayfish populations usually does not shift significantly even over relatively long period. Highly infected populations of North American crayfish (and nearby populations of susceptible native species) should remain a higher priority for management strategies, but low prevalences of *A. astaci* may still pose a threat to native crayfish and should not be underestimated. Therefore, regular screening for *A. astaci* should be of high priority at sites where native susceptible crayfish may encounter invasive crayfish species. The traditional screening methods of *A. astaci* may be supplemented by eDNA methodology, which we confirmed once again to be a useful tool in monitoring programs.

The same applies to chronically infected populations of native European crayfish, which have either become more common in Europe or at least we have been recognising more and more of them in recent years. Although the existence of persistent native crayfish populations may offer some future hope for their conservation, it must be remembered that they are still reservoirs of *A. astaci* infection that can spread the disease further, either through anthropogenic transport or contact with other species.

Although we did not discover chronically infected native crayfish populations in Czechia, their potential occurrence cannot be ruled out, as the existence of such populations are a plausible explanation of occasional crayfish plague outbreaks caused by genotype group A. These also occurred in at least two Czech watercourses in the recent decade, but we found out that the recent outbreaks in Czechia were caused by four known *A. astaci* genotype groups (A, B, D, E) plus still enigmatic genotype Up, which has caused mass mortality in this area already in the past. Such a wide range of genotypes suggests different sources of *A. astaci* infection. The recent detection of two different *A. astaci* haplotypes d1 and d2 indicated two independent sources of *A. astaci* group D in the country, possibly of aquarium origin. The crayfish plague pathogen (coincidentally, of this genotype group) has already been documented in several North American crayfish species sold in pet shops. Our subsequent study, which focused on the distribution and prevalence of the crayfish pathogen in urban waters of Budapest, indirectly supports the hypothesis that established ornamental crayfish populations are spreading this pathogen in open waters. While populations of the three most widespread crayfish species (*P. clarkii*, *P. leniusculus*, *F. limosus*) in Europe remain the primary source of *A. astaci* infection, the findings of our research suggest that the aquarium trade represents another significant pathway for the introduction of *A. astaci* into freshwater ecosystems.

It seems that *A. astaci* genotype groups are associated with their original North American crayfish hosts in invaded areas as Europe. This is probably due to the founder effect, as such narrow pathogen-host specificity

does not seem to occur in the native range of *A. astaci*. Our routine country-wide screening for *A. astaci* presence with subsequent *A. astaci* genotyping in populations of their original North American hosts in Czechia supported the link between *P. leniusculus* and *F. limosus* and genotype groups B and E, respectively. This specific *A. astaci* association may be therefore a useful indicator for tracing the sources of the pathogen in crayfish plague outbreaks in Central and Western European countries. We have also used it when investigating recent crayfish plague outbreaks in Czechia. However, when potential *A. astaci* hosts come into contact, the resulting combinations of host taxa and *A. astaci* genotypes may become unpredictable, which may hinder the reliable genotyping of infection sources in local crayfish mass mortalities. Furthermore, we found out that when different genotypes of *A. astaci* encounter, some individuals can carry co-infection, but it seems that over time one more successful pathogen genotype may outcompete another, and become dominant in the population.

The *A. astaci* genotype group E, which is typically carried by *F. limosus* in Europe, has not been detected in the region from which *F. limosus* was supposedly successfully introduced. It is most likely that it has only a restricted range and might eventually be found in other parts of *F. limosus* range, but we cannot rule other scenarios, such as disappearance of original host populations, its replacement with some more successful *A. astaci* genotype over time.

Furthermore, when investigating the variability of *A. astaci* in its native range using mtDNA markers, we found seemingly low variation of strains, with only two *A. astaci* haplogroups (which are also common in Europe). However, using other genotyping methods, we found significantly greater variability in *A. astaci* and confirmed that in fact none of these genotypes are known from Europe. The unusual combination of mitochondrial haplotypes and nuclear markers (b/C) raises intriguing questions regarding the evolutionary history, dispersal patterns, and potential gene flow between different *A. astaci* strains. This data highlights the importance of using various complementary genotyping methods (in our case, mtDNA markers, qPCR genotyping and microsatellite markers) for more comprehensive characterisation of *A. astaci* variability. However, it is not always possible to use multiple methods because chronic carriers often have low levels of *A. astaci* DNA. Therefore, it would be advisable in the future to develop a reliable genotyping method to consistently differentiate *A. astaci* variability even at low DNA concentrations, which are typical for original *A. astaci* hosts.

To sum up:

- 1) Populations with low *A. astaci* prevalence should not be underestimated when planning conservation strategies.
- 2) It is important to implement routine country-wide screening for *A. astaci* and its hosts, ideally using traditional capture-based monitoring combined with eDNA screening.
- 3) It is necessary to educate the public, especially hobbyists and pet shop owners, about the problems associated with releasing pets, not only because they may become invasive themselves but also of potential release of harmful pathogens.

- 4) The identification of *A. astaci* genotypes in Europe is a suitable tool for tracing the source of infection, but not in places where several *A. astaci* original hosts are in contact.
- 5) The combination of genotyping methods is essential to study the diversity of (not only) *A. astaci*.

FUTURE PERSPECTIVES

There are still many gaps in our knowledge of *A. astaci* that need to be filled in order to develop coherent strategies for the conservation of endangered crayfish species. Here, I outline several future directions that I believe could provide valuable insights and inspiration that would be of interest to a crayfish research community:

Investigation of A. astaci axenic cultures from its native range in more details:

In general, data on *A. astaci* in its native range is still scarce. In the study by Martín-Torrijos et al. (2021b) investigated the variability *A. astaci* in its native range using only mtDNA markers, which have relatively low resolution. As a result, the interpretation of *A. astaci*'s genetic variability may have been underestimated. Axenic cultures of *A. astaci*, especially those representing novel genotypes could be studied in more detail using variable markers or whole-genome sequencing, which would provide a more accurate and reliable assessment of *A. astaci* genetic diversity, and open new research avenues.

Development of sensitive genotyping method(s) with high resolution:

In studies examining the diversity of *A. astaci* within its original hosts, we often encounter low levels of *A. astaci* DNA. This often limits the applicability of genotyping methods, such as microsatellite markers, when working with mixed genome samples. While alternative non-culture-based genotyping methods can be utilized, these tend to offer lower resolution in distinguishing *A. astaci* variability. One potential solution is the preparation of axenic cultures from crayfish exuviae, as demonstrated by Martín-Torrijos et al. (2021b). However, the process of preparing axenic cultures can be both time-consuming and resource-intensive, requiring the isolation and cultivation of the pathogen, which can be challenging and may not always yield sufficient material for comprehensive analysis. Therefore, it would be advisable in the future to develop alternative markers to microsatellites that are sensitive and reliable even at low pathogen DNA concentrations and that would distinguish *A. astaci* genotypes directly from mixed genome samples.

Screening of apparently healthy P. leptodactylus populations for the potential occurrence of A. astaci chronic infections in Central and Western Europe:

Several studies (including our own) have confirmed the presence of chronic *A. astaci* infections in *P. leptodactylus* populations, particularly in Eastern Europe, Turkey, and Armenia. However, data from Central and Western Europe remain scarce. This phenomenon appears to be more frequent in *P. leptodactylus* than in other native European crayfish species, suggesting that *P. leptodactylus* may exhibit a lower susceptibility to *A. astaci*. Chronically infected individuals pose a significant threat to more susceptible native crayfish species by facilitating the spread of the pathogen. While we have not observed chronically infected

populations of *A. astacus* or *A. torrentium* in Czechia, the potential for their existence cannot be excluded. The presence of chronically infected *P. leptodactylus* populations may explain occasional crayfish plague outbreaks in Czechia caused by genotype group A or the enigmatic genotype Up. Investigating the role of *P. leptodactylus* in the spread of *A. astaci* in Central and Western Europe, a task that we have planned but not managed to realize yet, could provide crucial insights into the epidemiology of *A. astaci* and its impact on native crayfish populations.



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