Estimating the threat posed by the crayfish plague agent *Aphanomyces astaci* to crayfish species of Europe and North America

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Introduction pathways, distribution and genetic diversity

by

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TABLE OF CONTENTS

1. LIST OF PUBLICATIONS

I. **Panteleit, J**., Keller, N. S., Kokko, H., Jussila, J., Makkonen, J., Theissinger, K., and Schrimpf, A. (2017): Investigation of ornamental crayfish reveals new carrier species of the crayfish plague pathogen (*Aphanomyces astaci*). *Aquatic Invasions* 12(1): 77–83. DOI: 10.3391/ai.2017.12.1.08.

II. Pârvulescu, L., Togor, A., Lele, S., Scheu, S., Şinca, D., and **Panteleit, J**. (2017): First established population of marbled crayfish *Procambarus fallax* (Hagen, 1870) f. *virginalis* (Decapoda, Cambaridae) in Romania. *BioInvasions Records* 6(4): 357–362. DOI: 10.3391/bir.2017.6.4.09.

III. Makkonen, J., Jussila, J., **Panteleit, J.**, Keller, N. S., Schrimpf, A., Theissinger, K., Kortet, R., Martín-Torrijos, L., Sandoval-Sierra, J., Diéguez-Uribeondo, J., and Kokko, H. (2018): MtDNA allows the sensitive detection and haplotyping of the crayfish plague disease agent *Aphanomyces astaci* showing clues about its origin and migration. *Parasitology* Feb 26, 1–9. DOI: 10.1017/S0031182018000227.

IV. **Panteleit, J**., Keller, N. S., Diéguez-Uribeondo, J., Makkonen, J., Martín-Torrijos, L., Patrulea, V., Pîrvu, M., Preda, C., Schrimpf, A., and Pârvulescu, L. (2018): Hidden sites in the distribution of the crayfish plague pathogen *Aphanomyces astaci* in eastern Europe: relicts of genetic groups from older outbreaks? *Journal of Invertebrate Pathology*. DOI: 10.1016/j.jip.2018.05.006.

V. **Panteleit, J.**, Jussila, J., Keller, N. S., Kokko, H., Makkonen, J., Schulz, R., Theissinger, K., and Schrimpf, A.: Three haplotypes of the crayfish plague agent *Aphanomyces astaci* found to be responsible for recent crayfish plague outbreaks in Germany and Austria. Submitted to Knowledge and Management of Aquatic Ecosystems.

VI. **Panteleit, J**., Horvath, T., Jussila, J., Makkonen, J., Perry, W., Schulz, R., Theissinger, K., and Schrimpf, A. (2019): Invasive rusty crayfish (*Faxonius rusticus*) populations in North America are infected with the crayfish plague disease agent (*Aphanomyces astaci*). *Freshwater Science* 38(2). DOI: DOI: 10.1086/703417.

2. ABSTRACT

Invasive species often have a significant impact on the biodiversity of ecosystems and the species native to it. One of the worst invaders worldwide is *Aphanomyces astaci*, the causative agent of the crayfish plague, an often fatal disease to crayfish species not native to North America. *Aphanomyces astaci* originates from North America and was introduced to Europe in the midst of the 19th century. Since then, it spread throughout Europe diminishing the European crayfish populations. The overall aim of this thesis was to evaluate the threat that *A. astaci* still poses to European crayfish species more than 150 years after its introduction to Europe. In the first part of the thesis, crayfish specimens, which are available in the German pet trade, were tested for infections with *A. astaci*. Around 13% of the tested crayfish were clearly infected with *A. astaci*. The study demonstrated the potential danger the pet trade poses for biodiversity through the import of alien species and their potential pathogens, in general. In the second part of the thesis, the *A. astaci* infection prevalence of crayfish species in wild populations in Europe was tested. While the stone crayfish, *Austropotamobius torrentium*, showed high susceptibility to different haplogroups of *A. astaci*, the narrow-clawed crayfish, *Astacus leptodactylus*, was able to survive infections, even by haplogroup B, which is considered to be highly virulent. In the last part of the thesis, *A. astaci* was traced back to its original distribution area of North America. While the crayfish plague never had such a devastating effect on crayfish in North America as it had in Europe, the reasons for the success of invasive crayfish within North America are not yet fully understood. It is possible that *A. astaci* increases the invasion success of some crayfish species. Several populations of the rusty crayfish, *Orconectes rusticus*, in the Midwest of North America were confirmed to be infected with *A. astaci* and a new genotype was identified, possibly indicating that each crayfish host is vector of a unique *A. astaci* genotype, even in North America. Overall, the present thesis provides evidence that *A. astaci* is still a major threat to the crayfish species indigenous to Europe. Crayfish mass mortalities still occur in susceptible crayfish species like *A. torrentium* even 150 years after the first introduction of *A. astaci*. While there are some indications for increased resistances through processes of co-evolution, the continuous introduction of crayfish species to Europe threatens to cause new outbreaks of the crayfish plague through the parallel introduction of new, highly virulent *A. astaci* strains.

2.1 Zusammenfassung

Eine der weltweit schlimmsten invasiven Arten ist *Aphanomyces astaci*, der Auslöser der Krebspest, eine für Flusskrebse oft tödliche Krankheit. *Aphanomyces astaci* stammt ursprünglich aus Nordamerika und wurde in der Mitte des 19. Jahrhunderts nach Europa eingeführt. Von da an verbreitete sich der Erreger über ganz Europa und führte zum Rückgang vieler Flusskrebspopulationen. Das Ziel dieser Arbeit war es zu evaluieren, wie groß die Bedrohung der europäischen Flusskrebsarten durch den Krebspesterreger mehr als 150 Jahre nach dessen Einführung nach Europa immer noch ist. Im ersten Teil der Arbeit wurden Flusskrebse, die im Tierhandel erhältlich sind, auf Infektionen mit *A. astaci* getestet. Rund 13% der getesteten Tiere waren eindeutig mit dem Erreger infiziert. Dieses Ergebnis zeigt die potentielle Gefahr auf, die der Tierhandel für die Biodiversität durch die Einfuhr gebietsfremder Arten zusammen mit ihren Krankheitserregern darstellt. Im zweiten Teil der Arbeit wurde der Infektionsstatus von in Europa wild lebenden Flusskrebspopulationen untersucht. Während der Steinkrebs, *Austropotamobius torrentium*, eine hohe Anfälligkeit gegenüber *A. astaci* zeigte, war der Galizierkrebs, *Astacus leptodactylus*, in der Lage, Infektionen durch den Krebspesterreger zu überleben. Im letzten Teil der Arbeit wurde *A. astaci* zurück zu seinem ursprünglichen Verbreitungsgebiet Nordamerika zurückverfolgt. Obwohl die Krebspest in Nordamerika nie so eine verheerende Wirkung auf Flusskrebse hatte wie in Europa, sind die Gründe für den Erfolg von invasiven Flusskrebsarten innerhalb von Nordamerika bis heute ungeklärt. Es wäre möglich, dass *A. astaci* das invasive Potential von manchen Flusskrebsarten erhöht. Mehrere Populationen des amerikanischen Rostkrebs, *Orconectes rusticus*, aus dem mittleren Westen der USA wurden positiv auf *A. astaci* getestet. Ein neuer Genotyp wurde identifiziert, was darauf hindeuten könnte, dass jede amerikanische Flusskrebsart mit ihrem eigenen *A. astaci* Genotyp assoziiert ist. Zusammenfassend gibt die vorliegende Arbeit Hinweise darauf, dass *A. astaci* immer noch eine der größten Bedrohungen für europäische Flusskrebse darstellt. Selbst 150 Jahre nach der Einführung von *A. astaci* kommt es zu Massensterben von Flusskrebsen besonders von empfindlichen Arten wie *A. torrentium.* Während es einige Hinweise darauf gibt, dass manche Arten durch Koevolution eine erhöhte Resistenz gegen den Krebspesterreger ausgebildet haben, droht die ständige Einfuhr neuer Flusskrebsarten zusammen mit potentiellen neuen Stämmen von *A. astaci* weitere Massensterben unter den europäischen Flusskrebsen auszulösen.

3. ABBREVIATIONS

AFLP	Amplified fragment length polymorphism
bp	Base pair
COI	Cytochrome c oxidase I
CTAB	Cetyl trimethylammonium bromide
DD	Data deficient
DNA	Desoxyribonucleic acid
EN	Endangered
EU	European Union
EUR	Euro
ICS	Indigenous crayfish species
ITS	Internal transcribed spacer
LC	Least concern
LSU	Large subunit primer for Sequencing
mtDNA	Mitochondrial DNA
NGS	Next generation sequencing
NICS	Non-indigenous crayfish species
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
RAPD	Random amplified polymorphic DNA
rnnS	Ribosomal small subunit
rnnL	Ribosomal large subunit
SSR	Simple sequence repeats
SSU	Small subunit primer for sequencing
VU	Vulnerable

4. INTRODUCTION

4.1 Invasive species

The process of globalization has significantly contributed to the dispersal of plants and animals around the world, overcoming any barrier, which would have limited the natural expansion of species beforehand (Nentwig 2007). In most cases, the introduction of species into new regions is without consequences, but of all species that are introduced into new areas about 0.1% become invasive, in extreme cases also referred to as pests (Williamson 1993). A species is considered invasive if it has significant ecological, environmental or economic impacts in the area where it has been introduced (Nentwig 2007). In Europe alone, the extrapolated costs of invasive species are estimated as 20 billion EUR/year (Kettunen et al. 2008). Those costs include damages caused by invasive species, e.g. to agriculture, and costs of management actions to control the invasive species. But not only the economic costs of invasive species are immense. Biological invasions are the second largest contributor to the loss of biodiversity (Wilcove et al. 1998).

There are different hypotheses on why species become invasive. Invasive species are often closely associated, and thus well adapted, to human altered environment, which gives them an inherent superiority over indigenous species (Sax and Brown 2000). Another example is the enemy release hypothesis already mentioned by Darwin, which suggests, that invasive species lose their natural predators if transferred to a new environment, giving them a competitive advantage over indigenous species (Torchin and Mitchell 2004). Different invasion mechanisms are not necessarily exclusive and more than one can possibly be active at the same time. Once invasive species have become established in a new environment, it is difficult and expensive to prevent further range expansion or even eradicate them. In some cases, the eradication of an invasive species can even be harmful to the environment, if for example the invasive species already integrated into the food web of the invaded area (Kopf et al. 2017). Some authors even claim that invasive species might be more of a salvation than a hazard for nature, like it was done in the controversial book “The New Wild” (Pearce 2016). According to Pearce (2016) invasive species are in most cases the result of human induced deterioration of the environment and help to restore nature to a healthy state. The most common scientific opinion, however, still is that the spreading of invasive species must be prevented and controlled. The European Union (EU)

recently adopted a list of 37 invasive species, including five crayfish species, whose spread must be prevented, controlled or the species must be eradicated (EU regulation 2016/1141).

4.2 Freshwater crayfish in Europe

The conservation of freshwater biodiversity has, in the past, received less attention than the terrestrial biodiversity. However, current estimated extinction rates of freshwater species exceed those of terrestrial species (Ricciardi and Rasmussen 1999; Richman et al. 2015). Freshwater crayfish are keystone species in freshwater systems and have a high impact on their environment (Gherardi 2007). Especially non-indigenous crayfish species (NICS) can have high impacts on the ecosystem mainly through food web alteration (Creed and Reed 2004). In Europe five indigenous crayfish species (ICS) exist - namely the noble crayfish *Astacus astacus* (Linnaeus, 1758), the narrow-clawed crayfish *Astacus leptodactylus* Eschsholtz, 1823, the thick-clawed crayfish *Astacus pachypus* Rathke, 1837, the white-clawed crayfish *Austropotamobius pallipes species complex*, and the stone crayfish *Austropotamobius torrentium* (Schrank, 1803). These five ICS are currently outnumbered in terms of species and individuals by ten NICS (Kouba et al. 2014) (Table 1). The NICS species have been categorized as old and new NICS (Holdich et al. 2009). Old NICS were introduced before 1975, mainly intentionally for stocking purposes and the release was unwanted by scientists and policy. The new NICS have been introduced since 1980, through the aquarium trade and for aquaculture purposes. Both, old and new NICS, occasionally escaped into the wild or were intentionally released by their owners. The invasiveness of the North American crayfish in Europe was facilitated by different biological traits of the invasive species in comparison to the European species, e.g., higher fecundity, faster egg developement or higher tolerance towards unfavorable enivronmental conditions (Souty-Grosset et al. 2006). However, the main reason for the success of the North American crayfish species in Europe can most probably be attributed to the concurrent introduction of the Oomycete *Aphanomyces astaci* Schikora, 1906, the causative agent of the crayfish plague.

Table 1 Overview of the crayfish species currently present in Europe. Old NICS were introduced before 1975, new NICS after 1980. Conservation status according to the IUCN red list www.iucnredlist.org Abbreviations are: DD=data deficient, LC=least concern, VU=vulnerable, EN=endangered, ↓decreasing, ↑ increasing, → stable, ? unknown.

ICS	Conservation status with population trend	Origin
Astacus astacus	VU ↓	Europe
Astacus leptodactylus	LC ?	Europe
Astacus pachypus	DD?	Europe
Austropotamobius pallipes	EN ↓	Europe
Austropotamobius torrentium	DD ↓	Europe
Old NICS		
Orconectes limosus	LC →	eastern North America
Pacifastacus leniusculus	LC ?	western North America
Procambarus clarkii	LC ↑	southern USA/central North America
New NICS		
Cherax destructor	VU ?	Australia
Cherax quadricarinatus	LC ?	Australia
Orconectes immunis	LC →	North America
Orconectes juvenilis	LC →	eastern North America
Orconectes virilis	—	North America
Procambarus acutus	LC ?	North America
Procambarus virginalis	—	unknown

4.3 The crayfish plague agent *Aphanomyces astaci*

The crayfish plague agent *A. astaci* originates from North America, where it lives as a parasite on many crayfish populations (Unestam and Weiss 1970; Unestam 1972, **APPENDIX VI**). From North America, it was introduced to Europe in the midst of the 19th century, probably even before the first crayfish were imported to Europe. *Orconectes limosus* (Rafinesque, 1817) is, by some authors, suspected as the first vector for the *A. astaci* introductions (Kawai et al. 2015). However, the first reports of *O. limosus* introductions are dated in 1890, while the first mass mortalities suspected to be caused by *A. astaci* took place in Italy in 1859 (Kawai et al. 2015). Until around 1970 the pathogen had spread all over Europe, from Spain in the West to the Black Sea in the East (Alderman 1996). The crayfish plague devastated the European crayfish populations and at least three crayfish

species today have decreasing population trends or are even facing extinction (Table 1). By now, besides Europe, *A. astaci* has also been introduced to Asia and South America (Mrugała et al. 2016; Peiró et al. 2016; Martin-Torrijos et al. 2018). Due to its devastating effects on the indigenous crayfish populations of Europe, *A. astaci* is today considered as one of the world's 100 worst invasive species (Lowe et al. 2004). Due to extensive research, *Aphanomyces astaci* is also one of the best studied invertebrate pathogens (reviewed by Svoboda et al. 2017).

One of the main pathways of introduction especially for the new NICS, which have been introduced after 1980, is the aquarium trade (Holdich et al. 2009). Crayfish have recently become very popular as pets to be held privately in aquaria (Chucholl 2013; Patoka et al. 2014a). As a result, 120 NICS are available for sale in the German pet trade alone, with an import rate of seven new species per year in the time between 2005 and 2009 (Chucholl 2013). In a study by Patoka et al (2014b), keepers of crayfish were asked what they do with the offspring of their crayfish after unwanted reproduction. About 2.5% of the people answered that they release them into the wild (Patoka et al. 2014b). While this number may seem small, if NICS survive and establish viable populations they become an immediate threat to ICS. One of the most problematic crayfish species is the marbled crayfish *Procambarus virginalis* (Lyko, 2017). This species was first discovered in the German pet trade (Scholtz et al. 2003). It has since then become the most popular crayfish in the aquarium trade due to its extraordinary coloration and cheap price (Chucholl 2013). *Procambarus virginalis* is the only crayfish species known to reproduce parthenogenetically, meaning that all offspring are clones of the mother (Martin et al. 2007; Vogt et al. 2015). One single individual of *P. virginalis* is enough to establish a viable population in the wild, which makes it especially problematic for the conservation of ICS. *Procambarus virginalis* has additionally been identified as a carrier of *A. astaci* (Keller et al. 2014). This represents a general problem of the aquarium pet trade, as the health status of crayfish that are being imported are usually unknown (**Appendix I**).

While the European crayfish populations can succumb to infections by *A. astaci*, with mortalities of up to 100% (Alderman et al. 1987), crayfish of North American origin have coevolved defense mechanisms against the disease (Unestam and Weiss 1970; Unestam and Nylund 1972). While recent studies from Sweden and Finland have found indications that these defense mechanisms might be subsiding in some populations of NICS leading to significant abundance declines

in crayfish numbers (Edsman et al. 2015; Jussila et al. 2016), most NICS in Europe still seem to be resistant to infections by *A. astaci*.

Over the course of the last years some populations of ICS have been found with latent *A. astaci* infections, i.e., they did not die from an infection with the disease (Viljamaa-Dirks et al. 2011; Jussila et al. 2011; Schrimpf et al. 2012; Maguire et al. 2016; **Appendix IV**). These latent infections could be signs of co-evolutionary adaptations of the host species and the pathogen (Jussila et al. 2014). Especially *A. leptodactylus* seems to have an increased resistance towards different strains of *A. astaci* (Kokko et al. 2012; Svoboda et al. 2012; Schrimpf et al. 2012; Maguire et al. 2016; **Appendix IV**). The molecular mechanisms behind the latent *A. astaci* infections are not yet fully understood - whether it is a reduced virulence of *A. astaci*, an increased resistance of the crayfish or a combination of both. A recent study indicates that the adaptions might be regional, between specific populations of crayfish and *A. astaci* closely associated with one another (Jussila et al. 2017). This could be of great importance, not only to European ICS, but also to crayfish species in North America, the origin of *A. astaci*. Many North American crayfish species are invasive within the North American continent, e.g., the rusty crayfish *Orconectes rusticus* (Girard, 1852) (Hill and Lodge 1999; Reid and Nocera 2015). *Orconectes rusticus* outcompetes and replaces native species, however, the mechanisms behind this invasion process are yet unknown. If the mode of action and virulence of *A. astaci* depends on the different host species or populations, this could have a high impact on the invasion success of invasive species, also within North America (**Appendix VI**). The impact of *A. astaci* in North America has never been studied, because the possible impact has never been so obvious as it is in Europe with crayfish mass mortalities. To study the intra-species diversity of *A. astaci* and to be able to link virulence differences of *A. astaci* to different crayfish species, it is inevitable to use genetic methods like sequencing and microsatellite analyses.

4.4 Genetic methods to detect and characterize *Aphanomyces astaci*

Before the development of genetic methods to identify *A. astaci*, scientists had to rely mainly on cultivation followed by visual identification of either the spores or the hyphae of *A. astaci* (Oidtmann et al. 1999). These methods were time consuming and often unsuccessful. The cultivation of *A. astaci* is difficult, as frequently present water molds or bacteria often overgrow *A. astaci* during the

process (Oidtmann et al. 1999). Crayfish mass mortalities and melanized brown areas on the cuticle of the crayfish were also seen as reliable indications for *A. astaci* infections (Nylund and Westman 2000). This view, however, has since been revoked, as melanization is a non-specific response of the crustacean immune response and thus the presence of melanin is not a reliable indication for an infection with *A. astaci* (Söderhäll and Cerenius 1992; Kozubíková et al. 2009).

Only with the development of genetic methods it was possible to reliably identify *A. astaci* from tissue samples of dead crayfish. Conventional PCR and subsequent sequencing of the partial ITS region (Oidtmann et al. 2006) as well as application of quantitative real-time PCR (qPCR) (Vrålstad et al. 2009) were developed in relatively close succession. Today qPCR is the main tool to detect *A. astaci* from infected crayfish tissue in most studies, as it has been shown to be the most specific and sensitive method for this purpose (Vrålstad et al. 2009; Tuffs and Oidtmann 2011; Kozubíková et al. 2011a).

Even before the application of PCR and qPCR for the detection of *A. astaci*, random amplified polymorphic DNA-PCR (RAPD-PCR) was used to divide different *A. astaci* strains into RAPD-groups (Huang et al. 1994). The first three groups A, B and C, are often also referred to as As, PsI and PsII, respectively, due to the crayfish genus from which the groups were first isolated, *Astacus* (As) and *Pacifastacus* (Ps). The fourth genetic group of *A. astaci,* D (Pc) was identified by Diéguez-Uribeondo et al. (1995) and was isolated from *Procambarus clarkii* (Girard, 1852). The fifth group E (Or) was isolated from *O. limosus* (Kozubíková et al. 2011b). These RAPD-groups can also be differentiated by amplified fragment length polymorphism-PCR (AFLP-PCR) (Rezinciuc et al. 2014) and by applying microsatellite analyses (Grandjean et al. 2014). Sequence analysis of the nuclear chitinase gene (Makkonen et al. 2012b) and, most recently, also sequencing of mtDNA (**Appendix III**) have been used to characterize different *A. astaci* strains.

All these different genetic tools that are being applied when trying to differentiate different *A. astaci* strains have caused a lot of confusion in the scientific crayfish community. The genetic groups have often been referred to as "genotypes". However, in genetic expressions, genotype is the correct term only for data generated by microsatellite analyses. In this thesis, genetic groups that were determined by RAPD-PCR will be called RAPD-groups. Genotype will only be used when referring to microsatellite analysis and haplotype/haplogroup will be

used when referring to sequence analyses. Haplotypes are allocated to different haplogroups due to phylogenetic analysis of the different sequences. Further confusion is created, because all studies in which the methods were first applied stuck to the same naming of the different groups identified, i.e. RAPD-group, genotype, haplogroup "A" to "E". While it would make sense to stick to already established labeling when the results are congruent, this creates problems when the results from the various studies differ. For example, haplogroup A contains both RAPD-groups A and C (Fig. 1). To avoid confusion when referring to the different genetic methods, in the discussion of this thesis, RAPD-groups will be referred to as As, PsI, PsII, Pc and Or. Although these were originally the names of the strains tested with RAPD-PCR, this method is not applied anymore today and thus probably no new RAPD-groups will be defined for *A. astaci* in the future. Since AFLP-groups are so far congruent with the RAPD-groups, in this thesis they are assumed as identical to the RAPD-groups and receive identical naming. Haplogroups and haplotypes will be referred to as in the study in which they were established, A/a, B/b, D/d1/d2, and E/e (**Appendix III**).

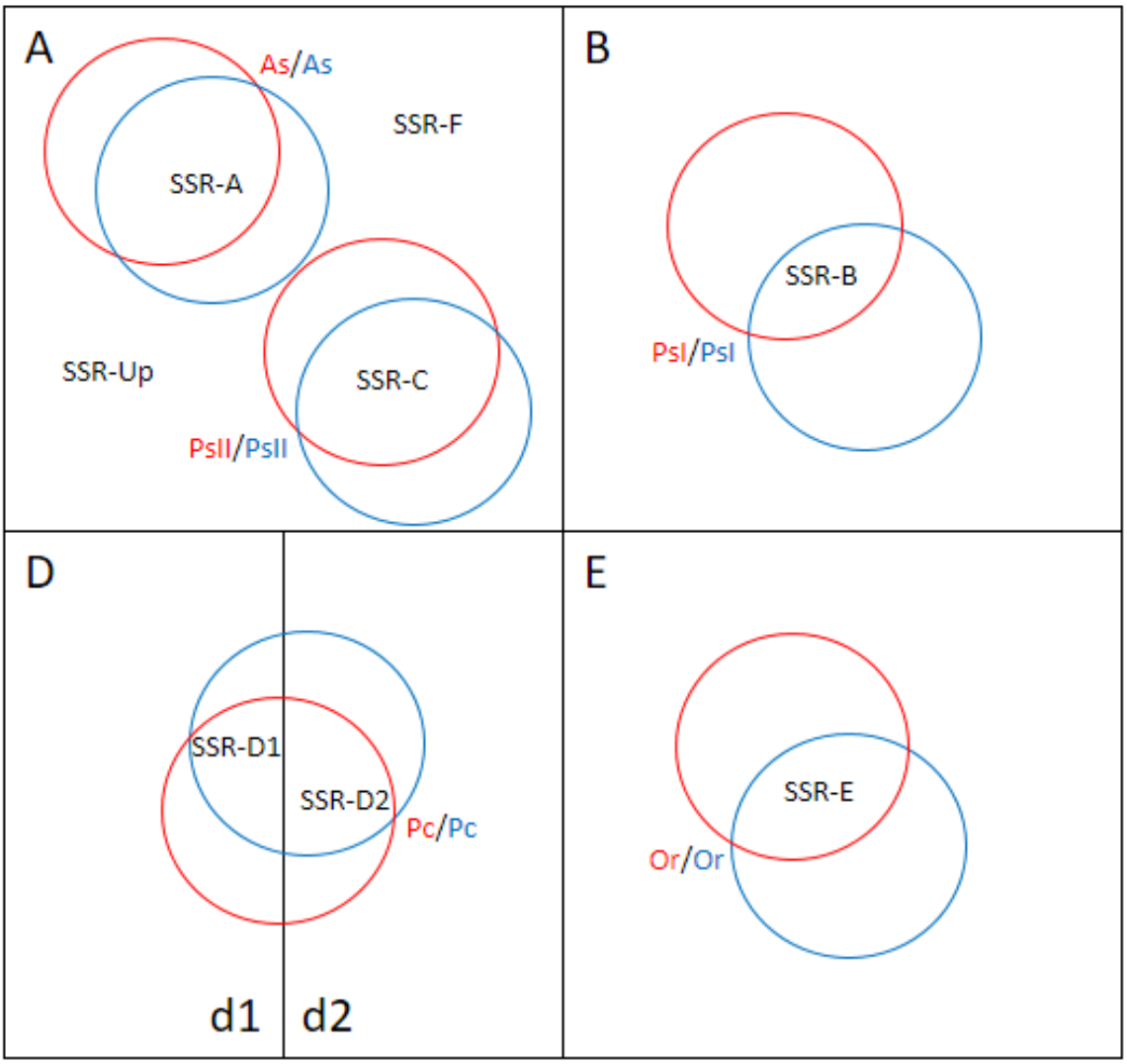

Fig. 1 Schematic depiction of the different genetic groupings and their relationship with each other. Black squares and rectangles are haplogroups/-types (**Appendix III**). Blue circles represent AFLP-groups (Rezinciuc et al. 2014) and red circles RAPD-groups (Huang et al. 1994). Black SSR-letterings are the different genotypes (Grandjean et al. 2014; **Appendix VI**). Not all known genotypes have been analyzed with sequence analysis, yet. Therefore, not all genotypes are included in the figure. Genotypes Up and F have not been tested with RAPD- or AFLP-analyses, for this reason they are not allocated to any of these groups.

5. OBJECTIVES OF THE THESIS

The overall objective of this thesis was to estimate the threat that *A. astaci* still poses to the ICS of Europe and to improve the knowledge of its genetic diversity and distribution in Europe as well as in North America. This was achieved by looking at three major aspects: i) the introduction of new crayfish species, along with *A. astaci*, from North America to Europe (**Appendix I**), ii) the infection prevalence of ICS and NICS and which haplogroups and genotypes of *A. astaci* are present in Germany, Austria and Romania (**Appendices II, III, IV and V**) and iii) the *A. astaci* haplogroups and infection prevalence of invasive crayfish within the Midwest of North America (**Appendix VI**) (Fig. 2).

Today, the trade of aquatic species for ornamental or hobby purposes is one of the largest threats to freshwater biodiversity due to intentional or unintentional release of the traded species in a new environment (Padilla and Williams 2004). Over 100 North American crayfish species are available in the pet trade in Germany alone. Those crayfish species are considered potential vectors for *A. astaci*. During this thesis, crayfish samples of 50 species of North American origin were tested for an infection with *A. astaci* (**Appendix I**). All of the tested species were available in the German aquarium pet trade. The aim of the study was to show that the potential threat that the introduction of NICS poses to ICS is even higher when considering not only the invasive potential of a species, but also the pathogens, i.e., *A. astaci*, which are imported along with the crayfish. One particular problematic NICS is the parthenogenetic marbled crayfish *P. virginalis*. This species was already positively tested for an infection with *A. astaci* (Keller et al. 2014). In this study, *P. virginalis* was discovered in the wild of Romania (**Appendix II**), probably after its introduction into the country via the pet trade. Microsatellite analyses were conducted to compare the allele pattern of the discovered *P. virginalis* from Romania with those previously found in Germany or the aquarium pet trade.

Over the course of the last years, new genetic tools to characterize *A. astaci* have been developed. Among those are microsatellite analyses (Grandjean et al. 2014) and sequence analyses (**Appendix III**). These methods were used to study the genetic diversity of *A. astaci* in the Romanian Danube and its delta, as well as in the Dniester River, which also flows into the Black Sea (**Appendix IV**). The Danube was already studied for crayfish plague infections by Schrimpf et al.

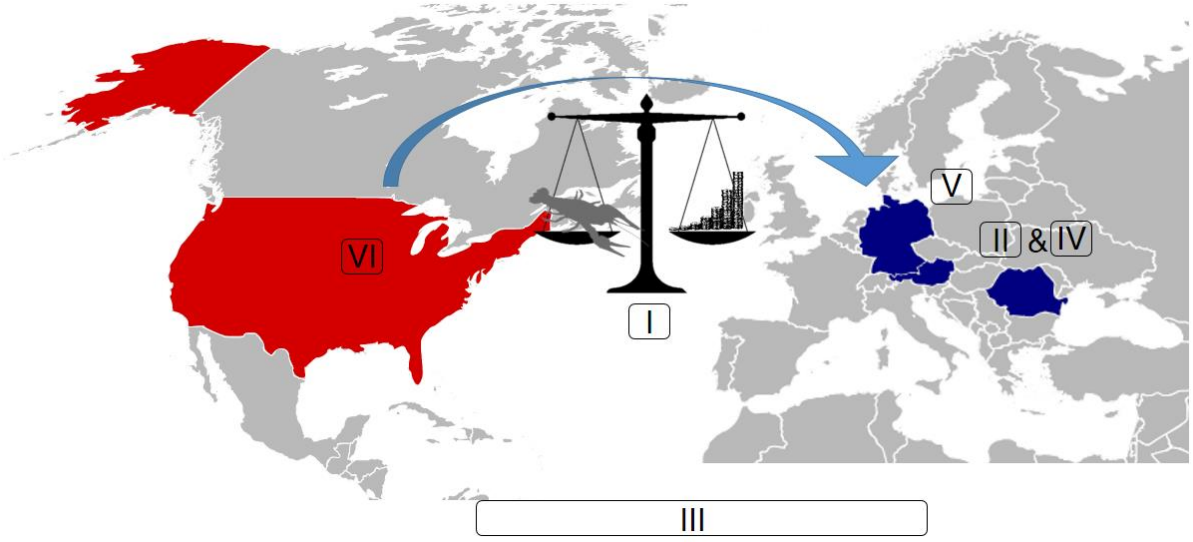

Fig. 2 Overview of the main topics of the thesis. Appendix I deals with the crayfish pet trade focusing on imports from North America to Europe. Appendix II is a study on marbled crayfish that were for the first time detected in the Romanian wild. In Appendix III primer for the identification of *A. astaci* haplotypes were developed, which were applied in the following studies. Appendices IV and V were screenings of wild ICS and NICS populations for infections with the crayfish plague. Appendix VI is a study on the *A. astaci* infection prevalence of invasive rusty crayfish within North America.

(2012). As a follow up study to Schrimpf et al. (2012), the aim in this thesis was to reevaluate the *A. astaci* infection prevalence of invading *O. limosus* populations and of *A. leptodactylus* in invaded parts of the Danube, as well as non-invaded parts, including the Danube Delta and the nearby Dniester River. Further, the haplogroups and genotypes of *A. astaci* were determined, which was not possible with the methods available in 2012. Identification of the haplogroups and genotypes would possibly allow for conclusion on the origin of *A. astaci* in the Danube and how it was introduced there.

A similar study to the one in Romania (**Appendix IV**) was conducted in Germany and Austria (**Appendix V**). Samples of ICS and NICS were tested for their *A. astaci* infection status and compared in regard to the infection prevalence among different species and populations. Samples were taken during crayfish mass mortalities, probably caused by the crayfish plague, to determine which haplogroups and genotypes were responsible for the crayfish mortalities.

In the last study of this thesis (**Appendix VI**) populations of American rusty crayfish *O. limosus*, which are invasive within North America were tested for their infection status with *A. astaci*. Haplogroups and genotypes of *A. astaci* were identified. The aim of this study was to get a first overview of the role that *A. astaci*

might play in the invasion success of crayfish species within North America and to draw attention to the potential problem of *A. astaci* in North America. The influence of *A. astaci* in its area of origin has never been studied, because crayfish mass mortalities have never been reported in North America. The study would also prepare future investigations on the virulence of *A. astaci* from different host species and thus its influence on the invasion success of invasive crayfish species like *O. rusticus*.

6. DESCRIPTION OF METHODS

6.1 DNA isolation

Aphanomyces astaci DNA from infected crayfish samples was isolated with two different DNA extraction protocols. For most samples we used a CTAB method according to (Vrålstad et al. 2009). It is a cheap and easy to use DNA extraction protocol. During the studies however, it was discovered, that the DNA quality notably improves when extracted with the E.Z.N.A. Insect DNA Kit (Omega Bio-tek, Norcross, GA; USA). Comparative data of DNA quality between the two protocols is not shown, however, subsequent sequencing and microsatellite analyses were more often successful when using the DNA Kit. DNA isolated with the CTAB method probably is much more fragmented, which is problematic when trying to sequence longer amplicons. One disadvantage of the DNA isolation kit is that it is more costly than the DNA extraction with the CTAB method.

6.2 *Aphanomyces astaci* detection

The two most commonly used methods to detect DNA of *A. astaci* from infected crayfish tissue are a conventional PCR with subsequent sequencing (Oidtmann et al. 2006) and a qPCR protocol (Vrålstad et al. 2009). They both have their own advantages and disadvantages. Both methods utilize partial amplification of the ITS region of *A. astaci*. The qPCR protocol was shown to be tenfold more sensitive than conventional PCR (Tuffs and Oidtmann 2011). While this higher sensitivity is useful and can improve detection rates (Kozubíková et al. 2011a), especially when analyzing North American crayfish species with usually low infection rates, it also sometimes leads to false positive results, which may have been caused by laboratory contamination during handling of the samples. Both methods exhibit good species specificity for *A. astaci*, with qPCR being more

specific than conventional PCR. However, it is assumed that the species diversity of oomycetes is very high and, in time, more species of the genus *Aphanomyces* will be discovered (Kozubíková et al. 2011a). The qPCR assay amplifies only a small range of the ITS region (59 bp), which might not include enough differences between yet undiscovered species and *A. astaci* to distinguish the species from one another. Because of this small size, subsequent sequencing of the qPCR product is usually not done to confirm the findings of *A. astaci*. The conventional PCR product of *A. astaci* is around 750 bp long (Oidtmann et al. 2004), increasing the chance to detect mutations between different *Aphanomyces* species by blasting the sequences with NCBI Genbank. In general, it is recommended to use both methods in parallel when studying unknown crayfish samples. For the studies in this thesis we always used the qPCR assay with an increased annealing temperature to further reduce the risk of false positives (Strand 2012). The conventional PCR was used additionally for single, highly infected samples to confirm the presence of *A. astaci* by comparing the sequences to reference sequences of known *A. astaci* cultures. As it is not possible to sequence samples containing very low amounts of *A. astaci* DNA, these samples were always interpreted with caution, as further genetic confirmation of the presence of *A. astaci* was not possible.

6.3 Genetic characterization of *Aphanomyces astaci*

Since 1994 different methods were developed to genetically characterize and analyze the relationship between different *A. astaci* strains, i.e., RAPD-PCR (Huang et al. 1994), AFLP-PCR (Rezinciuc et al. 2014), microsatellite analyses (Grandjean et al. 2014) and sequencing of different *A. astaci* genes (Makkonen et al. 2012; **Appendix III**) (see introduction). In this thesis, haplogroups of different *A. astaci* samples were identified using the recently developed primer pairs LSU and SSU (**Appendix III**). Additional sequencing of the chitinase gene (Makkonen et al. 2012) allowed for the further discrimination of samples of haplogroup A into those belonging to either RAPD-group A or RAPD-group C.

To identify genotypes of *A. astaci* the protocol by Grandjean et al. (2014) was used. Microsatellites are non-coding DNA sequences consisting of short, repetitive DNA motifs of 2-6 bp, often referred to as simple sequence repeats (SSRs). An important feature of microsatellites is that the number of sequence repeats is highly variable (Nordheim & Knippers 2015). This means that each *A. astaci*

genetic group has likely a different number of sequence repeats. Due to the obligatory asexual reproduction of *A. astaci*, the number of repeats within one population however are relatively stable, because genomes of different individuals are not mixed. This also explains the high degree of homozygosis of *A. astaci*. The use of microsatellites for the analysis of DNA is called genotyping, and the different genetic groups identified are called genotypes.

7. SUMMARY OF RESULTS

In the first part of the thesis the aquarium pet trade was investigated with regard to its role in the introduction of *A. astaci* together with imported crayfish species (**Appendix I**). Overall, 85 crayfish samples from North American as well as New Guinean origin were tested for an infection with *A. astaci* by using qPCR. An infection was detected in eleven samples (13%) - all of North American origin. All of the infected specimens were different crayfish species and nine of the species were identified as vectors of *A. astaci* for the first time. With sequencing analyses of the nuclear chitinase gene, no new haplogroups of *A. astaci* were discovered. In one sample the chitinase gene was identical to the one from RAPD-group D, while in another sample, it was identical to those of RAPD-group B, C and E. By sequencing the COI gene of the crayfish, a few mismatches between morphological species identification by the hobby breeder and genetic species identification through sequencing were discovered.

In the second part of this thesis, the *A. astaci* infection prevalence of wild crayfish populations in Romania, Germany and Austria was investigated. Additionally, a viable population of the marbled crayfish *P. virginalis* was for the first time detected in the wild in Romania (**Appendix II**). Probably originating from the crayfish pet trade, microsatellite analyses revealed the same allele pattern as in every other *P. virginalis* sample studied so far, providing further evidence for the parthenogenic reproduction of the species. The crayfish plague agent *A. astaci* was not detected in the studied population.

The genetic methods that were developed this thesis (**Appendix III**) were fundamental to all subsequent studies of this thesis. Two primer pairs were developed to amplify the mtDNA of ribosomal rnnS and rnnL subunits. The currently known strains were allocated to four different haplogroups, A, B, D, and

E. The haplogroups matched with the previously identified RAPD-PCR groups with one exception: haplogroup A contained the RAPD-groups A and C, originating from *A. astacus* and *P. leniusculus*. The results were also confirmed by sequencing samples directly obtained from infected crayfish tissue.

Aphanomyces astaci was detected in NICS and ICS populations from the Romanian Danube, where the current invasion front of the NICS *O. limosus* in the Danube is located (**Appendix IV**). *A. astaci* was also detected in samples from the ICS *A. leptodactylus* taken from the Danube Delta and the nearby Dniester River. The *A. astaci* infection prevalence of *A. leptodactylus* and *O. limosus* in the invaded part of the Danube was 21% and 8%, respectively, which was not statistically different. In the Dniester River however, the infection prevalence of *A. leptodactylus* populations was significantly lower (6%) than in the invaded part of the Danube or the Danube Delta. Marine decapods were negatively tested for *A. astaci* infections. Among the positively tested crayfish samples, two different *A. astaci* haplogroups were detected. Haplogroup A was detected in the Danube Delta and in the invaded part of the Danube in Serbia, close to the Romanian border. Haplogroup B was found in the Danube Delta and also in the River Dniester. The chitinase gene of the samples belonging to haplogroup A was identical to those of known strains of RAPD-group C, indicating that the samples were more closely related to RAPD-group C than A. One of the samples with haplogroup A was also successfully genotyped by microsatellite analyses, showing an identical allele pattern to genotype Up.

Investigations of the *A. astaci* infection prevalence in Germany and Austria showed a very high infection prevalence (69%) of *A. torrentium*, which died during mass mortality events probably caused by *A. astaci* (**Appendix V**). The *A. astaci* infection prevalence of NICS in Germany and Austria was between 6% and 21%. Three haplogroups were detected in *A. torrentium* samples during six events of crayfish plague mass mortalities. In Austria, haplogroup B was found in three populations. In the third population there was also haplogroup A, i.e., genotype Up, detected at the same time, confirming the presence of two different haplogroups in one ICS population. In Germany haplogroups B and D were detected in two different *A. torrentium* populations. Genotype Up was further also identified in a sample from Lake Attersee in Austria.

In the last part of the thesis, *A. astaci* was traced back to its original distribution area of North America. In 4 out of 10 populations of the rusty crayfish *O. rusticus*, a North American crayfish species which is invasive in parts of North America, *A. astaci* infections were detected (**Appendix VI**). The findings were confirmed by isolating the *A. astaci* strains and subsequent partial sequencing of the ITS barcoding region. Microsatellite analyses revealed a new genotype in two different crayfish populations. Sequencing of the chitinase, rnnL, and rnnS genes identified the *A. astaci* strain to be part of haplogroup A. One of the isolates had two deletions in the rnnL sequence, making it a unique haplotype within haplogroup A.

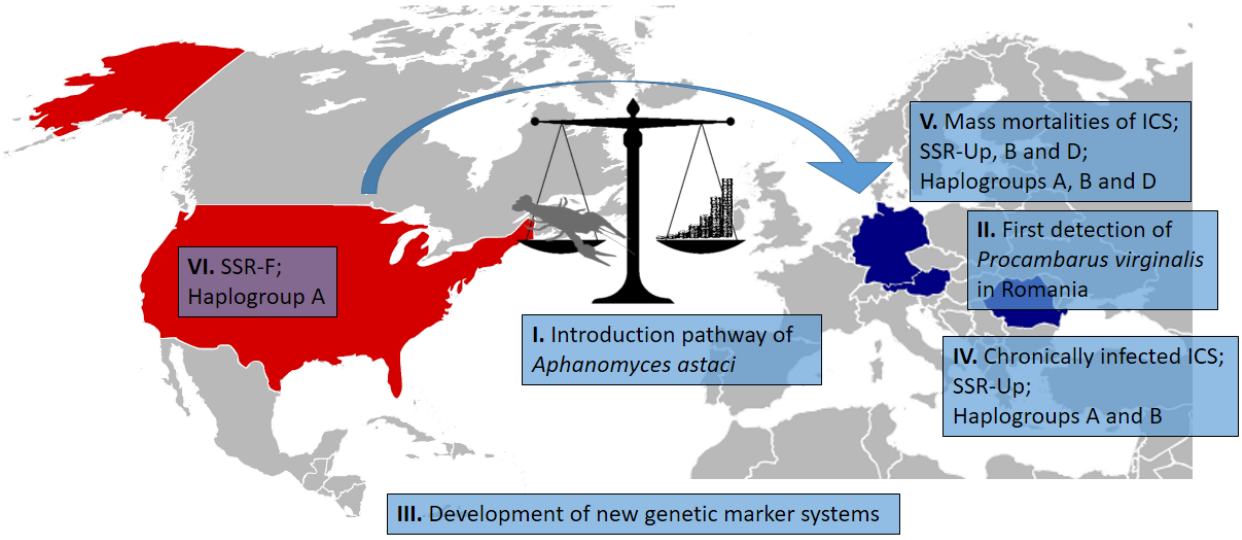

Fig. 3 Overview of the most important results of the thesis. Appendix numbers are written in bold. ICS are indigenous crayfish species. SSR labels indicate which *A. astaci* genotypers were found. Haplogroups are based on the methods from Appendix III.

8. DISCUSSION

8.1 Crayfish pet trade in Europe

In this thesis, it was confirmed that crayfish imported from North America to be sold as pets or kept in private aquaria, are often vectors of the crayfish plague pathogen *A. astaci* (**Appendix I**). Nine species were identified as vectors for *A. astaci* for the first time. This result is similar to a study by Mrugala et al. (2014), in which six other species were identified as vectors for the first time. While the horizontal transmission of *A. astaci,* i.e., the transmission of the pathogen between crayfish individuals kept in close proximity, could not be shown in this thesis, this

problem was indeed identified by Mrugała et al. (2014) and James et al. (2017). These results show, that ICS of Europe are not only threatened by the introduction of NICS, but also their associated pathogens, if specimens are released, intentionally or unintentionally, into the wild (Patoka et al. 2014a). The EU recently adopted a list of invasive species whose spread has to be prevented from now on (EU Regulation No 2016/1141). However, this list includes only five NICS which already have viable populations in Europe, namely *O. limosus*, *O. virilis*, P*. leniusculus*, *P. clarkii* and *P. virginalis*. The list does not include species that are imported through international trade, but are not yet invasive or established in the European wild. It is of very high importance that species which are known to have a high invasive potential or species which are known carriers of *A. astaci*, like *O. rusticus* (Olden et al. 2006; **Appendix VI**), are added to the invasive species list. Individual countries in Europe have stricter rules for the pet trade. Examples are Ireland and Scotland, where keeping alien crayfish is illegal and the crayfish pet trade is strictly regulated (Peay 2009).

One of the most problematic crayfish available in the pet trade is probably the marbled crayfish, *P. virginalis*, due to its parthenogenetic reproduction and its high popularity as a pet (Patoka et al. 2014b). The pet trade presumably led to the introduction of *P. virginalis* to Romania and other countries (**Appendix II**; Marten et al. 2004; Chucholl and Pfeiffer 2010). It is also available for sale in Ireland, despite the fact that importing and selling of alien crayfish is illegal (Faulkes 2015a). Laws and regulations can only be effective if they are also enforced (Faulkes 2015b). When this is not possible, other methods to reduce the negative effects of the pet trade need to be executed. Education of retailers and private owners of crayfish is an important aspect to alleviate the threat posed by the pet trade (Faulkes 2015b). An infection of *P. virginalis* with *A. astaci* could not be shown in this thesis. However, *P. virginalis* has already been identified as a vector of *A. astaci* in earlier studies (Keller et al. 2014; Mrugała et al. 2014).

8.2 Presence of different *Aphanomyces astaci* strains in Europe

Alderman (1996) estimated that *A. astaci* reached Romania through the Danube around 1879 and the Black Sea in 1890. The first genetic proof for the presence of *A. astaci* in the Romanian Danube was given in 2012 (Pârvulescu et al. 2012). In the same year, *A. astaci* was also shown to be present in the Danube Delta, Romania, in viable *A. leptodactylus* populations (Schrimpf et al. 2012). Due

to the absence of NICS in the Danube Delta the source of the *A. astaci* infections remained unclear (Schrimpf et al. 2012). *Orconectes limosus* is so far the only NICS detected in the Danube (Pârvulescu et al. 2009). The current invasion front of *O. limosus* is around 900 km upstream of the Danube Delta (Pârvulescu et al. 2009). In this thesis, it was possible to identify the haplogroups and genotypes of *A. astaci* in the invaded part of the Danube, the Danube Delta and also in the Dniester River (**Appendix IV**). In the invaded part of the Danube haplogroup A was found and in the Dniester River haplogroup B. In the Danube Delta, both haplogroups, A and B were present. It is unclear, how different *A. astaci* strains interact with each other when they come into contact in one population of crayfish. Sexual reproduction of *A. astaci* has not been confirmed, yet (Söderhäll and Cerenius 1999; Diéguez-Uribeondo et al. 2009) and other possible consequences of haplogroup mixing remain to be studied.

All haplogroups in Romania were determined from infected *A. leptodactylus* specimens. Despite the fact that strains of haplogroup B were shown to be highly virulent in several studies (Makkonen et al. 2012a; Jussila et al. 2013; Viljamaa-Dirks et al. 2016; Jussila et al. 2017), the *A. leptodactylus* populations in this thesis were latently infected by *A. astaci*, i.e., they did not die from the infections. It seems that *A. leptodactylus* in general has an elevated resistance to *A. astaci* (Kokko et al. 2012; Svoboda et al. 2012; Maguire et al. 2016). More and more studies report on the existence of latently infected and thus resistant ICS (e.g. **Appendix IV**; Maguire et al. 2016; Kokko et al. 2012), but some haplogroups seem to remain very harmful and virulent to most ICS, e.g., haplogroup B (**Appendix V**; Jussila et al. 2017). The ongoing pet trade and thus the import of North American crayfish species together with new, possibly species specific, *A. astaci* strains increases the risk that new NICS, together with highly virulent strains will be released into the European nature (**Appendix I, VI**; Mrugała et al. 2014).

While the common view in crayfish plague research is that *A. astaci* RAPD-group As was the first genetic group to be introduced into Europe, this thesis gives indications that RAPD-group PsII (both belonging to haplogroup A) has also been among the first genetic groups to be introduced to Europe (**Appendix IV**). Frequent shipping traffic from the north-eastern USA to Europe could have been the source of the first introductions of *A. astaci* haplogroup A to Europe. *Aphanomyces astaci* in the Danube Delta and the Dniester River seems to be a relic from these older outbreaks, probably even from the first infection waves that

spread through Europe (Alderman 1996; Schrimpf et al. 2012). Due to the elevated resistance of *A. leptodactylus* to the infections, *A. astaci* was able to remain in the populations as chronic infections. The genotype of the samples with haplogroup A found in the Danube and its Delta was genotype Up, which was until then only once detected in the Czech Republic, where it caused mass mortalities in an *A. torrentium* population (Grandjean et al. 2014). Genotype Up was later also found to be responsible for crayfish mass mortalities at two locations in Austria (**Appendix V**). This was the third finding of this genotype in Europe so far. This genotype, which is part of haplogroup A and RAPD-group C, thus seems to have a broader distribution in Europe than expected when it was first discovered (Grandjean et al. 2014). It thus seems that haplogroup A has a vast distribution all over Europe and that it is is a very diverse haplogroup, containing a variety of different haplotypes especially from the genus *Orconectes* (**Appendix III**). Rusty crayfish from northern USA were also infected by *A. astaci* haplogroup A (genotype F) (**Appendix VI**). The eastern and northern USA harbors a high diversity of crayfish species (Holdich 2002), which in turn increases the chance for a high diversity of *A. astaci* (Jussila et al. 2015), because *A. astaci* most probably coevolved on different crayfish species.

The *A. astaci* haplogroup of the infected *O. limosus* population in the Danube could not be identified. However, it seems unlikely that *O. limosus* is the source of the *A. astaci* infections in the Danube Delta, because *O. limosus* has previously been associated with *A. astaci* RAPD-group Or, belonging to haplogroup E, (**Appendix III**; Kozubíková et al. 2011b), which was not found on infected *A. leptodactylus*.

In **Appendix V** it was shown that three haplogroups, A, B, and D, were responsible for mass mortalities of *A. torrentium* populations in Germany and Austria. Most importantly, it was for the first time shown that haplogroup D has a high virulence towards *A. torrentium*. While *A. leptodactylus* exhibits a resistance against some haplogroups, *A. torrentium* seems to be highly susceptible to most haplogroups of *A. astaci*. However, there are some reports of chronically infected *A. torrentium* populations in Slovenia and Croatia (Kušar et al. 2013; Maguire et al. 2016). These chronically infected populations were shown be to carriers of the genotypes A_1 and A_2, while the mass mortalities in Germany and Austria (**Appendix V**) were caused by genotype Up. All three of these genotypes are part of haplogroup A (**Appendix III**). The virulence of strains belonging to this

haplogroup thus seems to vary significantly, which was also observed in an earlier study by Makkonen et al. (2012a). This highlights that the identification of haplogroups is not sufficient and an identification of genotypes should, if possible, always be conducted additionally.

8.3 Presence of *Aphanomyces astaci* in North America

To find out more about the origin of different *A. astaci* strains that are currently present in Europe, crayfish samples directly from North America need to be studied for their *A. astaci* infection status. Large-scale data on the genetic variability of *A. astaci* in North America is currently missing. A first study on the *A. astaci* distribution was done in this thesis (**Appendix VI**). Invasive *O. rusticus* were tested positive for infections with *A. astaci*. The crayfish had a low infection prevalence, which is an indication that the crayfish are resistant to the disease, because they can retain the growth of *A. astaci* in their tissue (Oidtmann et al. 2006). Sequence analysis showed that the detected *A. astaci* strain has not been identified before and belongs to haplogroup A. Other crayfish species that are originally from the north-eastern USA and are currently invasive in Europe, e.g. *O. immunis*, have also been shown to be infected with haplogroup A, indicating that this haplogroup might actually be native to this area of the USA (**Appendix III**). Furthermore, the microsatellite analysis of one of the samples revealed a new genotype. This is a further indication, that genotypes of *A. astaci* might be species specific (Grandjean et al. 2014; Maguire et al. 2016), although interspecific transmissions are possible (James et al. 2017). It is yet unclear how virulent an *A. astaci* strain of a specific crayfish species is towards another crayfish species from North America, if they got into contact. It is possible that this would reduce the fitness of the newly infected crayfish species, even if it was resistant to its own *A. astaci* strain (Edsman et al. 2015; Jussila et al. 2016). It was shown in an infection experiment that American crayfish species can exhibit increased mortality when they are artificially infected with a second strain from a different crayfish species (Aydin et al. 2014). Some recent studies even indicate that the resistance of NICS towards *A. astaci* is starting to decrease even against their own *A. astaci* strain (Edsman et al. 2015; Jussila et al. 2016). This could be of high importance for crayfish species that are threatened by invasion of other crayfish within North America. If crayfish species with their specific *A. astaci* strain are translocated to areas where other crayfish species live, the displacement of the indigenous species

could be facilitated by the presence of *A. astaci*. This should be considered in future crayfish conservation programs.

9. CONCLUSION AND FUTURE PERSPECTIVE

This thesis provides strong evidence that *A. astaci* still provides a significant threat to the indigenous crayfish populations of Europe. Population trends of at least some ICS are still decreasing and crayfish mass mortalities caused by *A. astaci* are still being reported (**Appendix V**). While there are some indications for an evolving relationship between *A. astaci* and some ICS populations (**Appendix IV**; Maguire et al. 2016; Martín-Torrijos et al. 2017; Jussila et al. 2017), the constant introduction of new crayfish species together with new *A. astaci* strains will likely prevent an equilibrium between ICS and the pathogen (**Appendix I**). The international trade is one of the most important reasons for the introduction of NICS around the world (Holdich et al. 2009). Regulations and legislation to mitigate the effects of the pet trade have so far proven to be ineffective Europe-wide as well as on a national level (Faulkes 2015b). International polices like EU regulation 2016/1141 need to be extended to cover more species with high invasive potential and known *A. astaci* vectors. Additionally, public education is probably one of the key factors to reduce the risk of NICS released into the wild.

It has recently been suggested that non-indigenous species in general might be more helpful than harmful, because non-indigenous species could, e.g., occupy open ecological niches or help to recover nature through higher tolerance towards degraded ecosystems which have been severely harmed or polluted by anthropogenic activity (Pearce 2016). These claims made by Pearce (2016) are in strict contrast to the situation of invasive crayfish in Europe. North American crayfish were imported along with *A. astaci*, which has obliterated many ICS populations leaving ecological niches open for American crayfish to fill. The natural equilibrium of the invaded ecosystems can further be disrupted by NICS through, e.g., increased burrowing and grazing activity (Ott 2014; Souty-Grosset et al. 2016). While it may be difficult and even harmful to eradicate already established NICS populations in Europe, if they have integrated into the food webs (Kopf et al. 2017), at least the further introduction of NICS and spreading of populations must be prevented.

Continuous development of the genetic methods to detect *A. astaci*, e.g., eDNA detection (Strand et al. 2014), might be necessary for future research questions. If more ICS with latent infections of *A. astaci* will be detected, eDNA detection could help to sample these latently infected ICS populations without the need to kill crayfish specimen if the species is protected. With eDNA detection it is also possible to identify 'crayfish plague free' water bodies that could be used for restocking measures or as receptor habitats for ICS populations that are threatened by crayfish plague epidemics (Collas et al. 2016). A problem of eDNA sampling is that conventional sanger sequencing of eDNA samples is very difficult. A species confirmation of the sample is therefore often impossible. More research on the biodiversity of the *Aphanomyces* genus is needed (Diéguez-Uribeondo 2009). Over the last years, new *Aphanomyces* species have been described that can create false positives when detected with sequence analysis of the ITS region (Oidtmann et al. 2006; Diéguez-Uribeondo 2009; Diéguez-Uribeondo et al. 2009).

The genetic methods developed during this thesis (**Appendix III**) will allow for more detailed analyses of different *A. astaci* strains in the future. The different genetic methods used in the field of crayfish plague research, however, have made the situation even more complicated. It has become increasingly difficult for stakeholders in crayfish conservation to understand the new discoveries made by studying the genetics of *A. astaci.* The application of these methods requires a careful definition of the different results achieved by these methods. Haplotypes and haplogroups need to be distinguished from RAPD-groups, AFLP-groups, and genotypes. A review concerning these problems should be outlined to create a universal nomenclature for the different groups in the future. One suggestion would be to use the naming applied in this thesis: A, B, D, and E as names for the different haplogroups, while As, PsI, PsII, Pc, and Or could be used as names for the RAPD-groups.

To improve the understanding of the co-evolutionary processes between crayfish species and *A. astaci*, large-scale screenings for crayfish plague infections, including determination of the haplotypes and genotypes of *A. astaci*, should be done in North America (**Appendix VI**). High crayfish species diversity in North America has probably facilitated the development of different *A. astaci* strains. This could also be problematic for the North American crayfish species, if crayfish are translocated within North America.

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12. APPENDIX

12.1 Appendix I

Investigation of ornamental crayfish reveals new carrier species of the crayfish plague pathogen (*Aphanomyces astaci*)

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Abstract

Several North American crayfish species have so far been identified as carriers of the crayfish plague agent *Aphanomyces astaci*. The pathogen is responsible for the declines of thousands of European crayfish populations. Currently, one of the introduction pathways of North American crayfish species is the aquarium trade which may sometimes be followed by intentional release or unintentional escape of the pet species into the wild. We investigated 85 samples of North American and New Guinean species, available through aquarium trade, for their possible infection with *A. astaci*. Crayfish plague infection was examined by applying real-time PCR. Besides morphological identification, we sequenced a fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene for crayfish species confirmation. Additionally, sequence analysis of nuclear DNA was conducted to identify the *A. astaci* lineage of moderate to highly infected crayfish. A total of 11 of the 85 analyzed crayfish individuals were tested positive for an *A. astaci* infection, of which nine species are for the first time identified as carriers of *A. astaci*. No new genetic lineages of *A. astaci* were identified. The results confirm that due to the positive carrier status of tested crayfish, the aquarium trade in Europe can facilitate the spread of *A. astaci* and can thus be a significant threat to the indigenous crayfish and the environment.

Keywords:

Freshwater crayfish, crayfish plague, invasive species, aquarium trade, real-time PCR

Introduction

Translocations and range expansions of non-indigenous species often disturb the ecological balance of the natural communities and native ecosystems by increased predation, competition and habitat alteration (Reynolds 2011). In the past, non-indigenous crayfish species (NICS) have intentionally been introduced and stocked into European waters to compensate for the native crayfish populations that were on decline due to mass mortalities caused by *Aphanomyces astaci* SCHIKORA, 1906 infections, also known as crayfish plague epidemics (Alderman 1996). Old NICS (i.e., NICS introduced before 1975), which are the signal crayfish *Pacifastacus leniusculus* (DANA, 1852), the spiny-cheek crayfish *Orconectes limosus* (RAFINESQUE, 1817) and the red swamp crayfish *Procambarus clarkii* (GIRARD, 1852), have long been established in European waters (Holdich et al. 2009), and today occur in many European countries in higher densities than indigenous crayfish species (ICS). This replacement can mainly be attributed to competition and crayfish plague transmission (Reynolds 2011), leading to habitat losses of ICS (Westman et al. 2002; Holdich et al. 2009). The crayfish plague disease is probably the most important factor regarding population declines of ICS. The oomycete *A. astaci* is a parasite which can be transmitted via infected crayfish (reviewed by Longshaw 2011), but also via infected crab species, in particular *Eriocheir sinensis* H. MILNE EDWARDS, 1853 (Schrimpf et al. 2014; Svoboda et al. 2014). Fish as well as transport of fishing gear, traps and fish stockings can be responsible for the further spread of *A. astaci* (Alderman 1996; Oidtmann et al. 2002).

In contrast to the Old NICS, there are the New NICS (i.e., NICS introduced after 1980) which were introduced mainly through aquarium trade and for aquaculture purposes (Holdich et al. 2009). Although animal trade has long been known to cause an opportunity for biological invasions, crayfish have only recently experienced an increased popularity as exotic pets (Holdich et al. 2009; Chucholl 2013; Patoka 2014). As a result, releases and escapes from aquaria and aquaculture are currently among the main pathways for invasions of New NICS into Central Europe (Alderman 1996; Holdich et al. 2009; Peay 2009). The main factors which increase the probability for releases into nature from aquaria are large body size and high availability in aquarium trade (Chucholl 2013). The threat arising from the crayfish aquarium trade is high, particularly in Germany where at least 120 alien crayfish species can be purchased. About seven new crayfish

species per year were imported for the aquarium trade during 2005 and 2009 (Chucholl 2013).

Since its first discovery in Europe in the late 19th century, *A. astaci* seems to have been introduced into Europe repeatedly numerous times, resulting in the introduction of different lineages of *A. astaci* from different locations in North America (Huang et al. 1994; Diéguez-Uribeondo et al. 1995; Kozubíková et al. 2011; Viljamaa-Dirks et al. 2013). So far, five lineages of *A. astaci* have been identified. Signal crayfish (*P. leniusculus)* of American and Canadian origin have been shown to carry the lineages PsI or PsII, respectively. American red swamp crayfish (*P. clarkii*) carry the lineage Pc and spiny-cheek crayfish (*O. limosus*) the lineage Or (Huang et al. 1994; Diéguez-Uribeondo et al. 1995; Rezinciuc et al. 2013; Kozubíková et al. 2011). Lineage As was first isolated from European noble crayfish *Astacus astacus* (LINNAEUS, 1758) while its original American host species remains unknown (Huang et al. 1994; Makkonen et al. 2012a; Viljamaa-Dirks et al. 2013). Following up on the allocation of these different lineages into different genetic groups as first done by Huang et al. (1994), Rezinciuc et al. (2013) revealed through AFLP-PCR that there is some genetic variation within these different genetic lineages of *A. astaci*. This was further confirmed through the development of microsatellite markers, showing differences in allele sizes within genetic lineages of *A. astaci* (Grandjean et al 2014; Maguire et al. 2016). Thus, the genetic variation of *A. astaci* is probably higher than revealed by RAPD analysis (Huang et al. 1994). It is therefore reasonable to assume that different genetic lineages of *A. astaci* consist each of numerous different genotypes.

While native European crayfish, i.e. the stone crayfish (*Austropotamobius torrentium* SCHRANK, 1803) and the as vulnerable rated, IUCN Red-List species noble crayfish (*A. astacus*) (IUCN 2015), are highly vulnerable to *A. astaci* infections and as a consequence can undergo mass population declines (Alderman 1996; Kozubíková et al. 2011; Filipova et al. 2013), North American crayfish can usually resist an *A. astaci* infection due to their coevolved immune system, unless they are exposed to additional stress (Söderhäll and Cerenius 1992; Alderman 1996; Cerenius et al. 2003; Aydin et al. 2014). However, a growing number of studies have found chronic infections in populations of noble crayfish (Makkonen et al. 2012b) stone crayfish (Kušar et al. 2013) or white-clawed crayfish (*Austropotamobius pallipes* LEREBOULLET, 1858) (Maguire et al. 2016), which

suggests that they might have developed an increased resistance against an infection with *A. astaci*.

Of 120 crayfish species available in German aquarium trade, 105 have been considered as potential *A. astaci* vectors because of their North or Central American origin (Chucholl 2013). Until 2014, six NICS have been identified as carriers of *A. astaci*: *Pacifastacus leniusculus* (Unestam and Weiss 1970), *O. limosus* (Vey et al. 1983), *P. clarkii* (Diéguez-Uribeondo and Söderhäll 1993), *Orconectes immunis* (HAGEN, 1870) (Schrimpf et al. 2013), *Procambarus fallax* f. *virginalis* MARTIN ET AL., 2010 (Keller et al. 2014) and *Orconectes virilis* (HAGEN, 1860) (Tilmans et al. 2014). Mrugała et al. (2015) recently identified seven crayfish species from the aquarium trade as new potential carriers of *A. astaci*, six of which originated from North America and one from Australia. As the recent identifications by Mrugała et al. (2015) were not confirmed by isolation of *A. astaci*, these results should be interpreted with caution. They also showed that frequent misidentification of crayfish species occurs which is why species might sometimes be sold under wrong species names.

As a complementary study to Mrugała et al. (2015), in this study we investigated a total of 85 crayfish individuals from 50 morphologically identified species, of mostly North American or Central American origin (USA, Canada, Mexico, Guatemala) for a possible *A. astaci* infection. Many of the studied species have never before been tested positive for an infection with *A. astaci*. All studied crayfish species can be bought in the German aquarium trade and are thus a potential threat for the native ecosystems, if released into the wild. Additionally, two of the studied species originate from Papua and West New Guinea, which allows for the testing of a possible horizontal transfer of *A. astaci* in the aquarium trade (Mrugała et al. 2015), as species from Australasia can be assumed to be *A. astaci*-free in their natural environment (Unestam 1976).

Material and Methods

Crayfish samples and species identification

The 85 individual crayfish from 50 different ornamental species, based on morphological identification, were obtained from a German hobby breeder. All studied species belonged to seven genera (*Barbicambarus, Cambarus, Cherax, Fallicambarus, Orconectes, Pacifastacus* and *Procambarus*). Thirteen of the

crayfish individuals could not be identified by the hobby breeder, however he assumed they were 10 different species, based on morphological characterization. After their death they were stored in 70% ethanol, for one month to five years. Individuals from the same species were stored in the same containers. For additional verification of the crayfish species identification a fragment of the mitochondrial cytochrome c oxidase subunit I (COI) gene was sequenced. DNA was extracted from muscle tissue using a standardized protocol ('High Salt DNA Extraction Protocol for removable samples'; Alijanabi and Martinez 1997). The reaction mixture contained 5x PCR buffer, 0.03125 u TaqMan® Taq (all Promega, Mannheim, Germany), 1.5 mM $MgCl_2$, 0.5 mM dNTP mix (all Fermentas, St. Leon-Rot, Germany), 0.4 µM of primers LCO1490 and HCO2198 (Folmer et al. 1994) and 2 µL DNA template for a final volume of 20 µL. For the samples that created no results a second attempt was started containing 4 µL DNA and 16 µL master mix. PCR was performed using a Primus 96 Plus Thermal Cycler (PEQLAB Biotechnologie GmbH, Erlangen, Germany) under the following conditions: 4 min 94 °C, 35x (45 s 94 °C, 45 s 47 °C, 60 s 72 °C) and 10 min 72 °C. PCR products were sequenced on a 3730 DNA Analyzer capillary sequencer (Applied Biosystems, MA, USA) by the company SEQ.IT (Kaiserslautern, Germany). The sequences were edited with the program Geneious R7 (Drummond *et al*. 2011) and compared with reference sequences from the NCBI GenBank via the Basic Local Alignment Search Tool (BLAST). The truncated alignment of the sequences was 605 base pairs (bp) long. The reference sequence was the sequence with the highest match to the samples sequence, but at least 95%. We only assumed a species to be confirmed, if the hobby breeder named the same species and if there were no other sequences in GenBank with the same percentage coverage. Otherwise the samples were only assigned to the respective genus without providing a species name.

Aphanomyces astaci *infection status analysis*

The soft abdominal cuticle, the inner joints of two walking legs and parts of the uropods were cut off for DNA extraction using a CTAB-method (Vrålstad et al. 2009). To assess the *A. astaci* infection status of the exotic crayfish, an ITS region-targeting TaqMan® minor groove binder (MGB) qPCR was conducted after Vrålstad et al. (2009) with some modifications (Schrimpf et al. 2013). Based on the number of PCR forming units (PFU) infection status and agent levels from *A. astaci* specific qPCR were defined according to Vrålstad et al. (2009), where samples with agent

level A0 (0 PFU) and A1 (PFU_{obs} < 5 PFU) are considered uninfected and agent level A2 (5 PFU ≤ PFU_{obs} < 50 PFU) and higher (A3: 50 PFU ≤ PFU_{obs} < 10^3 PFU; A4: 10^3 PFU ≤ PFU_{obs} < 10^4 PFU; A5: 10^4 PFU ≤ PFU_{obs} < 10^5 PFU; A6: 10^5 PFU ≤ PFU_{obs} < 10^6 PFU; A7: 10^6 PFU ≤ PFU_{obs}) are considered infected with *A. astaci*.

Aphanomyces astaci *lineage identification*

For *A. astaci* lineage identification we amplified a 370 bp long fragment of the chitinase gene according to Makkonen et al. (2012a). The sequence from the chitinase gene only allows to determine the lineages As and Pc. The lineages PsI, PsII and Or have the same chitinase sequence and thus cannot be distinguished from one another. The amplification was checked on a 1.5% agarose gel containing 0.5 µg ml^{-1} ethidiumbromide and the amplified PCR products were then purified with QiaQuick PCR Purification Kit (Qiagen, Germany). The purified PCR products were premixed with AAChiF-primer (Makkonen et al. 2012a) and sent for sequencing to GATC Biotech (Cologne, Germany). The sequences were edited with the program Geneious R7 and the lineage was determined by comparison to reference sequences from GenBank.

Results

Crayfish species identification

The COI sequence analysis was successful for 55 of the 85 crayfish (Appendix 1; Genbank accession numbers: KU527854 - KU527891). For those individuals the species identification by the hobby breeder is given as well as the closest matching GenBank entry. The alignment was truncated to receive good sequences at the 3' as well as the 5' end. For 24 morphologically identified specimens no reference sequence was available in GenBank. For 27 out of the 31 individuals for which a reference sequence was available and the COI sequencing was successful (87.1 %) the closest GenBank match corresponded to the morphological identification of the hobby breeder. In four cases out of these 32 successfully sequenced individuals (12.9 %) with reference sequence, the genetic species determination deviated from the morphological determination. These were the morphological identified specimens *Cambarus striatus* HAY, 1902, *Orconectes hylas* (FAXON, 1890), *Orconectes eupunctus* WILLIAMS, 1952 and *Cherax holthuisi* LUKHAUP & PEKNY, 2006, which had a better genetic identity with the species *Cambarus halli* HOBBS, 1968,

Orconectes quadruncus CREASER, 1933*, Orconectes obscurus* (HAGEN, 1870) and one different *Cherax sp*., respectively.

Aphanomyces astaci *infection status analysis*

DNA of *A. astaci* was detected in 11 out of the 85 (12.9 %) samples, each of those 11 individuals belonging to a different species, and all originating from North or Central America (Table 1). Seven crayfish had low agent levels (A2), while two *Orconectes* species (morphological identification: *Orconectes eupunctus* and *Orconectes hylas*) had moderate agent levels (A3 and A4). *Procambarus llamasi* VILLALOBOS, 1954, originating from Mexico, had a very high agent level (A6). The two specimens from Papua New Guinea or West Guinea, *C. boesemani* and *C. holthuisi*, were tested negative for *A. astaci* infection.

Table 1 Number of samples for specific agent level of infected crayfish species. The level of infection ranges from A0 (not infected) to A7 (very high level of infection). Positive tested samples are presented in bold letters

Species (morphological determined) [n]	Agent level					
	A0	A1	**A2**	**A3**	**A4**	**A6**
Cambarus fasciatus (3)	1	1	**1**			
Cambarus manningi (4)	1	2	**1**			
Orconectes sp. (1)				**1**		
Orconectes sp. (1)					**1**	
Orconectes luteus (1)			**1**			
Orconectes neglectus (1)			**1**			
Orconectes ozarkae (1)			**1**			
Pacifastacus leniusculus (4)	2	1	**1**			
Procambarus llamasi (1)						**1**
Procambarus sp. (1)			**1**			
Procambarus simulans (2)		1	**1**			
Summary of all specimens	4	5	**8**	**1**	**1**	**1**

Aphanomyces astaci *lineage identification*

The sequencing of the chitinase gene was only successful for two infected crayfish species. *Procambarus llamasi* was carrier of the Pc-lineage. The *A. astaci* lineage carried by *O. hylas* could be narrowed down to be either the Or-, Ps-lineage or a different, yet unknown genetic lineage which has the same chitinase sequence as the above mentioned.

Discussion

In the study at hand we tested the *A astaci* infection status of different crayfish species which are sold in the German aquarium trade. We observed that 11 out of

85 (12.9 %) individuals, each belonging to a different species, tested positive for *A. astaci* (Table 1). Six species are for the first time identified as carriers of *A. astaci:* three *Orconectes* species (*O. ozarkae* WILLIAMS, 1952, *O. neglectus* (FAXON, 1885) and *O. luteus* (CREASER, 1933)), two *Cambarus* species (*C. fasciatus* HOBBS, 1981 and *C. manningi* HOBBS, 1981) and *Procambarus simulans* (FAXON, 1884). For three infected species a genetic confirmation of the morphological species determination was not possible due to reduced sequence similarity compared to the reference sequence obtained from GenBank (*Orconectes eupunctus* and *O. hylas*) or due to lacking COI reference in GenBank (*Procambarus pygmaeus* HOBBS, 1942). Finally, two species tested positive in our study have already been known to be carriers of *A. astaci* (*P. leniusculus,* Unestam and Weiss 1970; *P. llamasi,* Mrugała et al. 2015). As we received the samples already stored in ethanol, it was not possible to attempt an isolation of *A. astaci* into laboratory culture. This would have been the ultimate proof of a real infection of the crayfish with *A. astaci*. Detection of *A. astaci* DNA in this study might in principle, be due to spore attachment on the cuticles of the crayfish.

Thirteen crayfish samples could not be identified morphologically. This represents a general problem of the crayfish aquarium trade, as crayfish are often misidentified and sold under wrong names (Mrugała et al. 2015). We cannot rule out that some species might have been misidentified by the hobby breeder, however 27 of the morphological identified individuals showed a good match (sequence similarity > 95%) with the COI reference sequences from GenBank, if a reference was available. Previously published sequences on the genus *Orconectes* (Taylor & Hardman 2002; Taylor & Knouft 2006) did not always confirm the morphological identification by the hobby breeder, highlighting the difficult morphological identification especially in North American species. Additionally, a 5% divergence may also include some cryptic species in cambarid crayfish (Mathews et al. 2008; Filipová et al. 2010). In general, there is necessity for a larger effort on creating a reliable genetic database for crayfish species identification, as for ten species from this study no COI reference sequence was available, which can lead to the misidentification of the sample (Filipová et al. 2011).

Unfortunately, for 30 samples the COI sequence analysis was unsuccessful. This may be due to a low DNA quality as a result of suboptimal storing conditions. DNA degradation can also lead to an underestimation of the agent level during

screening for the pathogen presence. Another reason for COI sequencing failure could be mutations in the primer binding sites of the standard Folmer primers.

Our study comprises small sample sizes, often only one specimen per species and always less than five (Appendix 1), which makes a negative infection result difficult to interpret. We cannot rule out that the real number of infected individuals is even underestimated, caused by DNA degradation or the presence of inhibitors. However, those specimens which tested positive for *A. astaci* can be considered as *A. astaci* carrying species, with maybe even a high prevalence of carriers in the sampled stock. For negatively tested specimens, we can only draw conditional conclusions, as the small sample sizes result in a rather large possibility of missing a low prevalence *A. astaci* infection in the main stock itself. Mrugała et al. (2015) have shown that *Procambarus enoplosternum* can be carrier of *A. astaci* while the one individual of this species in our study tested negative. Further studies regarding the *A. astaci* infection rate of the other negative tested species are thus needed.

There are several possible scenarios how the studied ornamental crayfish got infected with *A. astaci*, either in their native environment before capturing or during holding in pet shop tanks. A horizontal transmission of the pathogen between the crayfish cannot be ruled out, since specimens were kept in adjacent aquaria by the crayfish breeder. Contaminated equipment could also have caused horizontal transmission of *A. astaci* between crayfish. As the sequence analysis of the chitinase gene only allows for the discrimination of the *A. astaci* linages As and Pc, while the remaining lineages Or, PsI and PsII are identical, we were only able to detect the Pc lineage in *P. llamasi.* However, one *Orconectes* species (morphologically identified as *O. hylas*) was either infected with the Or lineage or one of the Ps lineages. It might also be possible however that it is infected with an unknown genetic lineage that has the same chitinase sequence. Unfortunately, the microsatellite analysis (Grandjean et al. 2014) for this sample was unsuccessful, possibly due to a rather low agent level (A3). However, the sequencing result reveals that at least two lineages of *A. astaci* infected the investigated specimens independently. For the remaining infected crayfish of this study, the *A. astaci* lineage remained unclear. This was due to the low amount of *A. astaci* DNA in most of the samples, which compromises identification of the *A. astaci* lineage. For the infected specimens in our study, it cannot be concluded at which stage they got infected, whether before or after entering Germany. In any case, a horizontal

transmission of the disease may represent a serious problem in the crayfish aquarium trade (Mrugała et al. 2015) because it can facilitate the spread of *A. astaci*.

Of the species investigated in our study, only the signal crayfish, belonging to the Old NICS, has so far established populations in the wild in Europe and is also the most widespread NICS in Europe (Holdich et al. 2009). However, the availability of ornamental crayfish in Europe increases the probability of wild population establishment also for other alien crayfish species (Chucholl 2013) and thus the establishment of novel *A. astaci* reservoirs in the wild. Even if the release of individual crayfish does not lead to a population establishment, one individual infected with *A. astaci* (like the *P. llamasi* in this study with agent level A6) is a threat to indigenous crayfish species. Thus, uncontrolled spread of *A. astaci* throughout Europe is facilitated by the lack of import restrictions for exotic species, also between EU countries. Ireland and Scotland are two cases with strict national alien species policy, as the aquarium trade of crayfish is completely banned and keeping of alien crayfish is illegal (Peay 2009). However, these import restrictions are only effective if the existing laws are also enforced, which seems to be a problem for example in Ireland where the parthenogenetic marbled crayfish is available for sale in the pet trade (Faulkes 2015a). An alternative approach to the complete ban of live crayfish imports, was proposed by Padilla et al. (2004). They recommend to post bonds to ensure that the costs to repair damages and implement conservation measures that arise from the aquarium trade are covered by those who cause the problems, i.e. importers and traders of crayfish. A similar regulation is also implemented in the EU regulation 1143/2014, called the polluter pays principle, stating that the costs that arise from the introduction of alien species into Europe are traders' responsibility.

Our study highlights the potential threats of alien invasive species and the diseases they might be carrying (Holdich et al. 2009; Savini et al. 2010), especially in the case of the public having easy access to pet animals and the opportunity to release them into nature (Chucholl 2013; Mrugala et al. 2015). A serious concern is the pet status of the alien crayfish. Pets associated with emotional attitudes are not treated like invasive alien species, as dispensable individuals would not be terminated but released into the wild to continue their life. This constitutes a factor often ignored when analyzing risks of pet crayfish, as e.g. with the FI-ISK score

(e.g., Tricarico et al. 2010; Patoka et al. 2014). One should never underestimate the threat of the pet animals to the receiving ecosystem.

To conclude, we want to emphasize the threat that the crayfish aquarium trade may pose for nature conservation, since the health status of crayfish kept in private aquaria or tank systems is currently largely unknown. We identified nine new crayfish species as carriers and thus potential transmitters of *A. astaci.* Crayfish that are bought from hobby breeders could in many cases be *A. astaci* carriers, and thus an ecosystem hazard and threat to native European crayfish. This is especially true for imported species from North America. Import restrictions for live crayfish trade alone are not sufficient to decrease the uncontrolled spread of *A. astaci*, but also the education of owners and retailers may be necessary (Faulkes 2015b) to reduce the threat that the crayfish pet trade poses to the indigenous crayfish species. As Souty-Grosset (2016) stated, the trade regulation for NICS has to be of EU-level concern rather than single countries. Five NICS have very recently been included in the list of alien invasive species of Union concern (EU regulation 2016/1141) with the aim to restrict the import and further spread of these species in the European Union. Because crayfish trade through the internet is not easily regulated the EU aims at establishing early detection surveillance systems of invasive species.

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Appendices I

Appendix I.1 The agreement of the sequenced DNA fragment to the morphological determined species as available from GenBank (with GenBank accession number in brackets) and the closest matching taxon as available from GenBank. "-" indicates an unsuccessful sequencing and thus a confirmation of the species determination was not possible. "n/a" means that no reference sequence for this species was available in GenBank. Species, which morphological identification did not match the genetic identification are marked with *

Species (morphological determined) [n]	Sample accession number	Match with sequence of assumed species if available [%] (accession number)	Taxon with the closest match to the genetic sequence [%] (accession number)
Barbicambarus cornutus (1)		-	-
Cambarus brachydactylus (1)	KU527876	100 (DQ411783)	*Cambarus brachydactylus* (100) (DQ411783)
*Cambarus cf striatus** (1)	KU527854	94 (KF827991)	*Cambarus halli* (99) (JX514478)
Cambarus cumberlandensis (1)		-	-
Cambarus englishi (1)	KU527878	99 (JX514487)	*Cambarus englishi* (99) *(*JX514487)
Cambarus fasciatus (3)	KU527875	100 (JX514495)	*Cambarus fasciatus* (100) (JX514495)
Cambarus fasciatus (1)		-	-
Cambarus girardianus (2)		-	-
Cambarus hubbsi (1)		-	-
Cambarus maculatus (4)		-	-
Cambarus manningi (3)	KU527855	100 (JX514497)	*Cambarus manningi* (100) (JX514497)
Cambarus manningi (1)		-	-
Cambarus rusticiformis (1)	KU527874	100 (JX514488)	*Cambarus rusticiformis* (100) (JX514488)
Cambarus rusticiformis (2)		-	-
Cambarus scotti (1)	KU527877	n/a	*Cambarus angularis* (96) (KF437318)
Cherax boesemani (1)	KU527872	95 (KJ950507)	*Cherax boesemani* (95) (KJ950507)
*Cherax holthuisi** (1)	KU527873	90 (KJ950519)	*Cherax sp.* (98) (KM501043)
Fallicambarus fodiens (3)	KU527862	97 (KC163667)	*Fallicambarus fodiens* (97) (KC163667)
Fallicambarus fodiens (1)		-	-
Orconectes durelli (1)		-	-
*Orconectes eupunctus** (1)	KU527871	94 (AF474349)	*Orconectes obscurus* (98) (AF474355)
*Orconectes hylas** (1)	KU527863	95 (AY701232)	*Orconectes quadruncus* (98) (AY701246)
Orconectes hylas (1)		-	-

Species (morphological determined) [n]	Sample accession number	Match with sequence of assumed species if available [%] (accession number)	Taxon with the closest match to the genetic sequence [%] (accession number)
Orconectes lancifer (2)	KU527865	n/a	*Orconectes menae* (90) (AY701238)
Orconectes lancifer (2)		-	-
Orconectes luteus (1)	KU527867	100 (AY701235)	*Orconectes luteus* (100) (AY701235)
Orconectes marchandi (2)	KU527866	100 (AF474353)	*Orconectes marchandi* (100) (AF474353)
Orconectes medius (1)		-	-
Orconectes neglectus (1)	KU527864	99 (AY701240)	*Orconectes neglectus* (99) (AY701240)
Orconectes ozarkae (1)	KU527868	99 (AY701242)	*Orconectes ozarkae* (99) (AY701242)
Orconectes palmeri longimanus (1)	KU527869	100 (AY701214)	*Orconectes palmeri longimanus* (100) (AY701214)
Orconectes palmeri palmeri (3)	KU527870	n/a	*Orconectes hobbsi* (98) (AY701211)
Orconectes punctimanus (1)		-	-
Orconectes sp. (1)		-	-
Pacifastacus leniusculus (2)	KU527879	100 (JF437997)	*Pacifastacus* leniusculus (100) (JF437997)
Pacifastacus leniusculus (1)	KU527880	98 (JF437997)	*Pacifastacus leniusculus* (98) (JF437997)
Pacifastacus leniusculus (1)		-	-
Procambarus barbiger (2)	KU527859	n/a	*Procambarus clarkii* (92) (KJ645848)
Procambarus bivittatus (1)		-	-
Procambarus elegans (1)	KU527856	n/a	*Procambarus versutus* (93) (JF737745)
Procambarus enoplosternum (1)	KU527882	96 (KF944432)	*Procambarus enoplosternum* (96) (KF944432)
Procambarus llamasi (1)	KU527860	99 (KF944433)	*Procambarus cf llamasi* (99)(KF944433)
Procambarus ouachitae (3)	KU527883	n/a	*Procambarus spiculifer* (93) (JF737627)
Procambarus pygmaeus (1)	KU527861	n/a	*Procambarus clarkii* (91) (JN000905)
Procambarus simulans (1)	KU527881	97 (EU583575)	*Procambarus simulans* (97) (EU583575)
Procambarus simulans (1)		-	-
Procambarus tulanei (1)	KU527885	n/a	*Fallicambarus harpi* (93) (KC163656)
Procambarus vasquezae (2)	KU527884	100 (KF944431)	*Procambarus vasquezae* (100) (KF944431)
Procambarus vioscai (1)	KU527857	n/a	*Procambarus spiculifer* (93) (JF737627)
Procambarus vioscai (2)	KU527858	n/a	*Procambarus clarkii* (94) (JN000905)
Unknown (2)	KU527886	n/a	*Procambarus clarkii* (93) (JN000905)
Unknown (1)	KU527887	n/a	*Procambarus spiculifer* (93) (JF737690
Unknown (1)	KU527889	n/a	*Procambarus clarkii* (94) (KJ645854)

Species (morphological determined) [n]	Sample accession number	Match with sequence of assumed species if available [%] (accession number)	Taxon with the closest match to the genetic sequence [%] (accession number)
Unknown (1)	KU527890	n/a	*Procambarus llamasi* (99) (JX127969)
Unknown (1)	KU527888	n/a	*Procambarus paeninsulanus* (100) (JF737489)
Unknown (1)	KU527891	n/a	*Cambarus englishi* (98) (KP294449)
Unknown (6)		-	-
Summary of all individuals (85)			

12.2 Appendix II

First established population of marbled crayfish (Decapoda, Cambaridae) in Romania

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Abstract

The marbled crayfish, *Procambarus fallax* f. *virginialis*, is an obligate parthenogenetic crayfish species, its spread in the wild being linked to the aquarium pet-trade. Forty-two adult individuals were found for the first time in Romania in the semi-natural ponds in Băile Felix, Oradea, Romania. Nine ovigerous females were captured as evidence that the population is breeding in the wild. They probably originated from the pet trade and were released by hobbyists into the pond. Microsatellite analysis revealed the same allelic patterns as in a previous study, confirming that these marbled crayfish are parthenogenetic and originate from a single individual. The pathogen, *Aphanomyces astaci* was not present in the population. The site inhabited by this established marbled crayfish population is supplied with water by thermal underground streams, ensuring a constant high temperature. The nearby Peța Natural Reserve protects several endemic species that could be threatened by the further range extension of marbled crayfish. Special protection measures are therefore urgently needed.

Key words: Invasive species; Marmorkrebs; Parthenogenesis; Pet trade; *Procambarus fallax* f. *virginialis*

Introduction

Biological invasions, especially ones triggered by humans, cause damage to the environment (Dorcas et al. 2011, Buckley 2017), and to the economy of the "host" countries (Pimentel et al. 2005, Wittenberg et al. 2006). While some species were introduced for farming (Holdich 1993) or brought as pets (Chucholl and Wendler 2017), the long-term consequences were underestimated in most cases (Lenteren 1997). Such incidents included various crayfish species, such as *Orconectes limosus* (Rafinesque 1817), *Pacifastacus leniusculus* (Dana 1852) and *Procambarus clarkii* (Girard 1852), which escaped from aquaculture facilities or were introduced into the wild, thus leading to their successful on-going invasion in Europe (Gherardi 2006). All of these species possess impressive invasive prowess through higher growth rates than native species (Kozák et al. 2007), adaptive ability (Buřič et al. 2013), high fecundity (Pârvulescu et al. 2015), and food plasticity (Olsson et al. 2009). They are also resistant carriers of *Aphanomyces astaci* Schikora 1906 (Strauss et al. 2012, Schrimpf et al. 2013), an oomycete pathogen causing the crayfish plague (Jussila et al. 2014). Its virulence to indigenous crayfish species outside of North America has led to *A. astaci* being classified among the world's 100 worst invasive alien species (Lowe et al. 2004).

Marbled crayfish, also known as Marmorkrebs, are one of the most popular pet crayfish species in the world (Faulkes 2015, Patoka et al. 2017). Their origin is unknown, as the first record of their presence comes from the German aquarium trade (Lukhaup 2001). Martin et al. (2010) regarded this crayfish as *Procambarus fallax* (Hagen 1870) f. *virginalis*. Marbled crayfish is either a result of autopolyploidy (Martin et al. 2016) or hybridization between *P. fallax* (Hagen 1870) and other species of the genus *Procambarus*. Vogt et al. (2015) proposed elevation of marbled crayfish to the species level, *P. virginalis*.

Many individuals have reached wild environments through human negligence and have occasionally created stable populations (Lipták et al. 2016, Chucholl and Wendler 2017), because one individual theoretically can start a new population via parthenogenesis (Martin et al. 2010). Furthermore, their high spawning rate may be a great advantage in establishing and maintaining wild populations (Chucholl et al. 2012). Marbled crayfish can survive at low temperatures (Veselý et al. 2015, Kaldre et al. 2016), as shown by studies on its establishment in continental Europe (e.g., Chucholl et al. 2012, Patoka et al.

2016). They have been reported in many European countries: Croatia (Samardžić et al. 2014), the Czech Republic (Patoka et al. 2016), Germany (Chucholl et al. 2012), Italy (Vojkovská et al. 2014), Sweden (Bohman et al. 2013), Hungary (Weiperth et al. 2015, Lőkkös et al. 2016), Slovakia (Janský and Mutkovič 2010), Ukraine (Novitsky and Son 2016), and also in Madagascar (Jones et al. 2009) and Japan (Faulkes et al. 2012). This species is also a host to the crayfish plague pathogen, *A. astaci* (Keller et al. 2014).

In Romania, there are three native species of crayfish: *Astacus astacus* (Linnaeus 1758), *A. leptodactylus* Eschscholtz 1823, and *Austropotamobius torrentium* (Schrank 1803). The alien crayfish *O. limosus,* was first documented in the Romanian Danube in 2008 (Pârvulescu et al. 2009) and successfully competes against *A. leptodactylus* populations in occupied habitats (Pârvulescu et al. 2015). The recent growth of the pet trade in Eastern Europe, including Romania (Raghavan et al. 2013), suggests this is the source of many alien species in the country. Using a population of non-indigenous crayfish found in a semi-natural pond in Romanian territory, we investigated individual morphology; and conducted microsatellite analysis of the population to compare the allele pattern to individuals from previous studies. We also tested the population for presence of *A. astaci* so that a plan to prevent impact on indigenous crayfish species could be instigated if necessary.

Methods

Field sampling

Sampling was conducted after finding that one or more of the five ponds in Băile Felix, near Oradea, România (Fig. 1), could be populated by exotic crayfish. The investigated location is an urban area in Băile Felix-Sânmartin, Bihor County, Romania (Fig. 1), containing five semi-natural ponds named "Waterlily lakes" ("Lacul cu nuferi", in Romanian). The central pond is located at 46°59'20.1"N/21°58'43.3"E. The ponds are cement walled basins with 80-90 cm deep water and a surface varying between ~150-400 m^2. The basins are supplied by subterranean warm water springs (39.5 °C on the date of investigation). Water temperature (24.6 °C) and conductivity (595 µS cm^{-1}) were measured in sample site 3 (see the map in Fig. 1) using a Hach-Lange multi-parameter (Düsseldorf,

Germany). The lowest water temperature in this pond during winter was 15 °C (air temperature -16 °C), and small ice-sheets were observed only at the pond's margins (A. Togor, unpublished data).

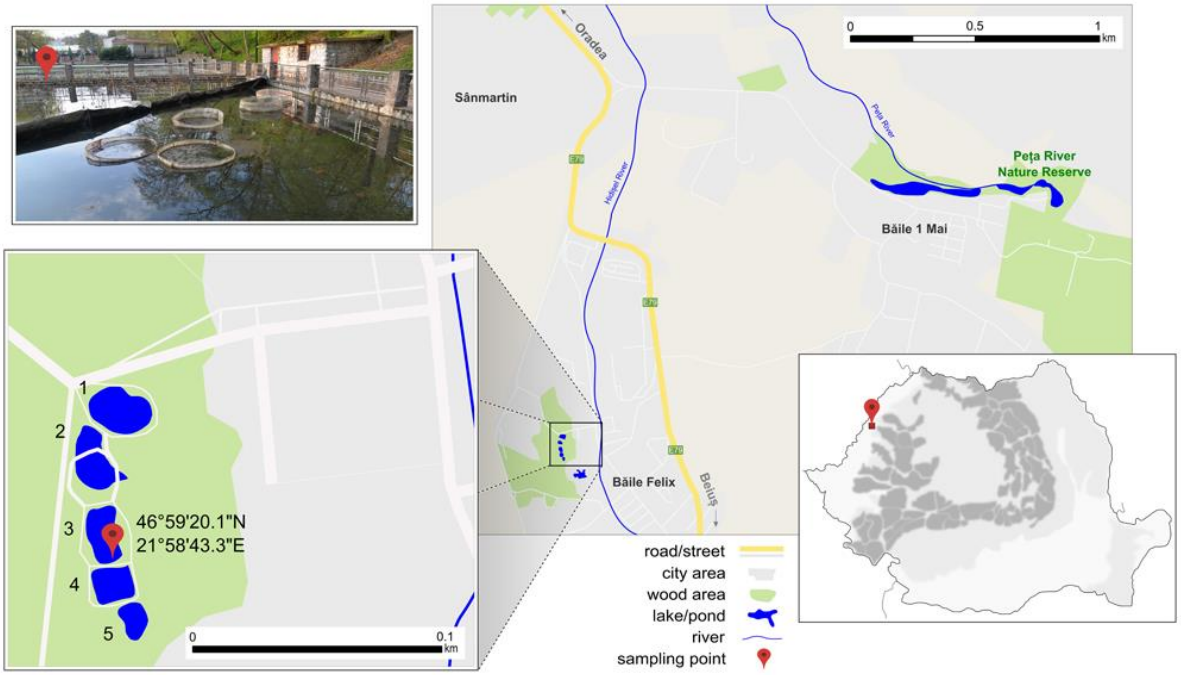

Figure 1. Map showing the location of the established population of marbled crayfish in the semi-natural ponds in Băile Felix, Oradea, Romania with GPS location of pond 3, detailed street-map and photography of the site. Street-map support by OpenStreetMap (https://www.openstreetmap.org).

Two traps were used to catch crayfish five times over five consecutive weeks in April and May of 2017 resulting in five groups of captured individuals. For each capture effort, the traps were baited with fish, left overnight, and checked the following day. The captured crayfish were marked and released in the same pond after a general inspection, except for nine specimens which were preserved and transported in the laboratory for the measurement of the total length (TL), cephalothorax length (CL) and width (CW) to the nearest 0.01 mm using a Black & Decker digital calliper. Wet weight (WW) was recorded using a Kern analytical balance to the nearest 0.01 g. Tissue from these nine crayfish was collected by detaching the last walking leg of each individual and preserving it in 96% ethanol for molecular analyses. Samples for the detection of *A. astaci* consisted of soft abdominal cuticle, walking legs, telson and uropods (Vrålstad et al. 2009), and were stored in 96% ethanol.

Species identification and population genetics

Species diagnosis followed the guidelines of Martin et al. (2010) and Vogt et al. (2015). The key feature for differentiating marbled crayfish and females of *P. alleni* (even at small sizes) is the morphology of the sperm receptacle, the *Annulus ventralis*. Moreover, microsatellites analysis was used to compare the allelic pattern of captured marbled crayfish to individuals from previous studies. Nuclear DNA was extracted from walking legs of nine collected specimens of marbled crayfish with the Qiagen Blood & Cell Culture DNA Kit (Hilden, Germany). The same five primer pairs (PclG-02, PclG-04, PclG-08, PclG-48, PclG-26) and methods as in Vogt et al. (2015) were used. PCR was carried out using a Primus 96 Cycler (Peqlab Biotechnologie, Erlangen, Germany) in two separate batches, A and B. The conditions were as follows: DNA was denatured at 95°C for 2 min, followed by 35 loops of denaturation at 95°C for 30 sec, annealing at 65°C or 55°C for batch A and batch B, respectively and elongation at 72°C for 1 min. A final elongation step at 72°C for 5 min concluded the PCR.. Fragment analysis was performed on a Beckman Coulter CEQ 8000 eight capillary sequencer (Beckman Coulter, Krefeld, Germany) using the Beckman Coulter DNA Size Standard Kit 400 bp. The microsatellite peaks were scored using the Software GeneMarker V. 1.95 (SoftGenetics, Pennsylvania, USA). Juvenile stages were scored according to Vogt et al. (2004).

Aphanomyces astaci *infection status analysis*

DNA from nine crayfish was extracted using the E.Z.N.A. Insect DNA Kit (Omega bio-tek, Atlanta, USA) according to the manufacturer's instructions. To assess the infection status of marbled crayfish, a TaqMan® minor groove binder (MGB) qPCR was conducted, targeting the ITS region as described in Vrålstad et al. (2009) with some modifications according to Schrimpf et al. (2013). An initial Pre-PCR decontamination step was done at 50°C for 120 sec followed by a polymerase activation and template denaturation at 95°C for 10 min. The PCR itself consisted of 50 cycles of denaturation at 95°C for 15 sec followed by an annealing at 62°C for 15 sec. A final cooling step was included for 60 sec at 40°C. Infection status and agent levels were defined according to Vrålstad et al. (2009) based on the number of PCR forming units (PFU), where samples with agent level A0 (0 PFU) and A1 (PFU_{obs} < 5 PFU) are considered uninfected and agent level A2 (5 PFU ≤ PFU_{obs} < 50 PFU) and higher are considered *A. astaci* positive.

Results

In this study, 42 crayfish individuals were captured in ponds 2 to 5, all indentified as marbled crayfish (Fig. 2a). No specimens were captured in pond 1 during the investigation. Six individuals carrying eggs and three carrying juveniles in the second developmental stage were found (Fig. 2c). The feature used to identify the species was the *Annulus ventralis*, which had a flatter, bell-shaped aspect, without scooped lateral wings on the lateral parts, and no peaked anterior portion (Fig. 2b). The microsatellite pattern of the studied samples confirmed the species identification being identical to the patterns found in Vogt et al. (2015), with clear triploidy (fragment length 267/271/303 bp, respectively) at the marker PclG02. This supports the notion that these marbled crayfish are obligatory parthenogenetic and originate from a single individual.

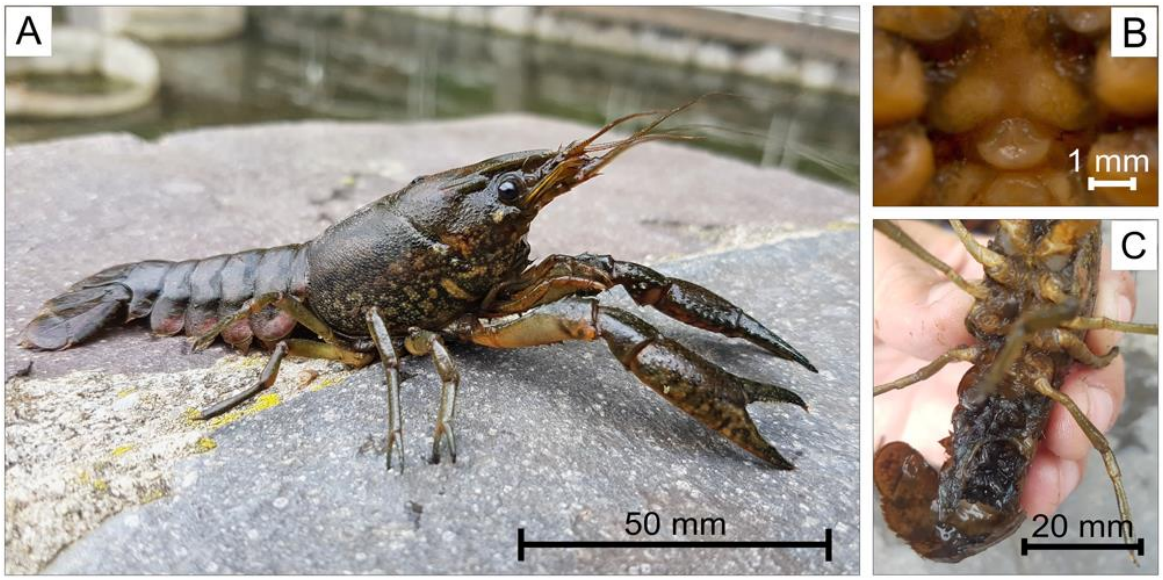

Figure 2. Pictures showing a general view of a specimen of marbled crayfish collected in the semi-natural ponds in Băile Felix, Oradea, Romania (A), a close up of the *Annulus ventralis* (B), and carried stage two juveniles (C).

The TL of the nine individuals analysed in the laboratory ranged from 71.8 to 94.9 mm, with 86.1 mm on average (SD = 8.5). The largest weight was 21.16 g (Table 1). The egg-carrying marbled crayfish in this capture measured 92.5 mm in TL, and weighed 17.95 g without its clutch. Other females presented dark and elongated pleopods, which suggests they had probably bred before (Hopkins 1967).

None of the nine samples analysed for presence of *A. astaci* tested clearly positive for DNA of the pathogen. All samples had the agent level A1, thus being below the limit of detection.

Table 1 Biometrical data measured for the batch of marbled crayfish individuals collected in the semi-natural ponds in Băile Felix, Oradea, Romania, and inspected in the laboratory. Abbreviations: TL - total length, CL - cephalothorax length, CW - cephalothorax width, WW - wet weight.

Order	TL	CL	CW	WW	General observations
1	94.25	43.57	20.60	20.91	fresh molted
2	84.06	38.91	18.44	15.12	dark & elongated pleopods
3	73.83	35.38	16.70	10.48	fresh molted
4	71.83	34.62	15.99	9.31	fresh molted
5	92.45	43.59	20.67	19.25	dark & elongated pleopods
6	92.05	42.36	20.04	17.95	116 juveniles, stage 2
7	94.93	44.39	20.77	21.16	dark & elongated pleopods
8	84.32	39.31	18.68	15.69	dark & elongated pleopods
9	87.44	40.69	19.71	17.32	dark & elongated pleopods
Mean	86.13	40.31	19.07	16.35	
SD	8.5	3.6	1.8	4.2	

Discussion

The established population of marbled crayfish in Romania is in a recreational promenade area, including five interconnected water bodies close to Hidișel River (Fig. 1), having a roughly constant temperature of around 25 °C given by warm springs (Tenu et al. 1981). The area is frequently visited by tourists because of the local attraction, the thermal lotus *Nymphaea lotus* f. *thermalis*. This lotus species is endemic in the thermal waters of the nearby Peţa River Nature Reserve (Fig. 1), while the semi-natural ponds are populated artificially. The crayfish species probably found its way into this pond by being abandoned there, like many other exotic species, such as *Trachemys scripta*, *Carassius* spp., *Colisa* spp, *Xiphophorus* spp. (A. Togor, pers. comm.). Alongside the thermal lotus, these warm waterbodies are inhabited by two other endemic taxa: the fish *Scardinius racovitzai* Müller 1958 and the mollusc *Melanopsis parreyssi* (Philippi 1847), both of which are critically endangered species (Freyhof and Kottelat 2008, Fehér 2011).

The ponds are not directly connected to natural river systems. Still, the risk of further expansion seems high because the area is frequently visited by uninformed public who could translocate specimens from the ponds to the nearby Hidișel and Peța rivers. As marbled crayfish consume plants (VanArman 2011),

they pose a potential threat to the thermal lotus, which has registered a decrease in population in 2017 (A. Togor, unpublished data). We suspect that crayfish might damage the lotus plants by eating the bulbs and roots, and/or the fragile sprouts in spring.

Considering the evidence found in other studies (Chucholl et al. 2012), this crayfish species seems less able to colonise large water courses. Consequently, we believe that the expansion of marbled crayfish does not represent a major threat for native crayfish populations, which are well represented in the mountain and submountain areas of the region by *A. astacus* and *A. torrentium*, the nearest at ~50 km, upstream on the Criș River (for the maps, we refer readers to Pârvulescu and Zaharia 2013, 2014). The mean multiannual temperature (Fick and Hijmans 2017) in the upstream area of Criș rivers inhabited by native crayfish species is 4 to 7 °C, much colder in comparison with the area of the pond inhabited by marbled crayfish at 10-11 °C.

Many pet crayfish species are carriers of the crayfish plague pathogen *A. astaci* (Mrugała el al. 2014, Panteleit et al. 2017). In this study, no infection with *A. astaci* could be detected in the marbled crayfish population. It should, however, not be assumed that the population is disease-free. Marbled crayfish can carry the pathogen and tolerate the infection like *P. fallax* (Keller 2014). The detection of agent level A1 in this study is not enough to confirm the absence of *A. astaci*. Thus, this population may be a latent reservoir for the pathogen. We suggest authorities take active measures against the introduction of animals into the ponds (Vrålstad 2011), but also warn tourists and local people not to transfer plants or animals from the site.

Acknowledgements

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12.3 Appendix III

MtDNA allows the sensitive detection and haplotyping of the crayfish plague disease agent Aphanomyces astaci showing clues about its origin and migration

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Abstract

The oomycete *Aphanomyces astaci* is listed as one of the 100 worst invasive species in the world, being the main reason for the reducing numbers of the native crayfish throughout Eurasia. The aim of this study was to examine the potential of selected mitochondrial (mt) genes to track the diversity of the crayfish plague pathogen *A. astaci*. The annotated mt genome (KX405004) of *A. astaci* was used as a reference for the primer design and the mt regions showing genetic differences were selected based on *A. astaci* transcriptomic data. Two sets of primers were developed to amplify the mtDNA of ribosomal rnnS and rnnL subunits. Based on the sequencing data, we confirmed two main lineages, with four different haplogroups and five haplotypes among 27 studied *A. astaci* strains, confirming the results from previous studies. The haplogroups detected were 1) the A-haplogroup with the a-haplotype strains isolated from *Orconectes* sp., *Pacifastacus leniusculus,* and *Astacus astacus* 2) the B-haplogroup with the b-haplotype strains isolated from the *Pacifastacus leniusculus*, 3) the D-haplogroup with the d1 and d2-haplotypes strains isolated from *Procambarus clarkii*, and 4) the E-haplogroup with the e-haplotype strains isolated from the *Orconectes limosus*. The described markers are stable and reliable and the results are easily repeatable in different laboratories with samples obtained from different crayfish host species. The present method has high applicability as it allows the detection and characterization of the *A. astaci* haplotype in acute disease outbreaks in the wild, directly from the infected crayfish tissue samples.

Keywords: Invasive species, oomycete, crayfish disease, single nucleotide polymorphism, ribosomal rnnS and rnnL subunits

Introduction

The crayfish plague, caused by *Aphanomyces astaci* (Schikora), is the most devastating crayfish disease known to date (Cerenius et al., 2009; Jussila et al., 2015). *Aphanomyces astaci* is listed among the 100 worst invasive species in the world by the Global Invasive Species Specialist Group of the International Union for Conservation of Nature (IUCN; Lowe et al., 2004), being the main reason for the reducing numbers of the native crayfish throughout Europe (Souty-Grosset et al., 2006). All the five European crayfish species*, i.e.,* noble crayfish *(Astacus astacus)*, stone crayfish *(Austropotamobius torrentium)*, white-clawed crayfish *(Austropotamobius pallipes)*, narrow-clawed crayfish *(Astacus leptodactylus),* and thick-clawed crayfish *(Astacus pachypus),* are susceptible to the disease, with catastrophic epidemics possible, and are listed in the IUCN Red list as threatened, with a declining population trend (IUCN, 2012).

During the past decades, phylogenetic studies on different *A. astaci* strains have been done to clarify the relationships within this species and the North American hosts that carry them. The Random amplification of polymorphic DNA–polymerase chain reaction (RAPD-PCR) techniques firstly revealed certain genetic diversity in *A. astaci* (Huang et al., 1994; Diéguez-Uribeondo et al., 1995; Rezinciuc et al., 2014). Thus, five genetic groups of *A. astaci,* named A, B, C, D, and E, have been identified in Europe by using RAPD-PCR (Huang et al., 1994; Diéguez-Uribeondo et al., 1995; Kozubíková et al., 2011). The RAPD-PCR group A (As) includes the strain of reference L1, which was isolated from native European crayfish *A. astacus*, and also a number of strains that seem to be related to the first invasion of *A. astaci* with an unknown host species in the 19th century. The RAPD-PCR group B (PsI) includes the strain of reference Pl, which was isolated from the North American crayfish species, *Pacifastacus leniusculus,* as well as other strains isolated from outbreaks in native European species and other *P. leniusculus* crayfish. The RAPD-PCR group C (PsII) is comprised of a single strain named Kv, isolated from an outbreak on signal crayfish in Kvarntorp (Sweden) that originated from Lake Pitt (Canada) (Huang et al., 1994). This group has not been detected since then (Söderhäll and Cerenius, 1999). The RAPD-PCR group D (Pc) includes the reference strain Pc and was first isolated from the red swamp crayfish (*Procambarus clarkii)* in Spain (Diéguez-Uribeondo and Söderhäll, 1993; Diéguez-Uribeondo et al., 1995) and a number of strains, *e.g.,* APO3 and Málaga5, isolated from outbreaks in native crayfish *A. pallipes* (Rezinciuc et al., 2014). Finally, the

RAPD-PCR group E (Or) comprises a reference strain Evira4805 isolated from the spiny-cheek crayfish *(Orconectes limosus)* naturalized in Europe (Kozubíková et al., 2011) and a few strains isolated from outbreaks in native crayfish *A. astacus*, *e.g.* Li10, after that (Kozubíková-Balcarová et al., 2013).

Later, studies based on Internal transcribed spacer (ITS)-regions indicated that *A. astaci* strains were genetically very similar since their intraspecific variation measured is close to zero (Diéguez-Uribeondo et al., 2009; Makkonen et al., 2011). It was postulated that this was a result of the clonal propagation via zoospores (Huang et al., 1994; Diéguez-Uribeondo et al., 2009; Rezinciuc et al., 2015). Additional studies exploring the *A. astaci* diversity in Europe were conducted on nuclear chitinase gene (Makkonen et al., 2012a), amplified fragment length polymorphisms (AFLP; Rezinciuc et al., 2014), and nuclear single sequence repeat microsatellite markers (Grandjean et al., 2014). Phylogenetic analyses using chitinase gene analyses (Makkonen et al., 2012a) and AFLP-PCR (Rezinciuc et al., 2014) indicate that all the strains of *A. astaci* split into two lineages: (i) one that comprises strains from RAPD-PCR groups A, B, C, and E, and (ii) a second one that comprises strains of RAPD-PCR group D. Furthermore, the chitinase gene sequencing and microsatellite markers have also been applied as a diagnostic tool to characterize the pathogen strains causing crayfish plague outbreaks (*e.g.,* Panteleit et al., 2017). However, the application of the chitinase gene as a marker has been found limited due to its incapability to separate the RAPD-PCR groups B and E (Makkonen et al., 2012a). For the microsatellites, the interpretation of the results is often hard due to the lack of possibility to confirm the amplification specificity and possible mixed infections from other Oomycetes species often present in crayfish (Kozubíková-Balcarová et al., 2013). Both methods also fail in cases when the pathogen quantities in samples are low, *i.e*., the quantitative PCR (qPCR) shows agent levels of mid-A3 or lower (Vrålstad et al., 2009). Therefore, only a limited number of crayfish plague outbreak cases detected with qPCR can be further characterized with these applications.

The diversity, distribution, and prevalence of *A. astaci* in its original distribution area in North America are still largely unknown. When novel species or crayfish from North America were introduced to Europe, likely also *A. astaci* has been repeatedly introduced with these animals (Makkonen et al., 2012a; Jussila et al., 2015; Rezinciuc et al., 2015). Nowadays, this threat should be minimized due to the EU regulation 1143/2014 on invasive alien species. The origin and

geographic migration of a broad variety of organisms, including oomycetes, have been tracked by using mitochondrial DNA (mtDNA) (Martin et al., 2007, 2008; Yoshida et al., 2013). When working with clonally reproducing organisms such as *A. astaci,* the mitochondrial (mt) genome provides a valuable marker for population studies (Makkonen et al., 2016). Thus, the aim of this study was to examine the potential applicability of mtDNA as a tool to track the origin and diversity of the crayfish plague pathogen *A. astaci* and provide a basis for developing an efficient tool to further characterize the disease outbreaks and their origins. The *A. astaci* diversity in Europe, as well as in its original distribution in North America, are currently rather poorly known, but hopefully intensively studied also in the future. As critical differences in the pathogen strains' virulence properties in Europe have also been observed (*e.g.*, Diéguez-Uribeondo et al., 1995; Makkonen et al., 2012b; 2014), the characterization of the strains causing the epidemics in the wild must be considered as a task with a high importance.

MATERIALS AND METHODS

Strains and species tested

A total of 27 *A. astaci* strains from the culture collections of the University of Eastern Finland (Finland), Evira (Finland), Charles University of Prague (Czech Republic), and Real Jardín Botánico-CSIC (Spain) were sequenced in this study. The strains were representing the five currently recognized RAPD-groups in Europe (Table 1). In addition, two Saprolegniales species, *Aphanomyces frigidophilus* and *Saprolegnia* sp., were sequenced as reference (Table 1).

Table 1 The *Aphanomyces astaci* strains sequenced in this study. The strains considered as reference strains for each genotype based on RAPD and microsatellite results are bolded. na indicates no result available for RAPD genotype.

Strain code[a]	Origin	Isol. year	RAPD group[c]	Microsat.genotype[d]	Mitoch. haplotype	Host	Reference
L1	**Lake Ämmern, SE**	**1962**	**A (As)**	**SSR-A_1**	**a**	***A. astacus***	**Huang et al. (1994)**
Upor4	Úpořský brook, CZ	2005	na	SSR-Up	a	*A. torrentium*	Grandjean et al. (2014)
UEF_AT1D	River Borovniščica, SI	2014	na	SSR-A_1	a	*A. torrentium*	Jussila et al. (2017)
UEF_VEN5/14	Lake Venesjärvi, Karvia, FI	2014	na	SSR-A_1	a	*A. astacus*	Makkonen et al. (unpublished)
UEF_T2B	River Kemijoki, Taivalkoski, FI	2007	na	SSR-A_1	a	*A. astacus*	Makkonen et al. (2012)
UEF_OI-1 (3)	Oxbow lake of River Rhine, Speyer,	2015	na	SSR-E	a	*O. immunis*	Makkonen et al. (unpublished)
Kv1 (VI03558)	**Lake Pitt, CAN (SE)[b]**	**1978**	**C**	**SSR-C**	**a**	***P. leniusculus***	**Huang et al. (1994)**
Pl	**Lake Tahoe, USA**	**1970**	**B (PsI)**	**SSR-B**	**b**	***P. leniusculus***	**Huang et al. (1994)**
EviraK047/99	Lake Korpijärvi, Mäntyharju, FI	1999	B (PsI)	SSR-B	b	*A. astacus*	Viljamaa-Dirks et al. (2013)
UEF_8866-2	Lake Puujärvi, Karjalohja, FI	2003	B (PsI)	SSR-B	b	*P. leniusculus*	Makkonen et al. (2012)
UEF_SATR (2)	Lake Saimaa, FI	2012	na	SSR-B	b	*P. leniusculus*	Jussila et al. (2013)
UEF_KTY3-4	Fish Research Unit, Kuopio, FI	2008	na	SSR-B	b	*A. astacus*	Makkonen et al. (unpublished)
UEF_T16 (3)	Lake Tahoe, CA, USA	2013	na	SSR-B	b	*P. leniusculus*	Makkonen et al. (unpublished)
UEF_7203 (3)	Lake Kukkia, Luopioinen, FI	2003	B (PsI)	SSR-B	b	*P. leniusculus*	Makkonen et al. (2012)
UEF_8140 (2)	Lake Pyhäjärvi, Säkylä, FI	2003	B (PsI)	SSR-B	b	*P. leniusculus*	Makkonen et al. (2012)
SAP-Pamplona 1	Pamplona, ES		B (PsI)	SSR-B	b	*A. pallipes*	Martin-Torrijos et al. (submitted)
AP03 (SAP2584)	**Cataluña, ES**	**2013**	**D (Pc)**	**SSR-D**	**d1**	***P. clarkii***	**Rezinciuc et al. (2014)**
SAP-Málaga5	Malaga, ES		D (Pc)	SSR-D	d2	*P. clarkii*	Martin-Torrijos et al. (submitted)
Li10	**Litavka River, CZ**	**2011**	**E (Or)**	**SSR-E**	**e**	***A. astacus***	**Kozubíková-Balcarova et al.**
T16-JR26A	*Saprolegnia* sp.						Makkonen et al. (unpublished)
SAP817	*Aphanomyces frigidophilus*						Diéguez-Uribeondo et al. (2009)

[a] With *n* of isolates sequenced from same location given in brackets.

[b] Isolated in Sweden from signal crayfish which originated from Pitt Lake, Canada.

[c] RAPD-genotypes published in Huang et al. (1994), Diéguez-Uribeondo et al. (1995), and Viljamaa-Dirks et al. (2013).

[d] Microsatellite genotypes published by Grandjean et al. (2014).

Primer design

The annotated mt genome (KX405004) of *A. astaci* was used as a reference sequence for the primer design (Makkonen et al., 2016) and the regions containing group specific differences were selected based on alignments with *A. astaci* transcriptomic data produced at the University of Eastern Finland (Kokko et al., unpublished). The alignments and manual editions of the sequences were conducted in Geneious 8.0 (Kearse et al., 2012). Primers were designed with the Primer3 program (Rozen and Skaletsky, 2000). Two primer pairs amplifying the mitochondrial ribosomal rnnS (AphSSUF and AphSSUR) and rnnL (AphLSUF and AphLSUR) genes were designed (Table 2).

Table 2 The primers.

Primer name	Sequence 5'-3'	Start pos.	Stop pos.	Length	Direction	%GC	T_m
AphSSUF	GGGCGGTGTGTACAAAGTCT	1 318	1 337	20	forward	55.0	60.3
AphSSUR	AGCACTCCGCCTGAAGAGTA	806	825	20	reverse	55.0	60.6
AphLSUF	AGGCGAAAGCTTACTATGATGG	2 849	2 870	22	forward	45.5	58.3
AphLSUR	CCAATTCTGTGCCACCTTCT	3 284	3 303	20	reverse	50.0	58.1

The species specificity of the target region was checked with sequence alignments to oomycetes that were either available in GenBank or sequenced in this study (Figure 1ab). The species from GenBank were *Aphanomyces invadans* (KX405005), *Saprolegnia ferax* (AY534144), *Pythium insidiosum* (AP014838), *Phytophthora infestans* (AY898627), *P. ipomoeae* (HM590420), *P. mirabilis* (HM590421), *P. phaseoli* (HM590418), *P. polonica* (KT946598), *P. ramorum* (DQ832718), *P. sojae* (DQ832717), and *Pseudosperenospora cubensis* (KT072718).

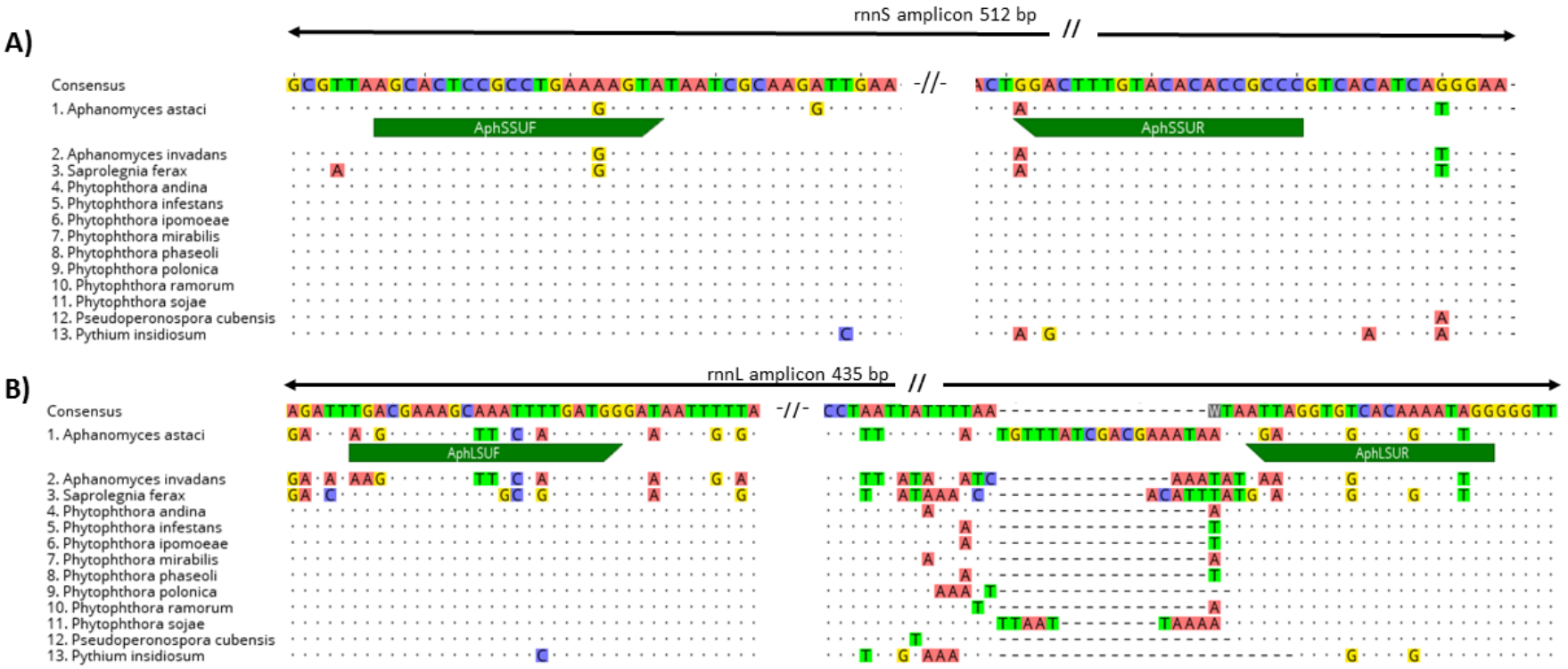

Figure 1 Primer regions aligned with other available oomycetes. A) rnnS region and primers AphSSUF and AphSSUR. B) rnnL-region and primers AphLSUF and AphLSUR.

PCR

The PCR reactions were carried out in 25 µL reaction volume containing 1 U of Dream*Taq* DNA polymerase (Thermo Fisher Scientific), 2X Dream*Taq* Green master mix (Thermo Fisher Scientific), 10 mM of primers, and 10-100 ng of template DNA. The reaction volume was filled with PCR-grade water. The amplification was conducted on a PTC-200 thermal cycler (MJ Research) with the following conditions: 95 °C, 3 min, 35x (95 °C, 30 s; 59 °C, 30 s; 72 °C, 30 s), and 72 °C 10 min. Each run contained a positive control (*A. astaci* DNA of strain UEF8866-2) and a blank reaction without a template. The amplification was checked on a 1.5% agarose gel containing 0.5 µM EtBr. Then, the samples were purified with GeneJET PCR Purification Kit (Thermo Fisher Scientific).

Sequencing, phylogenetics, and analysis on the genetic diversity

The Sanger sequencing reactions were performed in GATC Biotech (Germany) with the primers AphSSUF and AphLSUF, respectively (Table 2). Approximately half of the amplicons were confirmed by additional sequencing with appropriate reverse primers AphSSUR or AphLSUR. The resulting sequence data was manually revised and edited, and the low-quality reads filtrated out from the alignments in Geneious version 8.0 (Kearse et al., 2012) and the primer sites were cut off from the sequences before the further analyses. The sequences were entered to NCBI GenBank database with access numbers MF973121-MF973149 for rnnS and MF975950-MF975978 for rnnL.

Three phylogenetic approximations were employed to reconstruct the phylogenetic relationships: a Bayesian inference (BI), a maximum likelihood (ML), and a neighbor-joining distance based analyses (NJ). The BI was performed in Mr Bayes v.3.2.6 software (Ronquist et al., 2012) using the MCMC method with 10,000,000 generations, three runs (8 chains per run) with a burn-in of 25% trees and a standard deviation of split frequencies <0.01. Nodes with posterior probability (pp) values ≥0.95 were considered supported. The ML was performed using RAxML v.8 (Stamatakis, 2014) implemented in raxmlGUI v1.5b1 (Silvestro and Michalak, 2012), with 100 independents replicates and 1000 rapid bootstraps. Nodes with bootstrap values ≥75 were considered supported. The NJ analysis was performed utilizing MEGA v6.06 (Tamura et al., 2013) using Kimura 2-parameter distances between the sequences and bootstrap values determined by 1000 replications. All the resulting trees from the BI, ML, and NJ were visualized on

FigTree v1.4.2 (Rambaut, 2012). *A. frigidophilus* was used as the outgroup in all three phylogenetic approximations. We preformed the analyses for the independent rnnS and rnnL, as well as for the concatenated rnnS and rnnL regions (Figure 2) with the same parameters described above.

Genetic diversity was estimated calculating the number of polymorphic (segregating) sites (S), the number of haplotypes, the haplotype diversity (Hd), the average number of nucleotide differences (k), and the nucleotide diversity (π) utilizing the program DNAsp v.5.10.01 (Librado and Rozas, 2009). We used TCS v.1.21 (Clement et al., 2002) to represent the mutational changes between the sequences throughout the most parsimonious haplotype network and to visualize the genealogical relationships, we used PopArt v1.7.2 (Leigh and Bryant, 2015).

Microsatellite genotyping

To validate the results of the mitochondrial data with the methods currently in use and obtain grouping for the previously uncharacterized strains, microsatellite analyses of selected strains (Table 1) were conducted at the University of Koblenz-Landau (Germany) utilizing the nine co-dominant microsatellite markers according to Grandjean et al. (2014). The PCR reactions were carried out with Multiplex PCR Kit (Qiagen, the Netherlands) and 0.1 to 0.38 µM of each of the labeled primers Aast4, Aast6, Aast7, Aast14 for Batch A as well as Aast2 Aast9, Aast10, Aast12, Aast13 for Batch B were added. 1 µL DNA template was appended for final volumes of 5 µL and 5.5 µL, respectively. The fragment analyses were conducted on a Beckman Coulter CEQ 8000 eight capillary sequencer. Alleles were scored using the GenMarker software (version 1.95, SoftGenetics LLC) and compared to reference strains.

Direct A. astaci *haplotyping from infected crayfish cuticle samples*

Direct haplotyping from infected crayfish samples was conducted at the University of Koblenz-Landau (Germany). One sample was from an aquarium-held marbled crayfish, *Procambarus fallax* f. *virginalis*, already tested positively for crayfish plague infection by Keller et al. (2014). Moreover, *A. astaci* DNA was extracted from stone crayfish belonging to populations from three natural water bodies in Austria and Germany, which underwent mass mortalities at the time of sampling (Table 3).

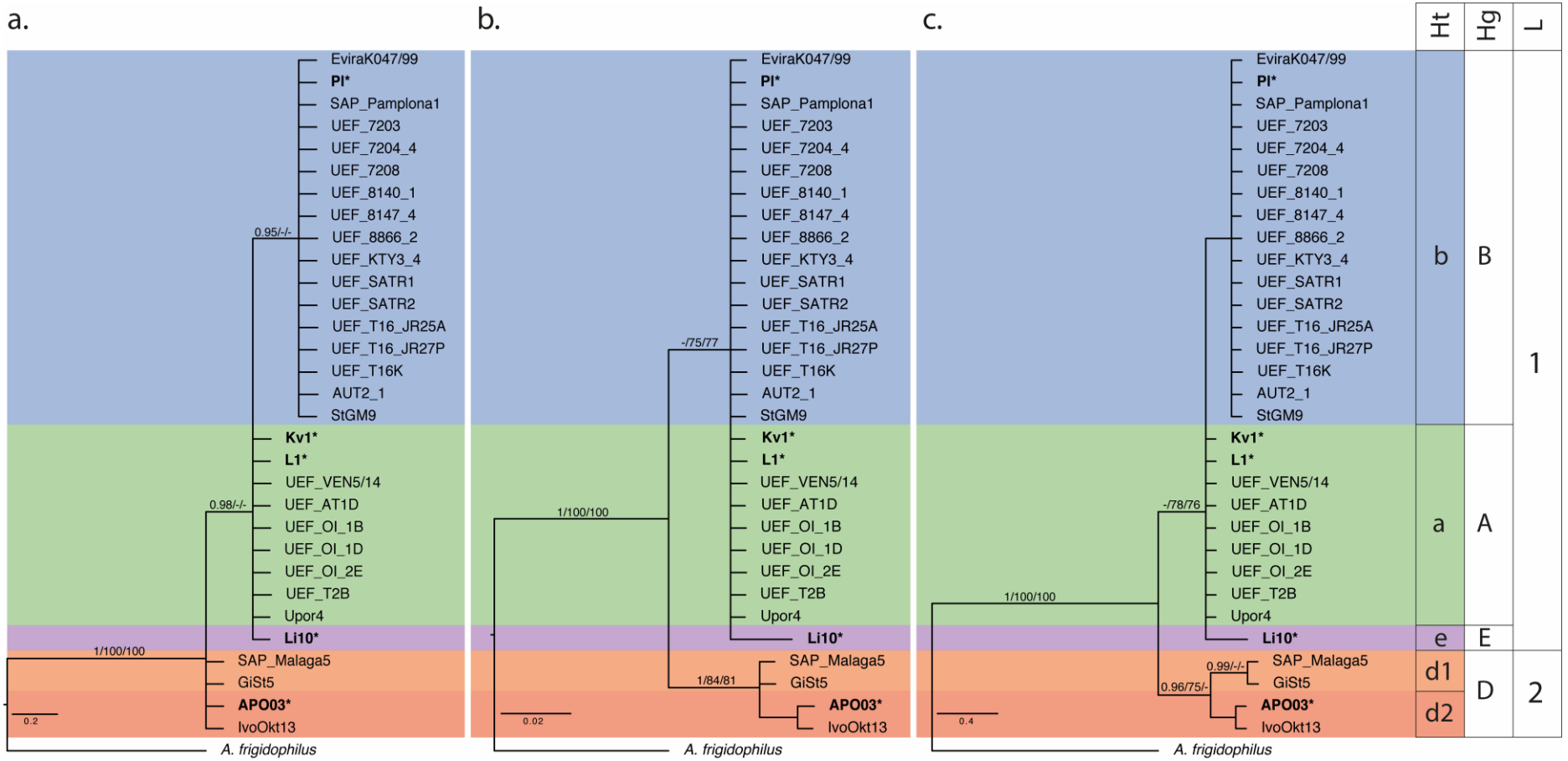

Figure 2 Bayesian inference analyses based on rnnS, rnnL, and concatenated rnnS + rnnL mtDNA sequences. **a.** Bayesian inference analysis was based on rnnS mtDNA sequences. **b.** Bayesian inference analysis was based on rnnL mtDNA sequences. **c.** Bayesian inference analysis was based on concatenated rnnS + rnnL mtDNA sequences. Values above the branches represent the posterior probabilities (>0.95) form Bayesian inference, and bootstrap support (> 75) from Maximum Likelihood and Neighbour Joining analyses. Scales bar for phylogenetic analysis indicates substitutions per site. The original strains used as references and identified in previous studies by RAPD-PCR technique (citas articulos) appear in bold and with a star key (*), correspond to group A: L1*, group B: Pl*, group C: Kv1*, group D: AP03*, group E: Li10*. Ht indicates haplotypes, Hp indicates haplogroups and L indicates lineages.

Table 3 Samples sequenced directly from crayfish tissue.

Sample code	Species	Origin	Year	Crayfish (n)	Agent level[a]	Microsat. genotype[b]	Mitoch. haplotype	Reference
AUT2_1	*A. torrentium*	Feeder of the Steyr Fluss, Austria	2013	7	A7	SSR-B	b	–
StGM9	*A. torrentium*	Schädlbach, Austria	2014	17	A7	SSR-B	b	–
IvoOkt13	*P. fallax* f. *virginalis*	Aquarium-held crayfish, the Netherlands	2014	33	A5	SSR-D	d2	Keller et al.
GiSt5	*A. torrentium*	Schwarzbach, Germany	2013	16	A7	SSR-D	d1	–

[a] The highest agent level (according to Vrålstad et al. (2009)) detected among population, from which the haplotyping was conducted.
[b]Microsatellite genotypes identified using the method published by Grandjean et al. (2014).

DNA of *A. astaci* was extracted from infected crayfish tissue using a CTAB method and the crayfish plague agent levels of them were examined with qPCR according to Vrålstad et al., (2009). The PCR reaction mixture contained 0.4 µM of each primer (Table 2), 0.75X Dream*Taq* Green master mix (Thermo Fisher Scientific), 0.5 U Dream*Taq* DNA polymerase (Thermo Fisher Scientific), 0.17 mM dNTPs, and 2.5 µl of the DNA template. The mixture was filled up to 12.5 µl with PCR-grade water. PCR was carried out on a Primus 96 Plus Thermal Cycler (PEQLAB Biotechnologies GmbH) with the following conditions: 95 °C, 3 min, 30x (95 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s), and 72 °C 10 min. Each run contained a positive control (*A. astaci* DNA) and a blank reaction without a template. The amplification was checked on an agarose gel with EtBr labelling. The PCR products were sequenced on a 3730 DNA Analyzer eight capillary sequencer (Applied Biosystems) by the company Seq IT GmbH & Co. KG (Kaiserslautern, Germany). The sequences were aligned and edited using the program Geneious R7 (Kearse et al., 2012) and then entered to NCBI GenBank database with the access numbers MF150010-MF150017. Microsatellite analyses of infected crayfish samples were conducted similarly as explained in chapter 2.5.

Results

Primer specificity

The two primer pairs developed to amplify the mitochondrial ribosomal rnnS and rnnL subunits (Table 2, Figure 1) produced amplicons with lengths of 512 bp and 435 bp (with primer regions included) from the tested *A. astaci* strains, respectively.

The primer pair AphSSU (Table 2, Figure 1A), developed for the rnnS subunit, also amplified some other aquatic oomycetes, *i.e., Saprolegnia* sp. However, the species were later easily identified based on their nucleotide sequences, as the sequence alignments and BLAST comparisons of the 512 bp rnnS region showed the sequence diversity to be high enough to separate *A. astaci* from closely related species. The differences for rnnS were 12 nucleotides (97.5% similarity) to *A. invadans*, 16 nucleotides (97.0% similarity) to *A. frigidophilus,* and 35 nucleotides (93.0% similarity) to *Saprolegnia* sp. (Table 1). Furthermore, the BLAST search to

NCBI GenBank showed a 93.3% similarity (32 nucleotides) to *Saprolegnia ferax* (AY534144).

The rnnL primers showed high species divergence at the primer regions (Figure 1B). At the 435 bp rnnL region, the diversity was slightly more variable, showing 94.6% similarity (20 nucleotides) to *A. frigidophilus,* but up to 100% similarity was detected against *Saprolegnia* sp. (Table 1), although the similarity to another parasitic *Saprolegnia* having rnnL sequence available, *S. ferax* (AY534144), showed only 116 bp matching region with 99.0% similarity.

Intraspecific diversity, phylogeny, and genetic diversity

For the diversity estimations, a 475 bp and 391 bp fragments of rnnS and rnnL amplicons were included, respectively (Supplementary Figure 1).The three approximations used to reconstruct the phylogenetic relationships (BI, ML, and NJ) (Figure 2) supported the differentiation of two linages for each of the data sets used in this study (rnnS alignment, rnnL alignment; and the concatenated rnnS and rnnL alignment): first lineage belong to the A, B, C, and E RAPD-PCR groups and a second lineage formed by D RAPD-PCR group (Figure 2).

According to the phylogenetic analysis of the rnnS alignment (Figure 2a), the first lineage includes only two subgroups. The first subgroup includes the sequences similar to the strains that belong to the RAPD-PCR groups A, C, and E; and the second subgroup includes the sequences similar to the strains that belong to the RAPD-PCR group B. The second lineage includes the sequences similar to the strains that belong to the RAPD-PCR groups D (Figure 2a). In contrast, the phylogenetic analysis of the rnnL alignment includes two subgroups for the first lineage. The first subgroup is composed by similar sequences to the strains from the RAPD-PCR groups A, B, and C; and the second subgroup by similar sequences to the strains from the RAPD-PCR groups E. The second lineage includes the similar sequences to the strains that belong to the RAPD-PCR groups D (Figure 2b).

As a result of the concatenated rnnS and rnnL sequences, we found four defined haplogroups within these two main lineages, grouping similar strains of the concatenated sequences rnnS and rnnL (Figure 2c). Lineage 1 includes three haplogroups (A-, B-, and E-haplogroup), each represented by different specific haplotypes. The A-haplogroup is formed by strains from RAPD-PCR groups A and C, including only one haplotype, *i.e.,* a-haplotype. The a-haplotype comprises

sequences from RAPD-PCR groups A, *i.e.,* L1 and Upor4, and sequences from RAPD-PCR group C, *i.e.,* Kv1 (Figure 2c). The B-haplogroup is formed by strains from RAPD group B, *i.e.,* Pl, EviraK047/99, SAP-Pamplona, which comprises the unique b-haplotype. The E-haplogroup is formed by strains from RAPD-PCR groups E, including one haplotype, *i.e.,* e-haplotype Li10 (Figure 2c). Lineage 2 includes only one haplogroup (D-haplogroup), confirmed by strains from the RAPD-PCR group D, *i.e.,* AP03 and SAP-Málaga5. This D-haplogroup has two haplotypes, *i.e.,* d1-haplotype (with the genome sequenced sequence AP03) and d2-haplotype (with the sequence SAP-Málaga5) (Figure 2c).

The genetic diversity analysis confirmed and supported the phylogenetic analysis. We found differences between the separated mitochondrial ribosomal rnnS and rnnL subunits (Figure 3, Supplementary Table 1). The amplicons corresponding to the rnnS subunit only registered 2 segregating sites (S) leading to 3 different haplotypes (Figure 3a). The haplotype diversity (Hd) was 0.598; with 0.744 average nucleotide differences (k) and a nucleotide diversity (π) of 0.0015. On the other hand, the amplicons corresponding to the rnnL subunit registered 8 segregating sites (S) leading to 4 different haplotypes (Figure 3b). The haplotype diversity (Hd) was 0.297; with 1.415 average nucleotide differences (k) and a nucleotide diversity (π) of 0.004. However, the concatenated sequences rnnS and rnnL showed a total of 10 segregating sites (S), where 8 of them were parsimony informative, confirming the existence of a total of 5 haplotypes (Figure 3c). The haplotype diversity (Hd) was 0.626; with 2.159 average nucleotide differences (k) and a nucleotide diversity (π) of 0.0025.

Direct haplotyping of infected crayfish samples

The specimens which showed the highest agent levels among the tested populations were selected for *A. astaci* haplotyping (Table 3). The qPCR agent levels of these successfully PCR amplified and sequenced samples varied between A5 and A7 (Table 3). The stone crayfish from the Schwarzbach (Germany) and the marbled crayfish sample (the Netherlands) were infected with the D-haplogroup of *A. astaci*. Here, the haplotype detected from the stone crayfish (GiSt5) was identical with the d2-haplogroup strain SAP-Málaga, while the DNA detected from marbled crayfish (IvoOkt13) grouped together with the d1-haplotype strain AP03 (Figure 2c). The remaining two samples from stone crayfish, *i.e.,* populations from

a feeder of the Steyr River (AUT2_1) and the Schädlbach (StGM9) in Austria, belonged to the b-haplotype.

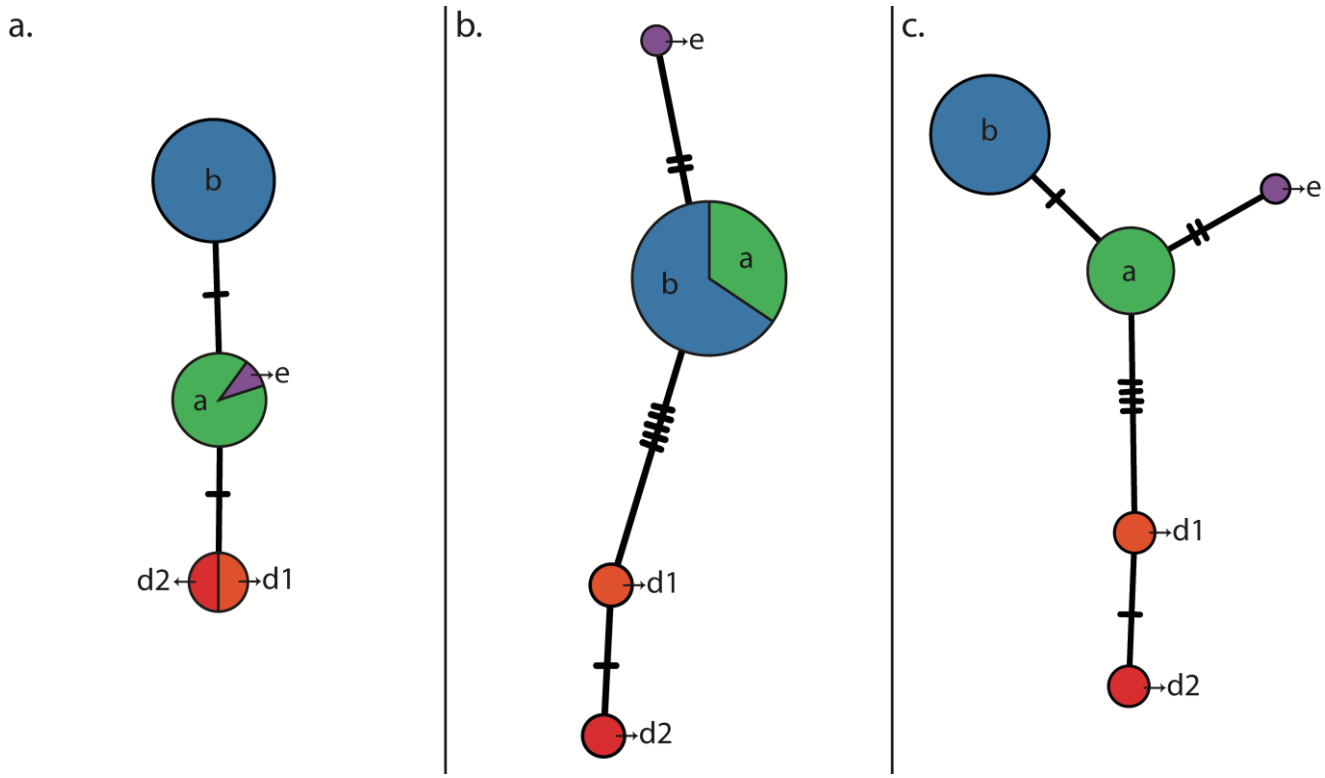

Figure 3 Haplotype network based on rnnS, rnnL, and concatenated rnnS + rnnL mtDNA sequences, generated by statistical parsimony. The area of the circles is proportional to the sequences number. **a.** Haplotype network based on rnnS mtDNA sequences. **b.** Haplotype network based on rnnL mtDNA sequences. **c.** Haplotype network based on concatenated rnnS + rnnL mtDNA sequences. Mutation steps between haplotypes are shown as hatch marks.

Discussion

In this paper, we have described a mitochondrial PCR and sequencing based approach that allows identifying the genetic diversity of *A. astaci* in mix genome samples*, i.e.,* clinical and preserved samples. These mitochondrial markers are stable and reliable and the results are easily repeatable in different laboratories with samples obtained from various crayfish host species revealing moderate to high infection levels.

The target regions rnnS and rnnL were selected based on the full mitochondrial genome of Spanish *A. astaci* strain AP03 (D-haplogroup) (Makkonen et al., 2016) and transcriptomics data of selected Finnish *A. astaci* strains representing the A- and B-haplogroups (Kokko et al., unpublished). The most commonly used mitochondrial barcoding gene (Hebert et al., 2003), cytochrome I oxidase (COI), contained no single nucleotide polymorphisms (SNPs) to distinguish

the different *A. astaci* strains and was therefore left out from further analyses and method development. Furthermore, a very low GC-content of the mtDNA, especially in the intergenic regions (Makkonen et al., 2016), and lacking data from intergenic regions limited the possible target regions for mtDNA haplotyping.

The specificity tests of the two primer pairs showed that the species level resolution of the mitochondrial ribosomal subunits (rnnS and rnnL) was high enough to separate closely related *Aphanomyces* species, *i.e., A. invadans* and *A. frigidophilus,* from *A. astaci*. The rnnL region exhibited higher specificity, although the overall sequence diversity of the whole PCR amplicon was more variable. However, a single *Saprolegnia* sp. showed 100% sequence similarity with *A. astaci*. Therefore, sequencing the rnnL region alone for species differentiation cannot be recommended. The rnnS region also amplified other aquatic oomycetes, such as *A. hypogyna* and *Saprolegnia* sp., but the species could be later separated based on the sequence data.

The method functionality was also compared to the microsatellite method developed by Grandjean et al. (2014). We conducted parallel analyses for studied *A. astaci* strains (Table 1) and infected clinical samples from infected crayfish (Table 3), and they grouped similarly with both methods. In some cases, when the agent levels detected in qPCR (Vrålstad et al., 2009) were mid-A3 or higher, the mtDNA markers showed slightly more sensitive amplification in comparison to the microsatellite markers (data not shown). The difference in the amplification sensitivity was likely caused by different copy number of the mtDNA in comparison to the nuclear DNA analyzed with the microsatellite markers. In future, the application of DNA extraction methods favoring the recovery of circular (mitochondrial) DNA for crayfish tissues will likely further increase the usability of the mtDNA markers in comparison to microsatellites. On the other hand, the diversity of *A. astaci* observed using our mtDNA markers was lower in comparison to the microsatellite markers. Here, the mtDNA markers were highly stable overall, the interpretation of the results from sequencing data was objective. Especially if new or unexpected species and strains will be detected, sequencing can be considered as the best choice to confirm the results in the aquatic environment with unknown microbial spectrum and diversity.

The phylogenetic and genetic diversity analyses of the two concatenated regions of the mitochondrial ribosomal subunits analyzed, *i.e.,* rnnS and rnnL,

based on reference strains of the groups (*i.e.,* L1 for RAPD-PCR group A, Pl for RAPD-PCR group B, Kv1 for RAPD-PCR group C, AP03 for RAPD-PCR group D, and Li10 for RAPD-PCR group E) showed similar results to those obtained by previous studies (Huang et al., 1994; Dieguez-Uribeondo et al., 1995; Kozubíková et al., 2011; Grandjean et al., 2014; Rezinziuc et al., 2014). Thus, these analyses allowed the identification of two main lineages and four haplogroups. Three of these haplogroups (*i.e.,* B-haplogroup, D-haplogroup, and E-haplogroup) corresponded to RAPD-PCR groups B, D, and E, and the fourth-one, *i.e.,* A-haplogroup, comprised the strains from group RAPD-PCR A and C. Each haplogroup was characterized by having a single, unique haplotype, except for the D-haplogroup that possessed two haplotypes, *i.e.,* d1 and d2. Therefore, the combination of both markers led us to clearly separate four haplogroups (A, B, D, and E) with five different haplotypes in them (a, b, d1, d2, and e).

The A-haplogroup and E-haplogroup were closely related haplogroups, although the haplotype network showed two SNPs between the groups of sequences. The number of isolates available from the E-haplogroup is currently limited (Kozubíková et al., 2011; Kozubíková-Balcarová et al., 2013). Moreover, the A-haplogroup comprise sequences identical to the strain of reference for RAPD groups A (L1 strain) and C (Kv1strain). The haplotype network generated no differences between these groups. Although this fact can be important and should be taken into account in the investigations, only one strain of *A. astaci* belonging to RAPD-PCR group C has been isolated so far (Huang et al., 1994; Kenneth Söderhäll, personal communication). Therefore, the limitation of strains corresponding to the RAPD-PCR groups C and E (Kozubíková et al., 2011) could have hindered the estimation of the real diversity within these haplogroups.

The *A. astaci* isolates for the A-haplogroup and E-haplogroup were obtained from several host species (Table 1) from Eastern and Northern USA, *i.e.,* genus *Orconectes* and genus *Pacifastacus*. However, Eastern and Northern parts of USA have broad and variable crayfish species diversities (Holdich, 2002). The host diversity likely increases also the pathogen strain diversity and accelerates the development of new lineages, haplogroups, and haplotypes, as a conseque nce of host-parasite coevolution (Jussila et al., 2015). Moreover, the translocation of species within North America occurs (Larson et al., 2012). For example, three *P. leniusculus* subspecies have been introduced in Lake Tahoe (John Umek, personal communication). The results observed here may be an indication of those crayfish

translocations. Furthermore, it can be assumed that also mixed crayfish species populations have been created with this kind of translocations. Although no evidence of this has been shown so far, these translocations could have allowed the pathogen host jumps, making possible the exchange of genetic groups (*e.g.,* Jussila et al., 2015). However, to investigate this possibility, further isolations from North American crayfish should be performed. In this kind of cases, using microsatellites would not be helpful, as distinguishing between a truly heterozygous locus or a combination of two different strains is impossible (Maguire et al., 2016). On the other hand, the mixture of pathogen strains might already be reality in Europe since several non-indigenous crayfish species, several members of the genus *Orconectes* as an example, have been introduced into Europe (Souty-Grosset et al., 2006), especially into Central European water bodies (Schrimpf et al., 2013; Panteleit et al., 2017). The oldest known *A. astaci* lineage in Europe, *i.e.,* RAPD-PCR group A was the basis of the a-haplotype in this study. If the RAPD-PCR group A of *A. astaci*, with its so far unknown original crayfish host, first arrived in Europe in the ballast water of an intercontinental ship, as speculated by Alderman (1996), the East coast of North America was the likely origin for the transport. Therefore, our results seem to be in line with these speculations.

The B-haplotype, commonly detected all over Europe due to signal crayfish *P. leniusculus* introductions, was traced back to Lake Tahoe, California (Souty-Grosset et al., 2006). Signal crayfish were first introduced to Europe, in particular, to Sweden (Abrahamsson, 1969) and Finland (Westman, 2000), from Lake Hennessey and from the Sacramento River, California. Based on this study, either the signal crayfish from these two locations were carrying similar strains belonging to the same *A. astaci* groups, or the stocking success of one of these populations was limited creating a founder effect, as the b-haplotype show high homogeneity in both nuclear markers (Diéguez-Uribeondo et al., 2009; Makkonen et al., 2011, 2012a) and also in mitochondrial markers used in this study.

The D-haplogroup seems to be introduced to Southern Europe (Spain) by introductions of *P. clarkii* (Rezinciuc et al., 2014). However, only two strains were tested in this study. The two Spanish *A. astaci* strains, AP03 and SAP-Málaga, split the D-haplogroup into two haplotypes, d1 and d2, respectively. Variation in this group was also observed with the microsatellite markers (unpublished data), where the first Pc-lineage has been typically connected to *P. clarkii* and the second

one to marbled crayfish *P. fallax* f. *virginalis,* a parthenogenetic species commonly available in the aquarium trade (*e.g*., Scholtz et al., 2003; Panteleit et al., 2017). In this study, the D-haplogroup mtDNA markers, which were detected from an infected crayfish tissue (Table 3), were exhibiting a similar grouping as the strains AP03 and SAP-Málaga, showing that both haplotypes are present in Spain and Germany (Figure 2).

Conclusion

The application of mitochondrial markers enables now the direct identification in clinical and preserved samples of the main *A. astaci* haplogroups responsible for crayfish plague outbreaks. The method provides an opportunity to characterize the diversity of *A. astaci* strains, facilitating investigations on disease history and its epidemiology. In this paper, we have described the technique's possibilities for a direct identification of *A. astaci*, responsible of the crayfish plague, which is still discovered in novel regions causing devastating outbreaks (*e.g.,* Peiró et al., 2016). We propose these mitochondrial rnnS and rnnL markers as an important tool to track the *A. astaci* diversity hindered among the original host species, *i.e.,* North American crayfish species, and in the future, it may help to define if the original distributional area of *A. astaci* groups is a reflection to its original host species distribution and North America. These results further improve our understanding of *A. astaci* evolution and assist in the protection and conservation of the native European crayfish.

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Appendices III

Table III.1 Haplotypes found in *Aphanomyces astaci* sequences for the mitochondrial ribosomal rnnS and rnnL subunits. The first line shows the relative position of rnnS and rnnL subunits and the second line the SNPs found in the concatenated sequence (rnnS + rnnL) of 866 pb. Columns 2 and 3 refer to the SNP position in the 512 pb sequence analysed for the rnnS mtDNA subunit. Columns 4-11 refer to the SNP position in the 355 pb sequence analysed for the rnnL mtDNA subunit. Bold letters indicate the transitions and the transversions at each relative position.

Haplotype	**148**	**367**	**546**	**570**	**582**	**652**	**661**	**663**	**691**	**841**	**849**
a	T	A	G	T	G	**T**	A	C	T	G	C
b	**C**	A	G	T	G	-	A	C	T	G	C
d1	T	**G**	G	**A**	**A**	**T**	**T**	**A**	T	**A**	C
d2	T	**G**	**A**	**A**	**A**	-	**T**	**A**	T	**A**	C
e	T	A	G	T	G	-	A	C	**C**	G	**A**

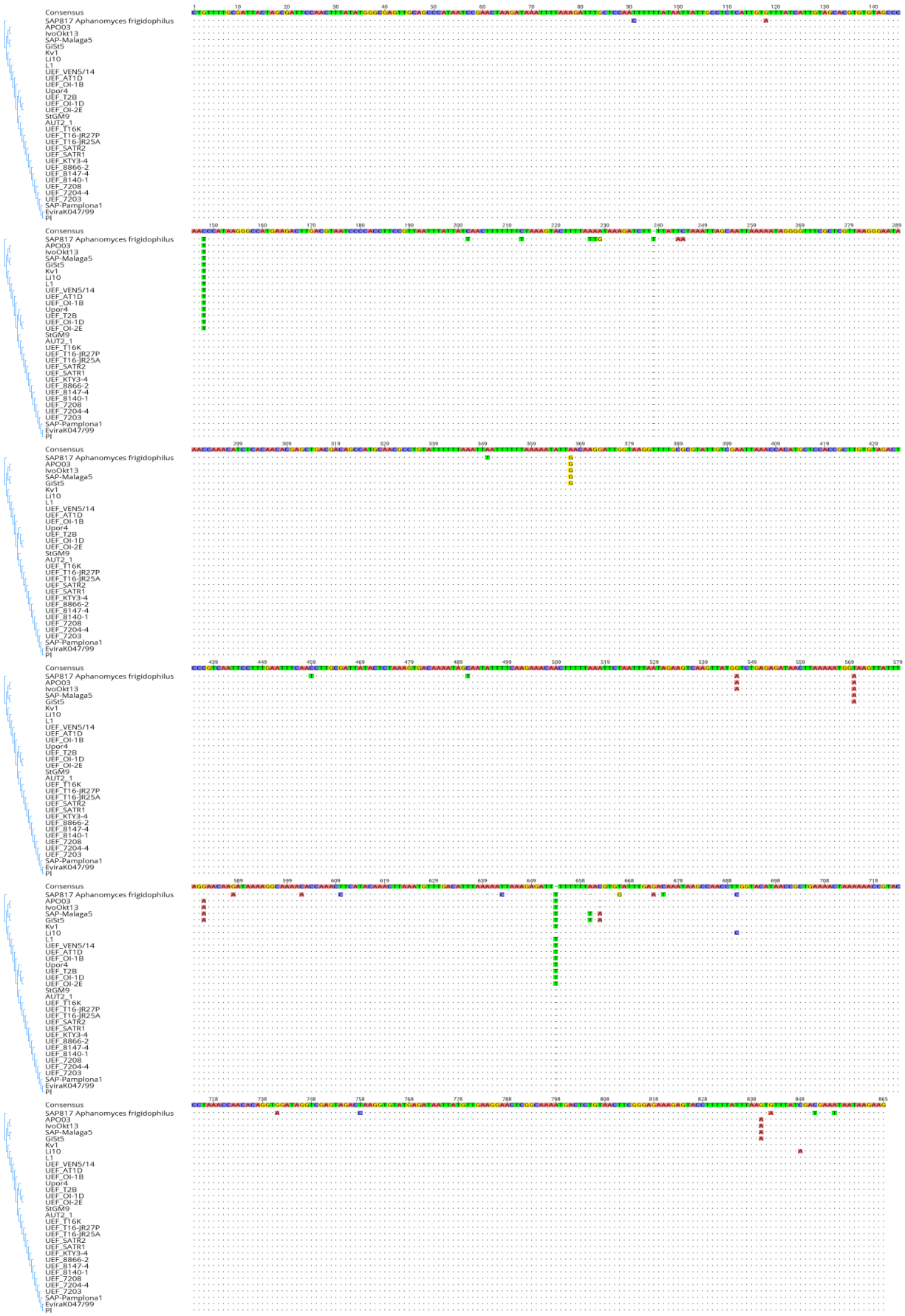

Figure III.1 The multiple alignment of the rnnS (1-475 bp) and rnnL (476-867 bp) regions.

12.4 Appendix IV

Hidden sites in the distribution of the crayfish plague pathogen *Aphanomyces astaci* in Eastern Europe: relicts of genetic groups from older outbreaks?

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Abstract

The crayfish plague agent *Aphanomyces astaci* is one of the world's most threatening invasive species. Originally from North America, the pathogen is being imported alongside American crayfish species, which are used for various purposes. In this study, we investigated the marginal, currently known distribution area of the pathogen in Eastern Europe by sampling narrow-clawed crayfish (*Astacus leptodactylus)* and spiny-cheek crayfish (*Orconectes limosus*) populations. In addition, using specific real-time PCR, we tested several marine decapod species, which also occur in brackish waters of the Danube at the West coast of the Black Sea and the Dniester River basin. By sequencing the nuclear chitinase gene, mitochondrial rnnS/rnnL DNA and by genotyping using microsatellite markers, we identified the *A. astaci* haplogroups of highly infected specimens. The *A. astaci* DNA was detected in 9% of the investigated *A. leptodactylus* samples, both in invaded and non-invaded sectors, and in 8% of the studied *O. limosus* samples. None of the marine decapods tested positive for *A. astaci*. The results revealed that narrow-clawed crayfish from the Dniester River carried the *A. astaci* B-haplogroup, while *A. astaci* from the Danube Delta belonged to the A- and B-haplogroups. In the invaded sector of the Danube, we also identified the A-haplogroup. Microsatellite analysis revealed a genotype identical to the genotype Up. It might be that some of the detected *A. astaci* haplogroups are relics from older outbreaks in the late 19th century, which may have persisted as a chronic infection for several decades in crayfish populations.

Keywords: Danube; Dniester River; invasive species; narrow-clawed crayfish; haplotypes; real-time PCR

Introduction

Usually, the relationship between a parasite and its host is in a natural equilibrium, which ensures the survival of both species under certain conditions (Anderson and May 1982). This relationship is influenced by the environment, the host immunity, and the parasite's virulence. If both the parasite and the host are translocated to a new environment, the host may benefit from the parasite's ability to also infect and weaken competitors of the original host (García-Ramos et al. 2015), as these may lack an evolutionary adaptation to the parasite. This may significantly increase the dominance of a natural host towards newly infected competitors (Strauss et al. 2012). The last century came with one of the most notable disasters for crayfish wild populations in Europe with episodes of mass mortalities driven by the crayfish plague (Alderman 1996; Lowe et al. 2004), a disease caused by the invasive oomycete *Aphanomyces astaci* SCHIKORA, 1903. While the pathogen's North American species specific origin is well known, as revealed by Unestam (1972) and by molecular analysis (Huang et al. 1994; Diéguez-Uribeondo et al. 1995; Makkonen et al. 2012a), its past and current distribution in Europe remains to be studied. This is especially true in boundary areas or regions not invaded by North-American crayfish species.

Aphanomyces astaci has coevolved with North American crayfish species (Unestam 1972; Söderhäll and Cerenius 1992). As a result, American crayfish do not usually die due to an infection with *A. astaci* (Cerenius et al. 2003). On the contrary, European crayfish species are highly susceptible and an infection usually leads to a quick death (Unestam 1969a; Alderman et al. 1987; Cerenius et al. 2009). Recently, it was shown that other freshwater crustaceans, specifically, the crab species *Eriocheir sinensis* MILNE EDWARDS, 1853 and *Potamon potamios* (OLIVIER, 1804) (Schrimpf et al. 2014; Svoboda et al. 2014) can serve as vectors for *A. astaci*.

Due to its virulence and devastating effects on indigenous crayfish species all over the world, *A. astaci* was classified among the world's 100 worst invasive alien species (Lowe et al. 2004). The first report regarding crayfish plague in Europe originates from the late 1850s, before the first documented introductions of North American crayfish species (Cornalia 1860). Alderman (1996) estimated the distribution of this disease based on the records of mass mortalities associating them with crayfish plague outbreaks. Repeated outbreaks were noted in the late

19th and early 20th centuries across substantial parts of Europe including the West, Central and East European to Eurasian river basins (Alderman 1996).

Based on RAPD-PCR analysis, five *A. astaci* genetic groups have been identified to date, which can be assigned to different host species according to their origins. Due to the multiple introductions of non-indigenous crayfish species and aggressive successful spreading of several species across Europe (Kouba et al. 2014), the current distribution of *A. astaci* is a mosaic of different genetic groups (Svoboda et al. 2017). RAPD-groups B and C were found on signal crayfish, *Pacifastacus leniusculus* (DANA, 1852) (Huang et al. 1994), group D on red swamp crayfish, *Procambarus clarkii* (GIRARD, 1852) (Diéguez-Uribeondo et al. 1995) and group E was identified on spiny-cheek crayfish, *Orconectes limosus* (RAFINESQUE, 1817) (Kozubíková et al. 2011). In contrast to these RAPD-groups, the original host of group A that was isolated from an indigenous European crayfish, i.e., *Astacus astacus* (LINNAEUS, 1758) and *Astacus leptodactylus* (ESCHSCHOLTZ, 1823), is still unknown (Souty-Grosset et al. 2006; Makkonen et al. 2012a; Viljamaa-Dirks et al. 2013). These RAPD-groups are often referred to as As, PsI, PsII, Pc, and Or. However, in this manuscript we will use the original lettering, A-E. The most recent development of primers targeting the mitochondrial small and large ribosomal subunits (rnnS and rnnL) divided the known RAPD-groups into four haplogroups (A, B, D and E) (Makkonen et al. 2018). The A-haplogroup contains the *A. astaci* RADP-PCR groups A and C, the B-haplogroup the strains from *A. astaci* RADP-PCR groups B, the D-haplogroup contains the d1 and d2-haplotypes from *A. astaci* RADP-PCR groups D, and the E-haplotype contains the strains from *A. astaci* RADP-PCR group E.

Moreover, differences in virulence were measured between groups (Diéguez-Uribeondo et al. 1995; Makkonen et al. 2012b; Jussila et al. 2013; Viljamaa-Dirks et al. 2016). In laboratory experiments, group B isolates caused the rapid and total mortality of noble crayfish *A. astacus*, while group A was less virulent in general (Makkonen et al. 2012b; Becking et al. 2015). Although European crayfish species are generally highly susceptible and usually die within a few days after infection, evidences for an evolutionary adaption of host and pathogen seem to be the reason for the survival of some crayfish populations. This has been shown for *A. astacus* (Jussila et al. 2011; Viljamaa-Dirks et al. 2011; Makkonen et al. 2012b; Viljamaa-Dirks et al. 2013) and *Austropotamobius pallipes* (LEREBOULLET, 1858) (Martin-Torrijos et al. 2017). Moreover, pathogen persistence in indigenous European

crayfish species has also been described for the narrow-clawed crayfish *A. leptodactylus* (ESCHSCHOLTZ, 1823) (Kokko et al. 2012; Svoboda et al. 2012; Schrimpf et al. 2012) and in the stone crayfish *Austropotamobius torrentium* (SCHRANK, 1803) (Kušar et al. 2013; Jussila et al. 2017).

Additionally, another technique that uses microsatellite markers allows the further identification of known genotypes of *A. astaci* not only from pure cultures, but also from infected crayfish tissue, which can be assigned to the different genetic groups. One group can consist of different genotypes, e.g., genetic group A, which contains at least the microsatellite genotypes A_1 and A_2 (Grandjean et al. 2014). The finer discrimination makes genotyping an important tool for the characterization of *A. astaci*, and may, in some cases, allow a reconstruction of the origin of one specific crayfish plague occurrence.

Range extensions of American crayfish species pose a permanent threat to indigenous crayfish (Holdich et al. 2009), because they can act as chronic *A. astaci* reservoirs (Kozubíková et al. 2009). One of the most invasive crayfish species and also carrier of *A. astaci* is *O. limosus*. This species has been documented in Europe since the 1890s (Souty-Grosset et al. 2006; Filipová et al. 2011). The reasons for the high invasive potential and spreading of *O. limosus* in Europe are higher fecundity and faster egg development in comparison to European species (Kozák et al. 2006; Souty-Grosset et al. 2006; Pârvulescu et al. 2015), as well as a wide habitat range and tolerance towards unfavorable environmental conditions, e.g., drought, cold, and low water quality (Souty-Grosset et al. 2006; Holdich and Black 2007). Although coexistence with European crayfish species, i.e., *A. astacus*, *A. leptodactylus* (Schrimpf et al. 2013a), white-clawed crayfish *A. pallipes* (Caprioli et al. 2013) and *A. torrentium* (Kušar et al. 2013) was noted, *O. limosus* mainly replaced indigenous crayfish species in Europe (Souty-Grosset et al. 2006).

For the first time, *A. astaci* was detected in the Romanian Danube River in 2011, specifically, in *A. leptodactylus* populations coexisting with the invasive *O. limosus*. Surprisingly, the pathogen was also confirmed in *A. leptodactylus* populations around 70 km downstream of the *O. limosus* invasion front (Pârvulescu et al. 2012). In 2012, the pathogen was also detected in one of the three branches of the Danube Delta, the Chilia Channel (Schrimpf et al. 2012). It is still unknown whether marine decapod species, which are highly abundant in the brackish waters of the Danube Delta (Petrescu et al. 2010), can also act as vectors of *A. astaci*.

The Danube Delta is a highly protected area where crayfish mass mortalities have not been reported to date. Due to the absence of *O. limosus* or other North American species in the region, the origin of *A. astaci* remained unknown. This study aims to extend the knowledge of the *A. astaci* distribution and genetic group assignment by investigating the estimated eastern range of the pathogen's distribution area. In addition to crayfish species, different marine decapod species were tested for a crayfish plague infection. Furthermore, in the case of *A. astaci* occurrence, we intended to identify the genetic group of crayfish plague pathogen by means of sequence analyses as well as microsatellite analysis.

Methods

Crayfish and marine decapods sampling

In 2015, we collected samples from indigenous *A. leptodactylus* populations in the Danube River as well as invaded populations of *A. leptodactylus* mixed with non-indigenous *O. limosus*. (Fig. 1; Table 1). In addition to the existing material from the Chilia Channel in the Danube Delta, 58 samples from the lake complex Roşu - Puiu - Lumina in the Danube Delta were collected in spring 2016. Outside of the Danube catchment, 104 *A. leptodactylus* were sampled in the Dniester River, Republic of Moldova, near Dubăsari in late spring between 2013 and 2015. Crayfish were captured by trapping or by fishing nets. All the samples consisted of soft abdominal cuticle, walking legs, telson and parts of the uropods stored in 96% ethanol. In addition to the newly collected and tested samples, we also include samples from an earlier publication, including 37 *A. leptodactylus* specimens from the Danube Delta that were tested for an infection with *A. astaci* (Schrimpf et al. 2012).

Table 1 The sampling sites and analyzed individuals for the qPCR detection of *A. astaci* DNA in populations according to the invasion status, NICS = non-indigenous crayfish species. The sites where sequencing and/or genotyping were possible are marked with an asterisk (*).

Sampling site (hydrographical basin)	GPS location (N/E)	NICS invasion status	No. of analyzed individuals	
			A. leptodactylus	*O. limosus*
Stara Palanka (Danube, Serbia)	44°49′37″/21°20′50″	*O. limosus*	2*	0
Dubova (Danube)	44°37′22″/22°16′23″	*O. limosus* since 2012 (Pârvulescu et al., 2012)	24	20
Drobeta-Turnu Severin (Danube)	44°37′17″/22°40′41″	Invasion front of *O. limosus* in 2015 (Pârvulescu et al., 2015)	17	18
Total specimens analyzed in invaded Danube			43	38
Calafat (Danube)	43°59′54″/22°56′02″	No NICS reported so far	8	-
Bechet (Danube)	43°44′48″/23°56′50″	No NICS reported so far	23	-
Giurgiu (Danube)	43°52′04″/25°57′48″	No NICS reported so far	30	-
Total specimens analyzed in non-invaded Danube			61	0
Roşu - Puiu - Lumina lakes (Danube Delta)	45°04′46″/29°31′49″	No NICS reported so far	58*	-
Dubăsari (Dniester River)	47°17′16″/29°08′19″	No NICS reported so far for the whole basin	104*	-

In order to test the hypothesis whether marine decapods, which can also appear in brackish waters, act as crayfish plague vectors, seven of the most common species were sampled at the western coast of the Black Sea, near Agigea (44°04′32″N/28°46′37″E) and Năvodari (44°18′48″N/28°49′27″E) (Fig. 1, Table 2). The samples included nine individuals of the rockpool shrimp, *Palaemon elegans* RATHKE, 1837, eight individuals of the jaguar round crab, *Xantho poressa* (OLIVI, 1792), the marbled crab, *Pachygrapsus marmoratus* (FABRICIUS, 1787), and the flying crab, *Liocarcinus holsatus* (FABRICIUS, 1798). Further, two individuals of the bristly crab, *Pilumnus hirtellus* (LINNAEUS, 1761), and one specimen of each the dwarf crab, *Rhithropanopeus harrisii* (GOULD, 1841) and the porcelain crab, *Pisidia longimana* (RISSO, 1816) were captured by hand in the summer months between 2013 and 2014. Pieces of the ventral carapax cuticle, abdomen and walking legs were dissected and stored in 96% ethanol.

Table 2 The marine decapods species collected from the West coast of the Black Sea, analysed for presence of *A. astaci* DNA using qPCR. GPS location for sampling sites: Constanta (44°14′18″/28°44′27″), Mangalia (43°48′43″/28°37′38″).

Species	Site locality	No. of analyzed individuals
Palaemon elegans, rockpool shrimp	Mangalia	9
Xantho poressa, jaguar round crab	Mangalia, Constanta	8
Pachygrapsus marmoratus, marbled crab	Mangalia, Constanta	8
Liocarcinus holsatus, flying crab	Constanta	8
Pilumnus hirtellus, bristly crab	Constanta	2
Rhithropanopeus harrisii, dwarf crab	Mangalia	1
Pisidia longimana, porcelain crab	Mangalia	1

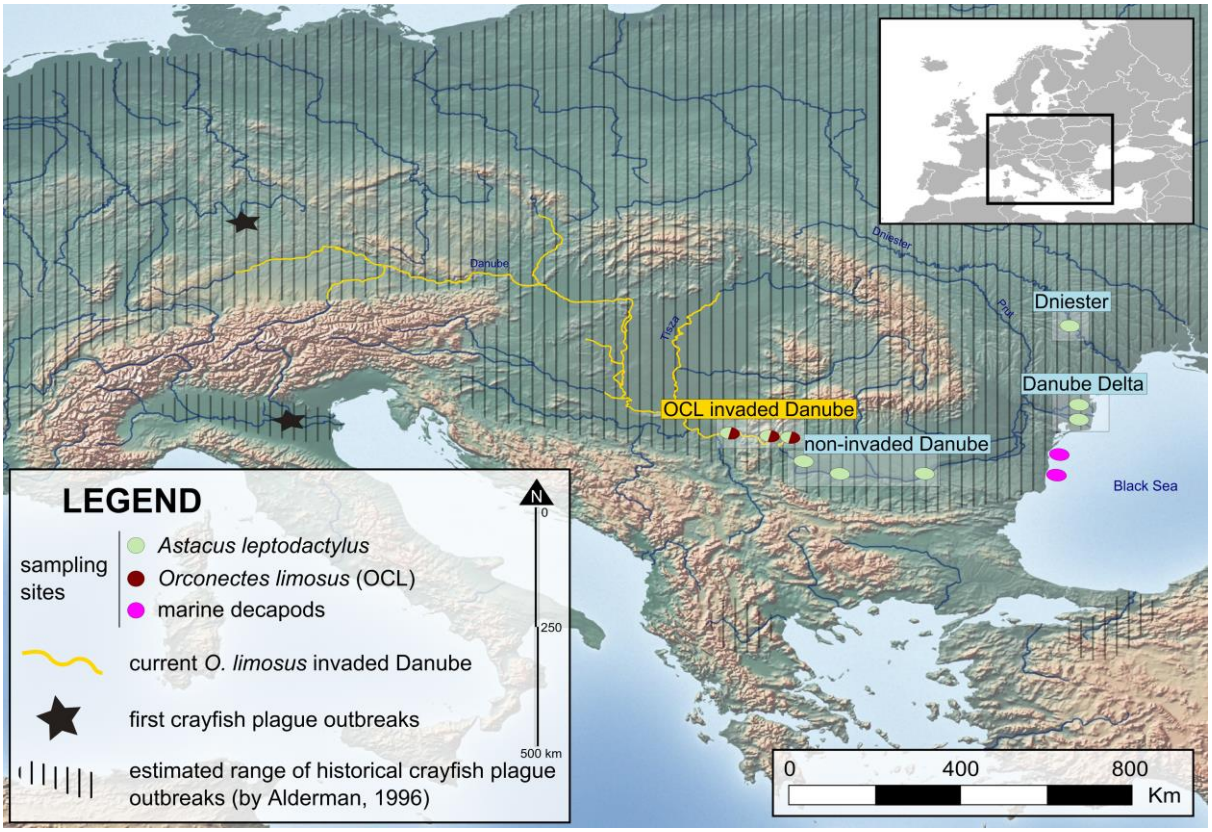

Fig. 1 The map shows crayfish and marine decapods sampling sites across the Lower Danube in the context of the last known invasion status of *O. limosus*. The map also presents the historical coverage area of crayfish plague as estimated by Alderman (1996).

Real-time PCR

DNA was extracted using a CTAB-method according to Vrålstad et al. (2009). To assess the infection status of crayfish and marine decapods, we conducted a TaqMan® minor groove binder (MGB) qPCR, targeting the ITS region, as described in Vrålstad et al. (2009), with some modifications (Schrimpf et al. 2013b). Infection status and agent levels were defined according to Vrålstad et al. (2009).

The proportions of infected crayfish in various river sectors were compared using Fisher exact tests. The 95% confidence intervals of infected specimens were calculated using the software RStudio V.1.0.44 (R Core Team 2016) with the package epiR V.0.9 (Stevenson et al. 2016).

Sequence analyses

If possible, the haplotype of *A. astaci* was identified for infected samples with high agent levels of A3, i.e., a PFU of at least 800, and above using sequence analysis of three different genes: the nuclear chitinase gene, and the mitochondrial ribosomal rnnS and rnnL subunits. The rnnS/rnnL mitochondrial haplogroup A contains RAPD-groups A and C. Use of the chitinase sequences allows for the discrimination of the two RAPD-groups A and C. First, we sequenced a 370 base pair (bp) long DNA fragment of the nuclear chitinase gene according to Makkonen et al. (2012a), with some modifications. The modifications were as follows: we used 5x PCR buffer, 0.025 U TaqMan® Taq (both Promega, Mannheim, Germany), 2 mM $MgCl_2$, 0.2 mM dNTP mix (both Fermentas, St. Leon-Rot, Germany), 0.2 µM primers AAChiF and AAChiR and added 3 µl DNA template for a final volume of 25 µl. The Two other genes were the mitochondrial ribosomal rnnS (512 bp) and rnnL (435 bp) subunits, according to Makkonen et al. (2018). Primer AphSSUF (5'-GGGCGGTGTGTACAAAGTCT-3'), AphSSUR (5'-AGCACTCCGCCTGAAGAGTA-3'), AphLSUF (5'AGGCGAAAGCTTACTATGATGG-3'), and AphLSUR (5'-CCAATTCTGTGCCACCTTCT-3') were used in the following reactions: The PCR reaction mixture contained 0.4 µM of each primer, 0.75X Dream*Taq* Green master mix (Thermo Fisher Scientific), 0.5 U Dream*Taq* DNA polymerase (Thermo Fisher Scientific), 0.17 mM dNTPs, and 2.5 µl of the DNA template. The mixture was filled up to 12.5 µl with PCR-grade water. PCR was carried out on a Primus 96 Plus Thermal Cycler (PEQLAB Biotechnologies GmbH) with the following conditions: 95°C, 3 min, 30x (95°C, 30 s; 60°C, 30 s; 72°C, 30 s), and 72°C 10 min. Each run contained a positive control (*A. astaci* DNA) and a blank reaction without a template. The amplification was checked on an agarose gel with EtBr labelling. Sequence analysis was also conducted for one sample with a high agent level (A6) from an earlier study (Schrimpf et al. 2012), because the haplotype of this highly infected sample had previously not been identified. The positive control consisted of DNA isolated from a pure culture of *A. astaci* strain UEF_SATR1, RAPD PCR group B and the negative control of pure reaction master mix. PCR products were sequenced on a 3730 DNA Analyzer eight capillary sequencer (Applied Biosystems,

MA, USA) by the company Seq IT GmbH & Co.KG (Kaiserslautern, Germany). All sequences were compared with pure cultured reference strains of *A. astaci*. The reference strains of pure culture isolates for RADP-PCR group A (UEF-AT1D, Jussila et al. 2017) and B (UEF-SATR1, Jussila et al. 2013) were obtained from the University of Eastern Finland, Kuopio campus, Finland. The reference strain Kv1 (RADP-PCR group C) was from Sweden (Huang et al. 1994) and reference strain AP03 (RADP-PCR group D) from Spain (Rezinciuc et al. 2014). Reference sequences of the RADP-PCR group E were generated from pure culture samples of the strains Li05 and Li08, isolated from *A. astacus* which had inhabited the Litavka stream (Kozubíková-Balcarová et al. 2013). The sequences were aligned and edited with the program Geneious R7 (http://www.geneious.com, Kearse et al. 2012).

Microsatellite analysis

We conducted microsatellite analysis using nine co-dominant microsatellite markers according to Grandjean et al. (2014) for samples with high agent levels of A3, i.e., a PFU of at least 800, and above. Only one sample was successfully analyzed with microsatellite analysis, sample DC18 from the Danube Delta. The other samples most likely did not contain enough *A. astaci* DNA for successful Microsatellite analysis. Amplification was done in two batches using the QIAGEN Multiplex PCR Kit (QIAGEN, Netherlands) according to the manufacturer's instructions, with 0.25 µl each of the labeled primers added Aast4, Aast6, Aast7, and Aast14 for Batch A, and Aast2 Aast9, Aast10, Aast12, and Aast13 for Batch B. Then, 1 µl DNA template was added for a final volume of 5 µl and 5.5 µl, respectively. PCR grade water was used as a negative control. PCR conditions were as follows: 95°C for 2 min, 35 cycles of 95°C for 30 s, 54°C for 90 s and 72°C for 60 s. The final elongation step was at 72°C for 5 min. PCR conditions were the same for both batches and PCR was performed on a Primus 96 Plus Thermal Cycler (PEQLAB Biotechnologie GmbH, Erlangen, Germany)

For the fragment analysis, 0.5 µl PCR product was mixed with 27.2 µl SLS buffer and 0.3 µl 400 bp standard (Beckman Coulter, Brea CA, United States). Analyses were conducted on a Beckman Coulter CEQ 8000 eight capillary sequencer. Alleles were scored using the GenMarker software (version 1.95, SoftGenetics LLC) and compared to reference genotypes from pure cultures. The reference genotypes were created from the same reference cultures as described

in section 2.3 and additionally a mixed DNA sample (Up4) was used as a reference for genotype Up. This sample was from a crayfish plague outbreak in the Uporsky Brook, Czech Republic.

Results

Overall, 25 out of the 266 (9%) *A. leptodactylus* samples tested positive via real-time PCR for *A. astaci* DNA (Table 3). Agent levels of the infected samples ranged from A2 to A6. Of the 38 *O. limosus* samples, 3 (8%) tested positive, all with agent level A2. Analysis revealed that 6 of 104 (6%) *A. leptodactylus* from the Dniester River were positive, showing very low (A2) to moderate (A4) levels of *A. astaci* DNA. The *A. astaci* infection prevalence among tested *A. leptodactylus* from the invaded part of the Danube was 9 out of 43 (21%), whereas no *A. astaci* DNA was detected in the individuals tested from the non-invaded sector of the Danube River. In the Danube Delta, one sample had very high DNA amounts (A6) of *A. astaci*. In the Delta, 10 out of the 58 (17%) tested samples contained at

least low (A2) amounts of *A.* astaci DNA. The Fisher tests showed that the proportions of infected crayfish were not significantly different for *A. leptodactylus* and *O. limosus* in the invaded part of the Danube ($p = 0.125$). There were no statistically significant differences between proportions of infected *A. leptodactylus* in the invaded Danube and the Danube Delta ($p = 0.797$). Nevertheless, the proportion of infected *A. leptodactylus* was significantly greater in the invaded part of the Danube and in the Danube Delta compared to Dniester River ($p = 0.013$ and $p = 0.027$, respectively). No DNA of *A. astaci* could be detected in any marine decapod sample. Finally, no significant differences ($p = 0.205$) were found comparing the proportions of infected *A. leptodactylus* in the Danube Delta in the present study to those recorded in the previous study by Schrimpf et al. (2012).

Sequence analyses of three genes (chitinase, rnnL and rnnS) were successful for four samples: the sample with agent level A6 (Sample ID DC18) collected from the Chilia Channel in 2012, the northern branch of the Danube in the Delta; two samples from the invaded part of the Danube River in Serbia with agent level A3 (Sample IDs ASLSRB 35 and 58); and one sample collected from the lake complex in the southern Danube Delta in 2015 with agent level A6 (Sample ID DD108) (GenBank accession numbers: MF740801–MF740809 and

MF774441–MF774443). Two different *A. astaci* haplogroups were detected. The A-haplogroup was detected in the samples from the Chilia Channel in the Danube Delta (DC18) and in the invaded part of the Danube River (ASLSRB35/58) and the B-haplogroup from samples collected from the Roşu - Puiu - Lumina lake complex in the Danube Delta (DD108) (Table 4). The four chitinase sequences were identical to each other. They corresponded to those of RAPD-groups B, C and E. These groups cannot be distinguished by the chitinase gene alone. Combining the results from the chitinase gene and the mitochondrial rnnL and rnnS genes allowed the haplogroup A samples, from the Danube Delta and the Danube River in Serbia, to be more closely identified as RAPD-group C, since the chitinase sequences were identical to those of RAPD-group C (Table 4). One of the *A. leptodactylus* samples with agent level A4 collected from the Dniester River was successfully sequenced only for the mitochondrial rnnL gene. This allowed the sample to be assigned to the *A. astaci* B-haplogroup (Sample ID LP_AlD26).

Microsatellite analysis was only successful for the sample with agent level A6 from the Chilia Channel in the Danube Delta collected already in 2012 (DC18). For this sample, loci Aast 2, Aast 7, and Aast 10 were heterozygous, while the remaining 6 loci showed homozygosity for alleles (Appendix A). The allele pattern was identical to the Up-genotype found in the Czech Republic (Grandjean et al. 2014).

Table 3 Number of tested specimen of *Astacus leptodactylus* and *Orconectes limosus* from the Danube River, Danube Delta and the River Dniester, respectively, associated agent levels (A0 (0 PFU) and A1 (PFU_{obs} < 5 PFU) are considered uninfected and A2 (5 PFU ≤ PFU_{obs} < 50 PFU), A3 (50 PFU ≤ PFU_{obs} < 10^3 PFU), A4 (10^3 PFU ≤ PFU_{obs} < 10^4 PFU), A5 (10^4 PFU ≤ PFU_{obs} < 10^5 PFU) and A6 (10^5 PFU ≤ PFU_{obs} < 10^6 PFU) infected), the absolute (n infected) as well as the relative (% infected) number of infected specimen and the 95% confidence intervals (95% CI) are shown. Highly infected specimens of Schrimpf et al. (2012) were used in this study for sequence analysis and genotyping.

Sampling site	n	Agent level							n infected	% infected	95% CI
		A0	A1	A2	A3	A4	A5	A6			
Danube channel, invaded sector (*A. leptodactylus*)	43	11	23	6	3	0	0	0	9	21	10 – 36
Danube channel non-invaded sector (*A. leptodactylus*)	61	51	10	0	0	0	0	0	0	0	0 – 6
Danube channel (*O. limosus*)	38	19	16	3	0	0	0	0	3	8	2 – 21
Danube Delta (*A. leptodactylus*)	58	25	23	3	6	0	0	1	10	17	9 – 29
Dniester River (*A. leptodactylus*)	104	94	4	2	1	3	0	0	6	6	2 – 12
Sum of all *A. leptodactylus*	266	181	60	11	10	3	0	1	25	9	6 – 14
Sum of all *O. limosus*	38	19	16	3	0	0	0	0	3	8	2 – 21
Sum of all samples	304	200	76	14	10	3	0	1	28	9	6 – 12
Danube Delta (*A. leptodactylus*) (Schrimpf et al. 2012)	37	23	3	5	3	1	1	1	11	30	16 – 47
Marine decapods	37	37	0	0	0	0	0	0	0	0	0 – 10

Table 4 Sequence analysis results for the chitinase, rnnL and rnnS genes. "n" is the number of successfully sequenced samples from each location. "Chitinase" indicates, which RAPD-groups are grouped together by the found sequence. "rnnL/rnnS Haplogroup" indicates which haplogroup was identified for each sample. Haplogroup A contains both RAPD-groups A and C, thus identifying the samples DC18 and ASLSRB45/58 to be RAPD-group C.

	Sample ID	n	Chitinase	Accession number	rnnL/rnnS Haplogroup	Accession number (rnnL)	Accession number (rnnS)
Danube Delta (2012)	DC18	1	B/C/E*	MF774442	A	MF740803	MF740807
Danube Delta (2015)	DD108	1	B/C/E*	MF774443	B	MF740804	MF740808
Danube River, Serbia (2015)	ASLSRB 35/58	2	B/C/E*	MF774441	A	MF740802/MF740805	MF740806/MF740809
Dniester River	LP_AlD26	1	–		B	MF740801	-

* RAPD-groups B/C/E cannot be distinguished through chitinase sequencing.

Discussion

Of the 304 crayfish samples collected from the marginal distribution of the invasive crayfish species *O. limosus* in the Danube, and also a non-invaded basin in the eastern range of estimated historical *A. astaci* distribution, the Dniester River, 28 of the samples tested positive for the crayfish plague agent *A. astaci*, specifically, 25 indigenous *A. leptodactylus* samples and 3 non-indigenous *O. limosus* samples. To extend the results further, this data is supplemented by additional *A. leptodactylus* samples (11 positive out of 37) from the Danube Delta, which had already been analyzed by Schrimpf et al. (2012). Our results show a low *A. astaci* infection prevalence of *A. leptodactylus* populations in both the absence and presence of the North American crayfish species *O. limosus*. The infection prevalence in *A. leptodactylus* is similar to earlier studies, where apparently healthy *A. leptodactylus* populations were found to be infected with *A. astaci* (e.g. Maguire et al. 2016; Kokko et al. 2018). In comparison to data from the Danube in 2012, the infection prevalence was a little lower (Pârvulescu et al. 2012), which could however be attributed to natural fluctuations of infection prevalence, like it was for example observed for *O. limosus* (Matasová et al. 2011). The reasons for the low infection prevalence in the Dniester River as well as the Danube River (6% and 20%, respectively), in general, remain unclear. One possibility might be that the indigenous *A. leptodactylus* populations are, to some degree, resistant to *A. astaci* (Unestam 1969b; Alderman et al.1987; Kokko et al. 2012, 2018; Maguire et al. 2016), which may result in reduced agent levels and the low prevalence of *A. astaci* in crayfish populations (Cerenius et al. 2003). The infection prevalence was similar to those of resistant North American crayfish, e.g., *P. leniusculus* (Filipová et al. 2013).

Applying sequence analysis, we identified the B-haplogroup (RAPD-PCR group B) in the Dniester River (Sample ID LP_AlD_26), Moldova, as well as in a sample from the Danube Delta in Romania. The finding of the B-haplogroup in the geographically separated Dniester River, i.e., an area where no *O. limosus*, or any other North American crayfish species, are present yet, supports the hypothesis that the infection is chronic to the tested *A. leptodactylus* populations. Chronically infected *A. leptodactylus* populations in Turkey showed an increased tolerance (balanced host-pathogen relationship), after the recovery of a crayfish plague collapse in the mid-1980s, due to lowered virulence of *A. astaci*, increased resistance of crayfish, or both (Kokko et al. 2012, 2018). Despite the known high

virulence of RAPD-group B (Jussila et al. 2013; Viljamaa-Dirks et al. 2016; Jussila et al. 2017), *A. leptodactylus* seems to be able to suppress an infection by this group (Maguire et al. 2016; Kokko et al. 2018). This may indicate an adaptation of *A. astaci* and indigenous European crayfish. The increased biotic resistance of a host species towards its pathogens due to the constant contact of the two is known to be characteristic during biological invasions (Faillace and Morin 2016).

Another haplogroup of *A. astaci* was found in the Danube Delta and the invaded part of the Danube River in Serbia. The mitochondrial sequences (Makkonen et al. 2018) assigned the three samples as part of the A-haplogroup. This haplogroup contains the RAPD-groups A and C and the uncultured genotype Up detected once in Czech Republic (Grandjean et al. 2014). In combination with analyses of the chitinase gene (Makkonen et al. 2012a), it was further possible to show similar grouping with RAPD-group C and with microsatellite analysis, one of these samples (DC18) was successfully analyzed, showing an identical allele pattern to the presumed genotype Up. The other samples could not be analyzed with microsatellite analysis, due to low agent levels of the samples. Grandjean et al. (2014) concluded that the genotype Up might originate from *P. leniusculus*, because of its high similarity to the genotypes B and C. However, these genetic groups and genotypes have not been detected on *O. limosus* to date, although identical chitinase and mtDNA sequence grouping was also detected in *A. astaci* strains isolated from German *Orconectes immunis* (Makkonen et al. 2018). *Orconectes limosus* is the only invasive crayfish species recorded in the lower Danube so far (Pârvulescu et al. 2012; 2015) and in Czech Republic it is known to be a carrier of the genetic RAPD-PCR group E (Kozubíková et al. 2011).

The infection prevalence of *O. limosus* in our study was very low (8%) in comparison to data from the Danube in 2012 with 32% of individuals infected (Pârvulescu et al. 2012). The general range of infection prevalence seems to be high in *O. limosus*, ranging between 0% and 100% (Kozubíková et al. 2009; Maguire et al. 2016). Temporal fluctuations might also be the reason for the low infection prevalence of *O. limosus* in the current study (Matasová et al. 2011). The haplotypes of the infected *O. limosus* samples in this study remained unknown due to the low agent levels found in the samples, which is characteristic of American crayfish species which can prevent the spread of *A. astaci* hyphae in their bodies (Cerenius et al. 2003).

When looking at the current distribution of crayfish species in the lower Danube, it could be hypothesized that *O. limosus* is the source of the *A. astaci* infection, which, however, cannot be proven in this study. If this was the case, *O. limosus* in the Danube would be a carrier of a typical *P. leniusculus* genetic group. Stockings of *O. limosus*, followed by natural diffusion, as well as uncontrolled spreading by anglers and water body owners, helped the species to extend its range and invade several European countries (Souty-Grosset et al. 2006; Kouba et al. 2014). During this human-mediated spread, the species might have been in contact with *P. leniusculus,* which could have provided an opportunity for the A-haplogroup and B-haplogroup to infect *O. limosus*. The species was introduced into the Hungarian Danube catchments in the late 1950s. From Hungary, *O. limosus* spread along the Danube (Maguire and Klobučar 2003; Pavlović et al. 2006) and reached the Romanian Danube in 2008 (Pârvulescu et al. 2009) where it coexists with, but slowly displaces, the indigenous *A. leptodactylus* (Pârvulescu et al. 2012; 2015). Another possible explanation might be that the populations of *O. limosus* in the Danube were not infected before they came into contact with *A. astaci* carrying *A. leptodactylus*. Non-infected *O. limosus* populations have been previously found in Europe (Kozubíková et al. 2009; Schrimpf et al. 2013a).

Due to the reasons mentioned above, we can only speculate about how both haplogroups, A and B, were translocated into the Danube region. Haplotype A might have originated from relic strains (Schrimpf et al. 2012), which might have spread across Europe with the first crayfish plague outbreaks and since then persisted in populations of *A. leptodactylus*. It was estimated that *A. astaci* first occurred in the lower Danube in 1879-1881 (Alderman 1996), ten years earlier than the first recorded *O. limosus* introductions took place in Poland (Souty-Grosset et al. 2006). Several episodes of mass mortalities of indigenous crayfish, which were probably caused by the disease agent *A. astaci*, have been reported in the Romanian literature (Băcescu 1967). Alderman (1996) estimated the spread of crayfish plague into the Dniester River around 1890-1892. The hypothesis that *A. astaci* in the Danube River could actually be a relic would also explain why the pathogen is found ~900 km downstream of the current invasion front of *O. limosus*. An alternative hypothesis is, that the pathogen was transferred to the Danube Delta in a step stone manner (Schrimpf et al. 2012). However, since *A. astaci* is absent from the non-invaded river sectors of the Danube River in Romania and only reoccurs in the Danube Delta, this hypothesis can probably be dismissed.

Although *P. leniusculus* populations have not been reported in the Romanian part of the Danube River, the unsuccessful introduction of *P. leniusculus* might also explain the presence of haplogroups B and A in the Danube Delta and the Dniester River.

The continuous expansion of infected *O. limosus* is a threat to indigenous *A. leptodactylus* in Romania. *Orconectes limosus* has not yet been noted in the Danube Delta, but is spreading downstream in the Danube River at a rate of around 15 km per year. It is expected that the species will reach this region in the 2060s (Pârvulescu et al. 2012). Currently, the haplogroup of *O. limosus* in the Danube River is not known. If it carries the RAPD-PCR group E similarly as *O. limosus* in Czech Republic, the simultaneous occurrence of three different haplogroups of *A. astaci* might increase the pressure on indigenous crayfish. Therefore, more samples with higher agent levels are needed to identify the group which *O. limosus* carries. Ideally, *A. astaci* should be isolated in pure culture.

An infection of the marine decapod species could not be detected in this study. These species are numerous in brackish waters of the Danube Delta (Petrescu et al. 2010; Skolka and Preda 2010) and might thus play a role in the spread of *A. astaci* if the pathogen was able to survive the salinity of the surrounding water. The Black Sea is known to have lower salt concentrations than the mean ocean salinity (Murray et al. 1991). However, many species of the Saprolegniaceae, which also belong to the oomycota, are unable to produce zoospores, even at low salt concentrations (Cerenius and Söderhäll 1985; Rantamäki et al. 1992; Harrison and Jones 1975). As we did not detect any A. *astaci* infections in the marine decapods in this study, we support the hypothesis that *A. astaci* is unable to spread within marine or brackish environments and is thus not able to infect marine decapods.

In conclusion, we tested indigenous crayfish of the Romanian Danube and the Danube Delta positive for *A. astaci* DNA, despite the fact that North American crayfish have not been recorded in the lowest parts of the Danube River. This work also provides evidence of *A. astaci* in an area inhabited by indigenous crayfish populations only, but which is presumed to be historically affected by outbreaks. Since the identified haplogroups of *A. astaci* do not correspond to the one previously detected in invasive *O. limosus*, it is possible that this species is not the original transmitter. In any of the discussed scenarios, it appears that the

pathogen and its host might be reaching a natural equilibrium, as the populations of *A. leptodactylus* tested here all coexist with *A. astaci*. This therefore provides hope for the survival of European indigenous crayfish populations.

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Appendices IV

Table IV.A Results of the microsatellite analysis using nine co-dominant markers (Grandjean et al. 2014). To compare allele sizes, the fragments from five pure-culture *A. astaci* strains of genotypes A, B, C, D and E, as well as from one mixed DNA sample (Up4) are shown. Sample DC18 was detected in this study and was found on *A. leptodactylus* from the Danube Delta. The allele pattern of sample DC18 is identical to the genotype Up. Allele sizes cannot be directly compared to the results from Grandjean et al. (2014) because they were generated on another sequencer with different color labels. Each reference sample was therefore tested in our own lab for the study at hand.

Code	SSR-A2	SSR-B	SSR-C	SSR-D	SSR-E	SSR-Up	SSR-Up (This study)
Strain	UEF-ATID	UEF-SATR1	Kv1	AP03	Li05 / Li08	Up4	DC18
Aast2	161	145	155	–	151/163	145/151	145/151
Aast4	105	89	89	133	89	89	89
Aast6	160	151	151	151	151/160	151	151
Aast7	207	215	191/215	203	207	205/215	205/215
Aast9	178	164/182	164/168	178	168/180	164	164
Aast10	145	135	135	157	135/145	135/141	135/141
Aast12	–	226/238	226	232	238	226	226
Aast13	195	203	203	195	203	203	203
Aast14	245	247	247	249	247	247	247

12.5 Appendix V

Three haplotypes of the crayfish plague agent *Aphanomyces astaci* found to be responsible for recent crayfish plague outbreaks in Germany and Austria

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Abstract

Recent development of novel genetic marker systems has created new opportunities to study the relationship between different *Aphanomyces astaci* haplotypes and also their the occurrence among wild crayfish populations. While some studies evaluated the infection prevalence of crayfish in the Central Europe, Germany and Austria have never been intensively studied in this regard. We applied real-time PCR on samples from 19 locations in Germany and from 10 locations in Austria to assess the *A. astaci* infection prevalence in native as well as non-native crayfish species. Additionally, we used sequencing and microsatellite analyses to identify the haplogroups and genotypes that may have caused crayfish mortalities mainly in populations of the stone crayfish, *Austropotamobius torrentium*. The infection prevalence of non-indigenous species was between 6% and 21% (specimen n = 380), while in cases, where mass mortalities of the European species, especially *A. torrentium* occurred, resulted in a significantly higher infection prevalence of 69% (specimen n = 100). We identified three different haplogroups, namely A, B and D, to be responsible for six crayfish plague epidemics. Haplogroup B was the most dominant group, as it was detected five times. Our results give new insights into the distribution of different *A. astaci* haplogroups in Germany and Austria, to be carefully considered when planning crayfish management projects and species protection programs.

Keywords: **stone crayfish, non-indigenous crayfish species, European crayfish species, haplotyping**

Introduction

The importance of crayfish as ecosystem engineers is well known and especially invaders can have high impacts on the ecosystem mainly through food web alteration (Gherardi, 2007; Creed and Reed, 2004). The management of non-invasive crayfish species (NICS) is therefore a crucial aspect of biodiversity conservation. Six NICS are currently present in Germany and Austria. The signal crayfish, *Pacifastacus leniusculus* (Dana, 1852), the red swamp crayfish, *Procambarus clarkii* (Girard, 1852), the spiny-cheek crayfish, *Orconectes limosus* (Rafiesque, 1817) and the narrow-clawed crayfish, *Astacus leptodactylus* Eschscholtz, 1823 in both countries, while the marbled crayfish, *Procambarus fallax* f. *virginalis* (Martin et al., 2010) and the calico crayfish, *Orconectes immunis* (Hagen, 1870) have been reported only from Germany (Kouba et al., 2014). *Astacus leptodactylus* is the only NICS not originally from North America, but from Eastern Europe. In contrast to the six NICS, three indigenous crayfish species (ICS) exist in Germany and Austria: the noble crayfish, *Astacus astacus* (Linnaeus, 1758), the stone crayfish, *Austropotamobius torrentium* (Schrank, 1803), and the white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet, 1858), the latter one being a species complex (e.g. Grandjean et al., 2002; Pedraza-Lara et al., 2010). The three species have been listed as vulnerable, data deficient, and endangered, respectively, all with decreasing population trends (IUCN Red List, 2018).

One of the main reasons for the population losses of ICS in Europe is the pathogen *Aphanomyces astaci* Schikora, 1903 (Alderman, 1996; Jussila et al., 2015). *Aphanomyces astaci* is an oomycete, which was introduced from North America to Europe in the midst of the 19th century and known to cause the fatal disease crayfish plague. Since then *A. astaci* has spread throughout Europe causing mass mortalities among ICS (Holdich et al., 2009). Due to its significant threat to ICS, *A. astaci* has received considerable scientific attention and is therefore also considered as one of the best-studied invertebrate pathogens (Souty-Grosset et al., 2006). Various studies have focused on studying the genetic variability of *A. astaci*, covering tools like random amplified polymorphic DNA (RAPD) –PCR (Huang et al., 1994), amplified fragment length polymorphism (AFLP) –PCR (Rezinciuc et al., 2014), microsatellite analysis (Grandjean et al., 2014), and sequence analysis (Makkonen et al., 2012a; Makkonen et al., 2018).

These methods allow for the differentiation of different genetic groups, genotypes and haplotypes, respectively. Differentiation of the genetic groups and haplogroups is important, as it allows conclusions on the origin, crayfish vector species and virulence of a specifically identified *A. astaci* strain (Svoboda et al., 2017).

Broad scale screenings of *A. astaci* infection prevalence have been carried out in Central Europe, i.e., the Czech Republic, France, and Croatia (Kozubíková et al., 2009; Filipová et al., 2013; Maguire et al., 2016). In the Czech Republic, *O. limosus and P. leniusculus* were identified as carrier of *A. astaci*, whereas the dominant and thus most threatening NICS was *O. limosus* (Kozubíková et al., 2009). Filipová et al. (2013) identified *A. astaci* in over 50% of the studied *P. leniusculus* populations in France, and Maguire et al. (2016) confirmed that *A. astaci* is in Croatia, even in indigenous *A. leptodactylus*, which also seemed to be resistant to *A. astaci*.

Latent infections of ICS with *A. astaci* have first been observed in *A. astacus* populations in Finland (Jussila et al., 2011, Viljamaa-Dirks et al., 2011). Since then the attention has shifted and also ICS were screened for *A. astaci* and several studies identified European crayfish populations with latent or chronic *A. astaci* infections (Kokko et al., 2012; Makkonen et al., 2012b; Svoboda et al., 2012; Schrimpf et al., 2012; Kušar et al., 2013; Viljamaa-Dirks et al., 2013; Jussila et al., 2017; Martín-Torrijos et al., 2017). The reasons why some ICS populations are resistant towards an *A. astaci* infection are not yet fully understood. While it has been shown in the laboratory that there are significant differences in the virulence between different *A. astaci* strains (Makkonen et al., 2012b; Jussila et al., 2013), at least *A. leptodactylus* seems to have a slightly elevated resistance towards various *A. astaci* strains (Jussila, unpublished), as healthy populations have been repeatedly found to be latently infected with different *A. astaci* strains (Kokko et al., 2012, 2018; Svoboda et al., 2012; Maguire et al., 2016; Panteleit et al., 2018). A recent study by Jussila et al. (2017) suggests that chronic infections might be the result of regional evolutionary adaptations of different crayfish populations and *A. astaci*. This could nevertheless be problematic if *A. astaci* is continues to be spread across Europe, also with the aid of humans via crayfish transfer, because chronically infected populations are constant *A. astaci* reservoirs (Jussila et al.,

2015) and a latent infection in one species or population could be detrimental to another one (Jussila et al., 2017).

Our aim was to get a further insight into which *A. astaci* strains are of most concern for biodiversity conservation issues. We first assessed the infection prevalence of sampled NICS and ICS in Germany and Austria, then performed sequence- and microsatellite analyses of highly infected samples to identify which genetic groups of *A. astaci* are abundant in freshwater systems in Germany and Austria and possibly involved in mass mortalities of ICS.

Material and Methods

Crayfish sampling

Crayfish sampling and analysis was conducted between 2013 and 2015. Whole crayfish specimens were directly frozen after sampling and then sent to the University of Landau from local stakeholders (mainly fisherman and local fisheries authorities) of the different sampling sites. Samples from different locations were always sent separately and in closed plastic bags to avoid cross contamination between populations. All samples were stored at -80°C upon arrival. Overall, we received 490 crayfish samples from 29 different locations (Table 1, Fig. 1). The sampling sites in Germany were from 19 locations in seven federal states (407 samples) and in Austria from 10 locations in four federal states (83 samples). Species composition for both countries combined was as follows: Crayfish from Europe: *A. astacus* (n=5), *A. leptodactylus* (n=5), *A. torrentium* (n=100) and crayfish from North America: *O. limosus* (n=84), *P. leniusculus* (n=238) and *P. clarkii* (n=58). Specimens of *A. torrentium* were sampled during mass mortality events or when single individuals died for unknown reasons. *Astacus astacus* specimens were also only collected dead, but the deaths were sporadic and no connections to mass mortalities were observed.

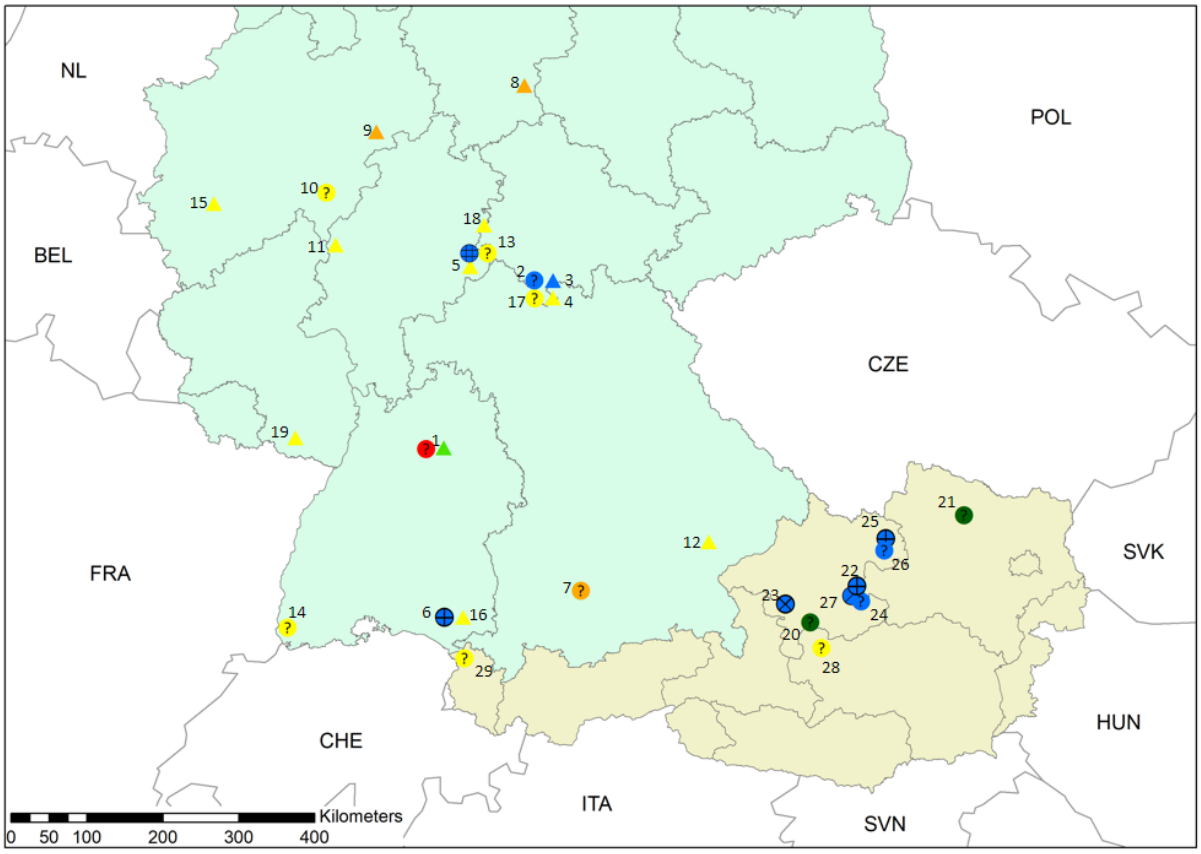
Table 1 Infection level of studied crayfish samples. Infected samples are written in bold letters. The asterisk indicates water bodies that belong to the Schädelbach stream system. For location IDs see also Fig 1 95% CI is the confidence interval of infected specimen within one population. Haplotype and genotype give the results for the respective population, if at least one sample was successfully sequenced or genotyped. Species abbreviations are as follows: Pl: *Pacifastacus leniusculus*, Ol: *Orconectes limosus*, Pc: *Procambarus clarkii*, Al: *Astacus leptodactylus*, At: *Austropotamobius torrentium*, Aa: *Astacus astacus*

Species	Location ID	Freshwater system in - Germany	n	Agent level							n infected	% infected	95% CI	Haplotype	Genotype
				A0	A1	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**					
Al	1	Breitenauer See	5	5								0	0	0–52		
At	2	Feeder of the Alster River	6	1	1		**2**	**1**	**1**			4	67	22–96	b	
At	3	Helling River	4	4								0	0	0–60		
At	4	Saarbach	1	1								0	0	0–98		
At	5	Schwarzbach	16				**1**	**1**	**4**	**4**	**6**	16	100	79–100	d1	D
At	6	Stiller Bach	26	17	1		**2**	**3**		**3**		8	31	14–52	b	
Pc	1	Breitenauer See	58	42	4	**9**	**3**					12	21	11–33		
Ol	7	Ammersee	27	16	6	**5**						5	19	6–38		
Ol	8	Okerstausee	6	6								0	0	0–46		
Ol	9	Pond near Bad Wünneberg	51	46	5							0	0	0–7		
Pl	10	Almbaumer Bach	23	17	5	**1**						1	4	0–22		
Pl	11	Ambach-Dillsystem	18	16	2							0	0	0–19		
Pl	12	Bina	20	20								0	0	0–17		
Pl	13	Haune	20	20								0	0	0–17		
Pl	14	Kander	20	11	5	**2**	**2**					4	20	6–44		
Pl	15	Pulheimer Bach	23	14	9							0	0	0–15		
Pl	16	Rösseler Weiher	13	11	2							0	0	0–25		
Pl	4	Saarbach	21	21								0	0	0–16		
Pl	5	Schwarzbach	20	2	7	**10**	**1**					11	55	32–77		
Pl	17	Streudorfer Kreck	3	2		**1**						1	33	1–91		
Pl	18	Ulster	20	15	5							0	0	0–17		
Pl	19	Wieslauter	6	6								0	0	0–46		
		All German samples	407	293	52	**28**	**11**	**5**	**5**	**7**	**6**	63	16	12–19		

Table 1 continued

		- Austria													
Aa	20	Offensee	3	1	1	**1**					1	33	1–91		
Aa	21	Teichwiesenbach	2	1		**1**					1	50	1–99		
At	22	Feeder of the Steyr Fluss	7	1			**1**	**1**	**1**	**2**	**1**	6	86	42–100	b	B
At	23	Inlet to Attersee Lake	5		1				**3**		**1**	4	80	28–100		Up
At	24	Lehnerbach*	4		1		**3**					3	75	19–99		
At	25	Lehnerbach West*	12	1	2	**3**	**2**		**2**	**2**		9	75	43–95	b	
At	26	Rechgraben*	2			**1**	**1**					2	100	16–100		
At	27	Schädelbach*	17				**2**	**2**	**4**	**6**	**3**	17	100	81–100	a & b	Up & B
Pl	28	Drainage near Krungel	11	1	2	**2**	**6**					8	73	39–94		
Pl	29	Tobelbach	20	5	6	**7**	**2**					9	45	23–69		
		All Austrian samples	83	10	13	**15**	**17**	**5**	**8**	**10**	**5**	60	72	61–82		
		Sum of all samples	490	303	65	**43**	**28**	**10**	**13**	**17**	**11**	123	25			

Fig. 1 Location of sampled crayfish and results of crayfish plague analysis. Triangle: not infected, circles: infected. The numbers give location IDs, colors indicate the crayfish species: blue: *A. torrentium*, yellow: *P. leniusculus*, red: *P. clarkii*, light green: *A. leptodactylus*, dark green: *A. astacus*, orange: *O. limosus*. Symbols in the circles indicate which *A. astaci* haplotype or genotype was found on the crayfish samples: ?: infection by an unknown haplotype or genotype, +: haplotype B, x: haplotype a (genotype Up), /: haplotype a (genotype Up) and haplotype b in one population of *A. torrentium* in the Schädelbach, #: haplotype d1.



Aphanomyces astaci *infection status analysis*

Aphanomyces astaci infection status was tested as in Panteleit et al. (2018). Based on the number of PCR forming units (PFU) infection status and agent levels from *A. astaci* specific qPCR were defined according to Vrålstad et al. (2009), where samples with agent level A0 (0 PFU) and A1 (PFU_{obs} < 5 PFU) are considered uninfected and agent level A2 (5 PFU ≤ PFU_{obs} < 50 PFU) and higher (up to A7 with PFU > 10^6) are considered infected with *A. astaci*. The 95% confidence intervals of infected populations were calculated using the software RStudio V.1.0.44 (R Core Team, 2016) with the package epiR V.0.9 (Stevenson et al., 2016).

Aphanomyces astaci *genotyping*

Identification of *A. astaci* genotypes was done using nine microsatellite markers after Grandjean et al. (2014). Laboratory procedures were identical to Panteleit et al. (2018). Peak scoring for multilocus genotype identification was done with GeneMarker V 1.95. Only crayfish samples with agent level A3 or higher were used for the genotype identification, as low agent levels usually lead to poor quality or lacking results. Peaks were compared to reference samples of different genotypes from *A. astaci* pure culture isolates and in case of genotype Up to a mixed DNA samples provided by Dr. Adam Petrusek from Charles University in Prague, Czech Republic.

Aphanomyces astaci *sequencing*

Sequencing of three different genes, the mitochondrial ribosomal rnnS and rnnL subunits (Makkonen et al., 2018) and the nuclear chitinase gene (Makkonen et al., 2012a), was done to identify the haplogroups of *A. astaci* from infected crayfish samples. Sequencing methods for the chitinase gene were identical to Panteleit et al. (2018) and for the rnnS and rnnL genes identical to Makkonen et al. (2018). Sequences were aligned and edited with the software Geneious R7 (Kearse et al., 2012). Haplotypes were compared to reference sequences from NCBI GenBank database. All sequences were uploaded to NCBI GenBank (accession numbers provided in Table 2). Like the microsatellite analyses, sequencing was also only done for samples with agent levels of A3 and above.

Results

Aphanomyces astaci *infection analysis*

Aphanomyces astaci was confirmed at 18 of the 29 locations and in five out of six crayfish species tested in this study, with *A. leptodactylus* being the only species where no DNA of *A. astaci* was detected (Table 1). Ten locations where *A. astaci* was detected were in Austria, and eight in Germany. The *A. torrentium* samples showed the highest infection prevalence (69% infected out of all samples), covering all agent levels from A0 to A7. Of the five *A. astacus* samples, two were infected (agent level A2). The American crayfish species, *P. leniusculus*, *O. limosus* and *P. clarkii,* showed overall infection prevalence of 14%, 7% and 21% of all samples, respectively.

Aphanomyces astaci *sequencing and genotyping*

The rnnS gene could be sequenced for 26 samples, in contrast to the rnnL gene where only six samples could be sequenced. With the mitochondrial rnnL and rnnS genes we detected three different haplogroups of *A. astaci*, A, B, and D, according to Makkonen et al. (2018) (Table 2). All haplogroups were detected on infected *A. torrentium* from six locations. Three locations were in Austria: In a feeder of the Steyr River (Location ID 22), haplogroup B was found. The other two locations belong both to the Schädelbach stream system. From the Lehnerbach stream (Location ID 24), an inlet of the Schädelbach, only the rnnL sequence could be used for haplogroup determination, resulting in haplogroup B. From the Schädelbach stream near Gmach (Location ID 27), two different haplogroups were detected in one population of *A. torrentium*: haplogroup A was found in one sample from this location, while haplogroup B was identified in nine other samples according to the rnnS gene. The chitinase sequence grouped the haplogroup A sample similarly with the RAPD-group C.

In Germany haplogroup B was found in the Stiller Bach (Location ID 6) and also in a feeder of the Alster River (Location ID 2). In the Schwarzbach (Location ID 5), 11 samples could be identified as haplogroup D by rnnS sequences, while one sample could be more closely identified as haplotype d1 based on the rnnL sequence (Makkonen et al., 2018).

By genotyping highly infected *A. torrentium* samples it was possible to determine the genotype of *A. astaci* from five locations: two samples from the Steyr River (Location ID 22) in Austria, for which the microsatellite pattern was identical to genotype SSR-B, three samples from the Schädelbach stream (Location ID 27) were also identical to genotype SSR-B. Genotype SSR-Up was found in the Schädelbach and also in an inlet to lake Attersee in Austria (Location ID 23). Three samples from the Schwarzbach (Location ID 5) in Germany, which belonged to haplogroup D, were highly similar to genotype SSR-D, reference strain AP03 (Rezinciuc et al., 2014). This assignment was also confirmed in a separate microsatellite analysis of the same sample by A. Petrusek and J. Svoboda from the Charles University in Prague (Czech Republic). However, these three samples varied slightly in their microsatellite pattern presumably due to allele drop outs at some loci (Appendix 1).

Table 2 Results of the sequence and microsatellite analyses from highly infected *A. torrentium* samples. Given are the country and the name of the water body from which the samples were taken. For location ID see also Fig. 1. HG/HT are the haplogroups/haplotypes for the three sequenced genes, rnnS, rnnL and chitinase with numbers of successfully sequenced samples (n). The chitinase groups together the the haplogroups B/C/E, therefore a combined grouping is indicated. GT indicates genotype. Loci sizes are shown in Appendix 1.

Country	Location	Location ID	rnnS HG (n)	rnnS accession numbers	rnnL HG/HT (n)	rnnL accession numbers	Chi HG (n)	Chi accession numbers	GT
Germany	Schwarzbach[+]	5	D (10)	MH180303 - 11, MF150015	D/d1 (1)	MF150014	D (1)	MH188846	
		5	D (1)	MH180312					D
		5							D
		5							D
Austria	Attersee	23							Up
Germany	Stiller Bach	6	B (2)	MH180300, MH180301	B (1)	MH181158	B/C/E (1)	MH188845	
Germany	Feeder of the Alster river	2	B (1)	MH180302	B (1)	MH181160			
Austria	Feeder of the Steyr river[+]	22	B (1)	MF150011	B (1)	MF150010			B
Austria	Schädelbach stream[+]	27	B (10)	MH180313 - 19, MH180321, 22, MF150013	B (1)	MF150012	B/C/E (3)	MH188847 - 49	B
	Schädelbach stream	27	A (1)	MH180320					Up
Austria	Lehnerbach	24			B (1)	MH181159			

[+]One sample of each of these locations was already analyzed and uploaded to Genbank in Makkonen et al. (2018)

Discussion

Our results give an important insight into the occurrence of different *A. astaci* haplotypes and the infection prevalence of some ICS and NICS populations in Germany and Austria. We investigated crayfish populations from 29 locations in Germany and Austria. Eight of the NICS populations had *A. astaci* DNA detected in crayfish tissues, including all three species tested with agent levels up to A3. The ICS populations on the other hand showed a wider range of agent levels between A0 and A7. The high agent levels were probably caused by the high susceptibility of the ICS to the haplotypes infecting them. Three different haplogroups, A, B and D, that probably caused mass mortalities of *A. torrentium* populations, were discovered.

In the Schädelbach stream system in Austria, we found two different *A. astaci* haplogroups, A and B, in one population of *A. torrentium*. The occurrence of two different haplogroups in one population of crayfish has so far only once been recorded before (Maguire et al., 2016). In this case, there is a possibility for *A. astaci* haplotype interaction, though such has not been reported and its possible consequences remain to be studied. Possible sexual reproduction of *A. astaci* has not been confirmed, yet (Söderhäll and Cerenius, 1999; Diéguez-Uribeondo et al., 2009). Still, it should be considered, that two different haplotypes might interact with each other, with unknown evolutionary outcomes. The presence of two different *A. astaci* strains in one population might also indicate the limitations of microsatellite markers, as a mixture of strains in a population, and possibly in one individual crayfish, could results in an artificial increase in hetereozygosity, as most loci are being described as homozygote.

Our findings of two different haplogroups of *A. astaci* in one crayfish population from the Schädelbach watercourse is interesting. No NICS that could have acted as a vector for *A. astaci* was found in the system or in the vicinity. It has been reported that people had attempted to remove crayfish from the streams in the Schädelbach watercourse illegally, these acts are known to act as means to spread *A. astaci* (Bohman et al., 2006; Ruokonen et al., 2018). Therefore, it can be speculated that an introduction of *A. astaci* happened through the transmission of spores via contaminated equipment during such events (Alderman, 1996; Souty-Grosset et al., 2006).

Microsatellite analyses revealed, that sample one from the Schädelbach (Location ID 27), which was identified as haplogroup A based on rnnS sequencing, had the identical allele pattern as the genotype Up. Genotype Up was first found in the Czech Republic in the Uporsky brook (Grandjean et al., 2014) and a second time in the Danube Delta, Romania (Panteleit et al., 2018) until our study. Austria and Romania are both part of the Danube basin, while the area of the Czech Republic near Prague, where genotype Up was first discovered is part of the Elbe basin, though very close to the border of the Danube basin. Despite the fact that this genotype has been found only three times to date, its distribution range seems to be quite wide, especially in the Danube basin. However, whether this wide distribution is a relic from early crayfish plague outbreaks or a result of more recent invasion processes remains to be studied (Panteleit et al., 2018).

Genotype Up has caused high mortalities among *A. torrentium* populations in the Czech Republic (Grandjean et al., 2014), while *A. leptodactylus* from the Danube Delta were only latently infected with this genotype (Panteleit et al. 2018). The *A. torrentium* mass mortalities in the Schädelbach, that we studied here, could have been caused by genotype Up or genotype B or by both. Earlier studies found that strains of haplogroup B are much more virulent and have caused a rapid death of crayfish in laboratory tests compared to strains that are part of haplogroup A (Jussila et al., 2011; Viljamaa-Dirks et al., 2013; Becking et al.,. 2015; Jussila et al., 2017). However, genotype Up has not been tested in controlled infection experiments, a study that should be carried out.

Our study indicates a high virulence of the haplogroup D against *A. torrentium,* as it has caused mass mortality in the Schwarzbach population. The *A. astaci* samples from this locality (Location ID 5) were identified as haplotype d1 (haplogroup D). Haplotype d1 (strain SAP_Malaga5) was first isolated from *P. clarkii* in the Turón River in South Spain near Malaga. There are no records on how or when *P. clarkii* was first introduced into the Turón River. First documented introduction into Spain occurred in the 1970's from Louisiana (Souty-Grosset et al., 2006; Rezinciuc et al., 2014). We detected the d1-haplotype in sampled *A. torrentium* from a location, where *P. leniusculus* inhabits the same water system, but roughly 10 km downstream of the *A. torrentium* population. We could not determine the *A. astaci* haplogroup infecting this *P. leniusculus* population from the Schwarzbach. We speculate, that another likely vector into the Schwarzbach was a *P. clarkii* population inhabiting a quarry pond about 12 km away, even

though these populations are not connected by water, as *P. clarkii* is assumed to be the original vector of haplogroup D (Diéguez-Uribeondo et al., 1995; Makkonen et al., 2018). *Aphanomyces astaci* spores may have been translocated from the quarry pond to the Schwarzbach via contaminated fishing gear or with animals. However, both NICS populations should be considered potentially harmful for any ICS populations remaining in the area.

The *A. astaci* infection prevalence was higher in populations of ICS (67%) than in NICS (13%). However, almost all ICS were sampled during mass mortalities most probably caused by *A. astaci*, with the exception of the *A. astacus* populations. The low infection prevalence of resistant North American crayfish species is similar compared to findings in earlier studies (Kozubíková et al., 2009; Pârvulescu et al., 2012; Filipová et al., 2013; Keller et al., 2014). However, infection prevalence seems to be subject to temporal fluctuations (Kozubíková et al., 2009; Matasová et al., 2011), making assumptions based on single sampling events, like in this study, difficult. Among the NICS, *P. clarkii* had the highest infection prevalence (21%), however, a single location (N=58) was tested and the result is thus representative for this population only. *Pacifastacus leniusculus* had a higher infection prevalence than *O. limosus*. From 14 locations 14% of the *P. leniusculus* samples were infected, while of the *O. limosus* samples, 6% from three locations were infected. This is contrary to what has previously been reported from France (Filipová et al., 2013) or the Czech Republic (Kozubíková et al., 2009), where, in both cases, *O. limosus* had a higher infection prevalence than *P. leniusculus*. It has been hypothesized that in the Czech Republic, only one introduction event of juvenile *P. leniusculus* with a low infection prevalence led to a generally low infection prevalence in the whole country (Kozubíková et al., 2009). The introduction history of *P. leniusculus* populations in Germany and Austria is much more complex and often poorly documented (Pöckl and Pekny, 2002). Multiple introduction events could have led to an elevated infection prevalence in comparison to the Czech Republic and also to the introduction of more than one genetic group of *A. astaci*.

Conclusions

The present study screened crayfish plague, *A. astaci*, status of indigenous and non-indigenous crayfish populations in Central Europe. The results are telling the story of the mixture of different alien crayfish present in Central Europe, together with their varying disease strains, all present in a single susceptible species, A. torrentium. Our results reflect the devastating effects of invasive crayfish species on *A. torrentium*. We want to emphasize the importance of proper disinfection of any equipment when fishing, crayfishing or working in the field to avoid transmission of *A. astaci* between water bodies. This is especially important, if ICS and NICS occur in close proximity to each other, which is often the case Central Europe. The awareness rising of the common public should also be intensified to promote correct behavior when visiting water bodies for the purpose of any recreational activities.

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Appendices V

Appendix V.1 Loci sizes for the microsatellite analysis of the 9 co-dominant markers (Grandjean et al. 2014). Given are the allele sizes of 9 samples from Austria and Germany from *A. astaci* DNA extracted from infected *A. torrentium*. For location IDs compare Fig. 1.

			SSR locus								
Location	Location ID	Geno-type	Aast 2	Aast 4	Aast 6	Aast 7	Aast 9	Aast 10	Aast 12	Aast 13	Aast 14
Schwarzbach Germany	5	D	**	133	151	203	178	**	232	**	249
Schwarzbach Germany	5	D	**	133	151	203	178	**	232	195	249
Schwarzbach Germany	5	D	**	133	151	203	178	**	**	**	249
Attersee Austria	23	Up	145/151	89	151	205/215	164	135/141	226	203	247
Feeder of the Steyr river Asutria	22	B	145	89	151	215	164/182	135	226/238	203	247
Schädelbach Austria	27	B	145	89	151	215	164/182	135	226/238	203	247
Schädelbach Austria	27	Up	145/151	89	151	205/215	164	135/141	226	203	247

12.6 Appendix VI

Invasive rusty crayfish (*Faxonius rusticus*) populations in North America are infected with the crayfish plague disease agent (*Aphanomyces astaci*)

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Abstract: The American rusty crayfish, *Faxonius rusticus*, is an invasive species in parts of North America where it displaces native crayfish species. In Europe, various invasive North American crayfish species are carriers of the crayfish plague disease agent *Aphanomyces astaci* that causes devastating population declines of European crayfish. Little is known about either the presence or the influence of *A. astaci* in North America where it originates. Here, we attempted to isolate *A. astaci* from North American *F. rusticus* for the first time and compare it to the strains detected in Europe. We tested the infection status of 84 *F. rusticus* samples from 10 different locations in the midwestern United States that are outside of the *F. rusticus* native distribution range. We used quantitative real-time PCR (qPCR) to assess the infection status in each individual, and we determined the mitochondrial haplotypes and multilocus microsatellite genotypes when it was possible. We detected *A. astaci* DNA in 9 individuals from 4 out of 10 locations. Analyses of the axenic culture isolates and the crayfish tissue samples by sequence analyses, haplotyping, and genotyping confirmed the results and revealed a novel *A. astaci* microsatellite genotype. Our results clearly identify *F. rusticus* as a host of *A. astaci* in North America. The threat of these strains to endangered crayfish species in North America remains unknown, but the potential of *A. astaci* infections should be considered when developing and implementing invasive species management plans for conservation purposes.

Keywords: freshwater crayfish conservation, quantitative real-time PCR, sequence analysis, microsatellites, invasion mechanisms, *Aphanomyces astaci* genotypes

Biological invasions affect more than 20% of all threatened species worldwide, and are the 2nd largest contributor to the loss of species biodiversity (Wilcove et al. 1998, Joppa et al. 2016). Invasions are facilitated when invaders have advantageous life-history traits, lack natural enemies in the novel environment, or carry pathogens and parasites (Nentwig et al. 2007). Contemporary freshwater crayfish assemblages are less abundant and less diverse than they were in the past (Edwards et al. 2014). About $^1/_3$ of the >400 freshwater crayfish species in North America are in need of conservation attention, and extinction rates are expected to increase by more than an order of magnitude by 2100 (Ricciardi and Rasmussen 1999, Crandall and Buhay 2008, Richman et al. 2015).

Freshwater crayfish often function as ecosystem engineers because of the major effect they can have on aquatic ecosystems, e.g., influence on detrital processing rates and the distribution of fine particulate matter (Creed and Reed 2004). These effects are strongest when particularly aggressive crayfish species, such as the rusty crayfish *Faxonius rusticus* (Girard, 1852) (formerly *Orconectes rusticus*), become established in a new area (Olden et al. 2006). The historic range of *F. rusticus* was centered in southwestern Ohio and northern Kentucky streams (Taylor et al. 1996). However, human-mediated translocations, both long and short, have led to the continuous spread of *F. rusticus* throughout the northeastern United States and southeastern Canada. *Faxonius rusticus* has displaced native crayfish such as *Faxonius propinquus* (Girard, 1852) and *Faxonius virilis* (Hagen, 1870) (Hill et al. 1993, Olden et al. 2006, Reid and Nocera 2015). *Faxonius rusticus* is larger and grows faster than *F. propinquus,* so *F. rusticus* is competitively superior and less susceptible to predation than *F. propinquus* (Hill and Lodge 1999). *Faxonius rusticus* also outcompetes native crayfish for preferred shelters, and therefore increases predation rates on native crayfish (Hill and Lodge 1999). Furthermore, range expansions of *F. rusticus* over

the last 50 years have been facilitated by their use as fish bait (Taylor et al. 1996), but regulations on the use of crayfish as fishing baits have decreased the rate of expansion more recently (Lodge et al. 2000, Puth and Allen 2004).

In addition to the direct effects of anthropogenic translocations, the transmission of pathogens, such as the oomycete *Aphanomyces astaci* Schikora 1906 (Class: Oomycetes, Order: Saprolegniales see Diéguez-Uribeondo et al. 2009), may also increase with the spread of *F. rusticus*. *Aphanomyces astaci* is a pathogen that causes the disease crayfish plague (Alderman 1996), and it is considered 1 of the 100 worst invasive species worldwide (Lowe et al. 2004). *Aphanomyces astaci* originates from North America, where it presumably lives as a parasite in the cuticle of freshwater crayfish species of both North American crayfish families, the Astacidae and Cambaridae (Unestam 1972, Martin and Davis 2001). American crayfish species are thought to be highly resistant to, or possibly tolerant of, *A. astaci* infections (Schäperclaus 1954, Unestam 1969). However, even American crayfish species may succumb to crayfish plague infections (Persson et al. 1987, Söderhäll and Cerenius 1992, Aydin et al. 2014, Jussila et al. 2014) or other opportunistic parasites (Edsman et al. 2015) if they are severely stressed or immunocompromised by other pathogens or environmental factors.

The known *A. astaci* strains can be allocated to 4 different haplogroups (A–E-haplogroups) through sequence analysis (Makkonen et al. 2018). Studies conducted in Europe have indicated that specific *A. astaci* strains may have adapted on one host species (Jussila et al. 2017, Makkonen et al. 2018). Infection experiments have also shown that healthy American crayfish species can die after being infected with specific *A. astaci* strains under laboratory conditions (Makkonen et al. 2012a, Aydin et al. 2014, Jussila et al. 2017).

Aphanomyces astaci could therefore have an impact on crayfish conservation in North America, where the crayfish diversity is much higher than in any other part of the world (Füreder 2009). In particular, if the high crayfish diversity in North America leads to high *A. astaci* diversity through coevolution, the virulence of *A. astaci* strains could vary greatly. Furthermore, the virulence of a given *A. astaci* strain might vary depending on the crayfish species it infects (e.g., Jussila et al. 2017). Therefore, translocations of North American crayfish species that carry a specific *A. astaci* strain into new habitats could have a significant impact on the population abundance of North American freshwater crayfish if the *A. astaci* strain is highly virulent to the native crayfish in that environment (e.g., Alderman 1996, Jussila et al. 2016a).

So far, only one study has confirmed the presence of *A. astaci* directly in North America (Huang et al. 1994). Previous information on the distribution and genetic variability of *A. astaci* in North America has been obtained either from studies of the crayfish pet trade (Mrugała et al. 2014, Panteleit et al. 2017) or areas invaded by North American crayfish species, such as Europe (reviewed by Svoboda et al. 2017), Japan (Mrugała et al. 2016, Martín-Torrijos et al. 2018), and Brazil (Peiró et al. 2016). However, with these studies from outside of North America, it is not possible to directly draw conclusions about the situation on-site. Therefore, in this study, we aimed to document the presence of *A. astaci* in North America by assessing its prevalence across populations of *F. rusticus* in the North American Midwest. We also cultured *A. astaci* and used sequencing and microsatellite analyses to genetically characterize the *A. astaci* strains present on this species. It is assumed that *A. astaci* genotypes are crayfish species specific (Grandjean et al. 2014). We therefore expected to find a new *A. astaci* strain representing unique genotypes to *F. rusticus*. Furthermore, *A. astaci* also could

be facilitating the invasion of crayfish species native to North America to areas outside of their native North American range – similar to what has been observed in Europe. Such facilitation would require swift considerations regarding the translocation of crayfish in the North American continent.

METHODS

Study design

To detect the possible *A. astaci* carrier status of *F. rustics*, we first analyzed tissue samples (*n*=84) from several populations (*n*=10) collected in the year 2015 within the region where *F. rusticus* is considered invasive. After positive detection of *A. astaci* in these samples, we then proceeded in year 2016 to isolate *A. astaci* strains from live *F. rusticus* crayfish specimens (*n*=41) from 4 populations. After successful isolations, we genetically characterized the strains by microsatellite genotyping and haplogroup sequencing of mitochondrial marker genes (Makkonen et al. 2018) of the *A. astaci* isolates with PCR amplification, sequence analyses, and microsatellite analyses. The data obtained were used to discuss the genetics of *A. astaci* isolates from *F. rusticus* and to hypothesize the possible role of the detected *A. astaci* strains during invasion attempts of *F. rusticus*.

Table 1. Infection status of selected *Faxonius rusticus* populations from exoskeleton samples collected in 2015. n is the sum of all samples at each location. State abbreviations are: MI = Michigan, WI = Wisconsin, IL = Illinois. The level of infection can range from A0 (not infected) to A7 (very high level of infection). Agent level A0 and A1 are both considered uninfected. Infected samples are written in bold numbers. The percent of infected individuals is also given.

Location, County, State	Latitude	Longitude	n	Agent level								% infected
				A0	A1	A2	A3	A4	A5	A6	A7	
Big Cisco Lake, Gogebic, MI	46°14'15"	-89°27'8"	3	2	1							0
Big Lake Outflow, Vilas, WI	46°8'39"	-89°45'9"	12	6	3	**1**	**1**		**1**			25
Boulder Outflow, Vilas, WI	46°6'35"	-89°41'38"	1		1							0
Des Plaines River, Will, IL	41°38'27"	-88°4'18"	13	6	4	**3**						23
DuPage River, Dupage, IL	41°50'38"	-88°11'55"	18	11	6	**1**						6
Hickory Creek, Will, IL	41°30'29"	-88°5'1"	10	7	3							0
Middle Branch Ontonogan, MI	46°24'39"	-89°7'57"	3	3								0
Prairie River, Lincoln, WI	45°14'7"	-89°38'60"	2			**1**	**1**					100
Vermillion, La Salle, IL	41°15'20"	-89°0'40"	4	3	1							0
Vermillion, La Salle, IL	41°11'33"	-88°54'13"	18	15	3							0
Sum across all locations			84	53	22	**6**	**2**		**1**			11

We define the terms strain, genotype, allele patterns, and haplotypes as follows. A strain is a certain laboratory culture of *A. astaci*, obtained from a single crayfish individual caught from one location. A genotype is a group of strains that share an identical microsatellite allele pattern. These allele patterns are identical with RAPD-genotyping (random amplification of polymorphic DNA) groups. A haplotype is a grouping based on the mitochondrial markers rnnS and rnnL.

Crayfish sampling and *A. astaci* detection

In 2015, we sampled *F. rusticus* in areas of the midwestern United States where it is invasive and has been found previously. *Faxonius rusticus* threatens native *F. virilis* populations in these locations (Olden et al. 2006 and Hill and Lodge 1999). We haphazardly collected 84 crayfish from 10 locations (minimum of 2/site, maximum of 18/site) in Illinois, Wisconsin, and the Upper Peninsula of Michigan, USA (Table 1). Crayfish were collected by hand, housed individually in plastic containers, and transported to the laboratory in a temperature-controlled cooler where they were later euthanized.

To assess the infection status and infection prevalence of crayfish samples with *A. astaci*, we collected exoskeleton samples from the euthanized crayfish. We cut pieces of the soft abdominal cuticle, the inner joints of 2 walking legs, and parts of the uropods off the exoskeleton and stored them, combined as a single sample, in 70% ethanol. We sent these samples to the University of Koblenz-Landau, Germany for further analysis. Storing the infected tissue in 70% ethanol inactivates viable hyphae (WHO 2004), so there was no risk of introducing new pathogen strains into Europe.

We extracted *A. astaci* DNA in one extraction per crayfish sample with a CTAB-method (Vrålstad et al. 2009). We used the ITS region targeting quantitative TaqMan® minor groove binder (MGB) with quantitative real-time PCR (qPCR) after Vrålstad et al. (2009), but we increased the annealing temperature to 62°C and decreased annealing time to 15 s to avoid false positives (Strand 2013). We determined infection status of each crayfish based on the number of PCR forming units (PFU) from *A. astaci* specific qPCR. We based these classifications on Vrålstad et al. (2009), where PFUs are translated into comprehensible, semi-quantitative agent levels. These agent levels are defined as follows: A0 (0 PFU) and A1 (PFU_{obs} < 5 PFU) are considered uninfected, and agent levels A2 (5 PFU ≤ PFU_{obs} < 50 PFU) and higher (A3: 50 PFU ≤ PFU_{obs} < 1.0×10^3 PFU; A4: 1.0×10^3 PFU ≤ PFU_{obs} < 1.0×10^4 PFU; A5: 1.0×10^4 PFU ≤ PFU_{obs} < 1.0×10^5 PFU) are considered infected with *A. astaci*.

Aphanomyces astaci isolation

Isolation of *A. astaci* on axenic cultures facilitates additional genetic analyses and further studies on physiological properties and virulence of the strains. Isolation trials of *A. astaci* were conducted from 41 live crayfish. We collected these *F. rusticus* in 2016 from 4 locations (Table 2) in Vilas County, Wisconsin, USA. Crayfish were transported live to Finland and processed under quarantine conditions at the University of Eastern Finland, Kuopio, Finland, in August 2016. Import permissions and veterinary border inspections were conducted as required by the Finnish Food Safety Authority Evira. Crayfish from different locations were stored separately in closed 2 L tanks containing tap water and monitored daily. The culturing process to isolate *A. astaci* from

crayfish tissue was initiated from moribund individuals within 24 h after they presented as moribund.

Table 2. *Faxonius rusticus* populations collected in year 2016 for *Aphanomyces astaci* isolation. WI = Wisconsin.

Location, County, State	Latitude	Longitude	n
Big Lake Outflow, Vilas, WI	46° 8' 39"	-89° 45' 9"	12
Boulder Outflow, Vilas, WI	46° 6' 35"	-89° 41' 37"	9
Trout Lake, Vilas, WI	46°01'50"	-89°40'31"	15
Plum Lake, Vilas, WI	45°59'32"	-89°33'31"	5
Sum across all locations			41

The isolation process that we followed is described in Viljamaa-Dirks and Heinikainen (2006). We added abdominal cuticles, walking legs, and other parts of the cuticle that contained visible melanizations to peptone glucose (PG1) culture plates (Unestam 1965, Söderhäll et al. 1978) that contained 10 mg/L of both ampicillin and oxolinic acid (Alderman and Polglase 1986). We incubated the culture plates in a 15°C incubator and examined the microbial growth on the plates daily. When *Aphanomyces*-like growth occurred on a plate (morphological identification according to Cerenius et al. (1988) and Oidtmann et al. (1999)), we transferred parts of the culture onto a fresh PG1 plate with a glass ring (Oidtmann et al. 1999) until an axenic culture of *A. astaci* containing only one species was achieved.

Aphanomyces astaci PCR amplification and sequence analyses

To genetically confirm the presence of *A. astaci*, we sequenced part of the ITS barcoding region with the primers 42 and 640 (Oidtmann et al. 2006). We then sequenced the isolated pure-culture samples as well as the crayfish tissue samples with agent levels ≥A3 (PFU > 800). The PCR reaction contained 0.5 µM of both primers (VBC-Biotech, Vienna, Austria), 1X DreamTaq buffer, 0.2 µM dNTP mix, and 0.025 u DreamTaq polymerase (all Thermo Fisher Scientific, MA,

USA). We added PCR-grade water to bring this mixture to a volume of 23 µL. Finally, we added 2 µL DNA with a concentration of ~20 ng/µL to the reaction mixture. PCR conditions were identical to that used by Oidtmann et al. (2006). The sequencings of the PCR products were done in Seq.IT GmbH & Co (Kaiserslautern, Germany) with a 3730 DNA Analyzer sequencer (Applied Biosystems, MA, USA). We aligned and edited the sequences with Geneious® 7.1.7 (Auckland, New Zealand) (Drummond et al. 2011). We confirmed species identity by applying the Basic Local Alignment Search Tool (BLAST) to the nonredundant database of National Center for Biotechnology Information GenBank (NCBI).

We identified the *A. astaci* haplogroups from the sequenced samples according to Makkonen et al. (2018). Additionally, we sequenced a 363-basepair (bp) long fragment of the nuclear chitinase gene according to Makkonen et al. (2012b) for further estimation of the genetic group present in the samples. We edited the sequences with the software Geneious® 7.1.7 and determined the *A. astaci* haplotypes by comparing them to reference sequences (MF973121-MF973149 for the small ribosomal subunits (rnnS), MF975950-MF975978 for the large ribosomal subunit (rnnL), and JQ173338, JQ173357, JQ173360, and JQ173351 for chitinase) in the NCBI GenBank database. We submitted the sequences produced in this study to NCBI GenBank with the accession numbers MG952474–MG952486 (for rnnS and rnnL), MG932070-MG932075 (for chi), and MG964326–MG964331 (for ITS).

The sample from the Big Lake Outflow, Vilas County, WI, with agent level A5 (Table 1) and axenic cultures were successfully amplified and sequenced with rnnS, rnnL, and chitinase primers. The other samples could not be amplified successfully because of the low amount of *A. astaci* DNA in the samples.

Aphanomyces astaci microsatellite genotyping

We genotyped the microsatellites of the axenic cultures and the tissue samples that had agent levels ≥A3 in the qPCR analysis. The multilocus genotype was determined by scoring 9 microsatellite markers (Grandjean et al. 2014). For PCR amplification, we used a Multiplex PCR Kit (QIAGEN GmbH, Germany). The reaction mixture (6 µL) contained 1 µL DNA template and 0.1 µM of each primer (Grandjean et al. 2014) with the exceptions of primers Aast 6 and Aast 9, of which we added 0.42 µM and 0.38 µM, respectively. The PCR conditions were as follows: initial denaturation at 95°C for 2 min, 35 cycles of 95°C for 30 s, 54°C for 90 s and 72°C for 60 s, followed by a final elongation step at 72°C for 5 min. We used a PeqStar 96X Cycler (Peqlab) for this PCR reaction. We analyzed the microsatellites with a CEQ 8000 (Beckman Coulter). Reaction mixture for fragment analysis contained 0.5 µL PCR product, 28.5 µL SLS Buffer and 0.3 µL DNA size standard 400 (both Beckman Coulter). We used the software GeneMarker v. 1.95 (SoftGenetics LLC, State College, PA, USA) to analyze the results of the microsatellite analyses.

The microsatellite genotyping was conducted for the axenic cultures *A. astaci* isolates from Trout Lake Vilas County, WI, USA and for 1 tissue sample from Big Lake Outflow, Vilas County, WI with agent level A5 in the qPCR.

RESULTS

Aphanomyces astaci detection

Nine of the 84 sampled *F. rusticus* tested positive for *A. astaci* infection (11%; Table 1, Fig. 1) based on qPCR. We found infected samples at 4 out of 10

locations. One sample, from the Big Lake Outflow, Vilas County, WI, was highly infected (agent level A5). The infections in the other samples with *A. astaci* were either very low (A2; *n* = 6) or low (A3; *n* = 2).

Fig. 1. Map showing the location of the different sampling sites in this study. Triangles indicate that no Aphanomyces astaci infection was detected in this *Faxonius rusticus* population, circles represent sites at which populations tested positively, and

Aphanomyces astaci **isolation and ITS sequence analysis**

We isolated *A. astaci* from 2 *F. rusticus* individuals from Trout Lake (Table 2). All 6 axenic cultures were confirmed to be *A. astaci* by ITS-sequencing – the sequences were 99.7-100% identical to those of *A. astaci* available in GenBank

(sequence AM947023 is an example) (Vrålstad et al. 2009). The ITS-sequences of these 6 isolates were also 100% identical to each other.

Sequence analyses and genotyping of *A. astaci*

The genotyping and the sequence analyses were conducted for tissues of 1 *F. rusticus* individual from Big Lake Outflow expressing high A5 agent levels of *A. astaci* in qPCR and for axenic cultures of *A. astaci* obtained from *F. rusticus* of Lake Trout. The rnnS and rnnL sequence analysis of the sample from Big Lake Outflow was identical to the A-haplogroup (Makkonen et al. 2018). The sequencing results of *A. astaci* from crayfish tissue in Big Lake Outflow corresponded to *A. astaci* A-haplogroup based on the rnnS and rnnL sequence analysis. The nuclear chitinase gene sequence, on the other hand, was identical to those of RAPD-groups B, C, and E. The sequences of the axenic cultures from Trout Lake also corresponded to the A-haplogroup. The rnnL sequences were 100% identical to each other and to the sequences of the A-haplogroup, with a single exception. Isolate UEF_Or #7b (MG952485) had 2 deletions compared with other isolates and the A-haplogroup. However, the deletions were not confirmed by independent secondary analysis.

The microsatellite genotyping of the samples gave consistent results, i.e. all samples had the same microsatellite allele pattern, henceforth called rust1-genotype. This multilocus genotype was different from all of the currently known *A. astaci* genotypes (Table 3). All microsatellite markers were homozygous except for Aast 9, which was heterozygous. The new multilocus genotype consisted of allele sizes already detected in other previously analyzed strains, but in a different combination.

Table 3. Allele sizes of the 9 different microsatellite loci (Aast2 to Aast14) used in the analysis. SSR-A to E are the reference strains. rust1 is the new genotype from this study. Results are from the mixed tissue sample from Big Lake Outflow Vilas, WI, as well as the 2 pure cultured *Aphanomyces astaci* isolates from Trout Lake, Vilas, WI are combined, as they were identical.

Genotype	Aast2	Aast4	Aast6	Aast7	Aast9	Aast10	Aast12	Aast13	Aast14
rust1	145	89	151	191	164/168	135	238	203	247
SSR-A_2	161	105	160	207	178	145	–	195	245
SSR-B	145	89	151	215	164/182	135	226/238	203	247
SSR-C	155	89	151	191/215	164/168	135	226	203	247
SSR-D	139	133	151	203	178	145	232	195	249
SSR-E	150	89/91	151/160	207	168/182	135/145	232/238	195/203	249

DISCUSSION

Our results confirm for the first time that *F. rusticus* is a carrier of the crayfish plague disease agent *A. astaci.* Furthermore, we report sequence analysis and genotyping of *A. astaci* of crayfish collected from a wild population in North America. We showed that the genotype of the obtained *A. astaci* isolates were different from the *A. astaci* genotypes that have been found in Europe. Finally, our study provides a first insight regarding the occurrence of *A. astaci* in wild crayfish in North America. Currently, we lack large-scale data on *A. astaci* infection prevalence and genetic variability in North America. These findings add to the existing knowledge about the hazards of transporting alien species to novel regions with the potential of also transporting their diseases and parasites, both of which might be detrimental to native species of that region (e.g., Jussila et al. 2016a).

The qPCR screening of *A. astaci* showed that infection prevalence was low (approximately ~11%) among the studied *F. rusticus* populations. Further, the infected individuals had small amounts of *A. astaci* DNA in their tissues – generally agent levels A2 and A3. These results are similar to some of the observations in other North American crayfish species that were sampled in Europe and Brazil (e.g., Pârvulescu et al. 2012, Filipová et al. 2013, Tilmans et al. 2014, Peiro et al. 2016, Panteleit et al. 2018), although there are also reports of heavily infected (e.g., Strand et al. 2012, Keller et al. 2014) or non-infected (Schrimpf et al. 2013b) North American species in Europe. Based on our results, *F. rusticus* can be added to the growing list of known vector species of *A. astaci* (Unestam and Weiss 1970, Vey et al. 1983, Diéguez-Uribeondo and Söderhäll 1993, Schrimpf et al. 2013a, Tilmans et al. 2014, Mrugała et al. 2014, Panteleit et al. 2017).

The microsatellite analysis revealed a new *A. astaci* genotype. The same genotype was detected in all the samples we analyzed (the 6 axenic cultures of *A. astaci* from Trout Lake and the tissue sample of 1 infected crayfish from Big Lake Outflow). Trout Lake and Big Lake Outflow are different water bodies, but are in close proximity with a distance of about 26 km through streams and lakes (Fig.1), which may explain why these samples share a genotype. We also may have identified an *A. astaci* genotype that is specific to *F. rusticus*, as almost all the described *A. astaci* genotypes to date are associated with 1 specific species of crayfish (Grandjean et al. 2014, Maguire et al. 2016), although interspecific transmission probably occurs (James et al. 2017). The overall genetic diversity of *A. astaci* in North America is probably considerably higher than detected so far in studies conducted with isolates of European origin given that the North American continent has the highest diversity of crayfish compared with the rest of the world (Crandall and Buhay 2008).

The sequences of the mitochondrial ribosomal subunits indicated that both the mixed tissue samples from Big Lake Outflow and the pure-culture isolates from Trout Lake belonged to the A-haplogroup. It includes the RAPD-groups A and C (Makkonen et al. 2018). The combined results of the rnnS, rnnL, and chitinase genes indicate a higher relatedness of the strain to RAPD-PCR group C than to RAPD-PCR group A. These results are in line with previous findings of the *A. astaci* A-haplogroup in the genus *Faxonius*, f.k.a. *Orconectes* (Makkonen et al. 2018).

Future studies of *A. astaci* in *F. rusticus* should concentrate on the original distribution area of this species in the Ohio River basin to determine the possible origin and other host species of the A-haplogroup in this area. The potentially frequent contacts of different, and especially invasive, host species could

influence the effects of so far host-specific *A. astaci* strains. For example, a particular strain of *A. astaci* associated with *F. rusticus* could reduce the fitness of competing species at the invasion front of *F. rusticus* (e.g., Edsman et al. 2015, Jussila et al. 2016b). In a European study, a population of the noble crayfish, *Astacus astacus,* was susceptible to an *A. astaci* strain isolated from latently infected stone crayfish, *Austropotamobius torrentium*, indicating different susceptibility of different European crayfish species to *A. astaci* strains (Jussila et al. 2017).

American crayfish species can exhibit mortality associated with *A. astaci* infection when they are artificially infected with a 2nd strain from another crayfish species host (Aydin et al. 2014). There are several studies on the invasion biology of *F. rusticus* (Hill et al. 1993, Perry et al. 2001, Olden et al. 2006, Loughman et al. 2009, Kilian et al. 2010, Bouchard et al. 2011), but the possible role of *A. astaci* in this context remains to be investigated. Furthermore, crayfish populations threatened by invasions of *F. rusticus*, or in the process of being invaded, should be sampled and screened for *A. astaci* infections. If possible, the pathogen should be isolated in single cultures as these isolates could then be used to evaluate its virulence in future infection experiments, assess host-pathogen interactions, and assess crayfish interactions in the absence or presence of *A. astaci*. Our results provide further warnings regarding the risks of introducing a species to new region, as these introductions often also result in introductions of parasites and diseases, which could be more detrimental than the introduced species itself.

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Author contributions. AS, JM, KT, TH, and RS designed the study. BP collected samples. JM, JP, JJ, and BP conducted biological lab work. JP and JM conducted genetic lab work. JP, JM, JJ, AS, and KT analyzed data. JP drafted the manuscript. BP prepared the figures. All authors participated in manuscript preparation and revision.

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13. DECLARATION

I hereby declare that I independently wrote this thesis with the title "Estimating the threat posed by the crayfish plague agent *Aphanomyces astaci* to crayfish species of Europe and North America — Introduction pathways, distribution and genetic diversity". All used resources and references are specified in this work. Contributions of coworkers and coauthors have been clearly identified.

I did not use the assistance of a doctoral consultant (or a similar person) in return for payment.

I did not, and have never, submit this PhD thesis in identical or similar form elsewhere for a scientific examination.

I am aware that a violation of the above mentioned points can lead to a withdrawal of the doctoral degree and additional legal consequences.

Landau, April 16, 2019 Jörn Panteleit

Authors' contribution to appendices I-VI

Appendix I

I did parts of the laboratory work and analyses of the samples and wrote the first draft of the manuscript.

Appendix II

I drafted the genetic research question and conducted the genetic laboratory work and analyses. Genetic parts in the manuscript were written by me.

Appendix III

I conducted parts of the genetic laboratory work and wrote the parts in the manuscript concerning my own laboratory contribution.

Appendix IV

I did parts of the laboratory analyses of the samples and wrote the first draft of the manuscript.

Appendix V

I did parts of the laboratory analyses of the samples and wrote the first draft of the manuscript.

Appendix VI

I was involved in the design of the study and conducted the genetic laboratory work on the samples and the data analyses. I wrote the first draft of the manuscript.

14. CURRICULUM VITAE

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Supervisor:	Prof. Klaus Lunau from the department for Sensory Ecology

Publications

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Conference Contributions

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