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Epidemiology of crayfish plague

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Epidemiology of crayfish plague

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Academic dissertation

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Abstract

Crayfish plague is a severe disease of European crayfish species and has rendered the indigenous crayfish populations vulnerable, endangered or even extinct in the most of Europe. Crayfish plague is caused by an oomycete *Aphanomyces astaci*, a fungal-like water mould that lives its vegetative life in the cuticle of crayfish and infects other crayfish by producing zoospores. Zoospores swim around for a few days in search of crayfish, and when they find one they attach onto its surface, encyst and germinate to start a new growth cycle as new growing hyphae penetrate the crayfish tissues. Unrestricted growth of *A. astaci* leads to the death of the infected animal in just a few weeks.

Crayfish plague induced mortalities started in Italy around 1860. Although the disease was known about since 1860 its cause remained unknown for several decades. Little was done to prevent the spread of the disease. A lively crayfish trade probably facilitated the spread of the crayfish plague, which reached Finland in 1893. The crayfish plague has remained the most important disease problem of the Finnish noble crayfish *Astacus astacus*, since then. The consensus was that the disease killed all infected animals in a short time, and it appeared almost impossible to restore the flourishing crayfish populations to the levels that existed before. Following the example of neighbouring Sweden, a North American crayfish species, the signal crayfish *Pacifastacus leniusculus* that appeared resistant to crayfish plague was introduced to Finland in 1960s. As expected, the signal crayfish slowly started to replace the lost populations of the noble crayfish to become an important part of the crayfish fisheries.

The introduction of the signal crayfish significantly added to the management problems of the noble crayfish stocks left. Signal crayfish appeared to be a chronic carrier of the crayfish plague agent, and spread the disease to the dwindling vulnerable noble crayfish populations. Later research showed that the crayfish plague agent is a parasite of North American crayfish that in normal circumstances does not harm the host animal. Intriguingly, the crayfish plague agent carried by the signal crayfish, genotype Ps1, is different from the pathogen originally introduced into Europe, genotype As.

The diagnosis of crayfish plague especially when based on the isolation of the pathogen is challenging and accordingly the genotype difference was mostly unrecognized until recently. In this study we determined the genotype of the causative agent from most of the detected Finnish crayfish plague cases between 1996 -2006. It appeared that most of the epidemics in the immediate vicinity of signal crayfish populations were caused by genotype Ps1, whereas genotype As

was more prevalent in the noble crayfish areas. Interestingly, a difference was seen in the outcome of the infection. The Ps1 infection was always associated with acute mortalities, while As infections were also frequently found in existing but weak populations. The persistent nature of an As infection could be verified in noble crayfish from a small lake in southern Finland. This finding explained why many of the efforts to introduce a new noble crayfish population into a water body after a crayfish plague induced mortality were futile.

The main conclusion from the field study data of this research was the difference in virulence between the Ps1 and As genotype strains. This was also verified in a challenge trial with noble crayfish. While the Ps1 strains did not show much variation in their growth behaviour or virulence, there was much more variation in the As strains. The As genotype arrived in Finland more than 100 years ago, and since that date it seems to have adapted to the novel host, the noble crayfish, to some extent. In order to gain insight into a possible vector of this genotype, we studied another North American crayfish species present in Europe, the spiny-cheek crayfish *Orconectes limosus* from a Czech pond. This crayfish species appeared to carry a novel genotype of *A. astaci*, named Orconectes genotype, designated “Or”. It seems possible that many of the North American crayfish species carry their own type of crayfish plague agent, with variable features such as virulence. These differences should be further tested in the future.

The results of this study alleviate the necessity to study the noble crayfish mortalities for the verification of crayfish plague, including the study for the genotype of the *A. astaci* strain. Crayfish fisheries and conservation management decisions should not be made without a prior control of the donating population and the receiving water body for the eventual presence of a low-virulent *A. astaci*.

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List of original publications

This thesis is based on the following publications

I Viljamaa-Dirks S, Heinikainen S, Nieminen M, Vennerström P, Pelkonen S (2011) Persistent infection by crayfish plague *Aphanomyces astaci* in a noble crayfish population- a case report. Bulletin of the European Association of Fish Pathologists, 31(5): 182-188.

II Kozubíková E, Viljamaa-Dirks S, Heinikainen S, Petrusek A (2011) Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague pathogen *Aphanomyces astaci*. Journal of Invertebrate Pathology, 108: 214-216.

III Viljamaa-Dirks S, Heinikainen S, Torssonen H, Pursiainen M, Mattila J, Pelkonen S (2013) Distribution and epidemiology of genotypes of the crayfish plague agent *Aphanomyces astaci* from noble crayfish *Astacus astacus* in Finland. Diseases of Aquatic Organisms, 103: 199-208.

IV Viljamaa-Dirks S, Heinikainen S, Virtala A-MK, Torssonen H, Pelkonen S (2016) Variation in the hyphal growth rate and the virulence of two genotypes of the crayfish plague organism *Aphanomyces astaci*. Journal of Fish Diseases, 39: 753-764.

The publications are referred to in the text by their Roman numerals. The original publications are reprinted with the permission of their copyright holders: European Association of Fish Pathologists (I), Elsevier (II), Inter-Research (III) and John Wiley and Sons (IV)

Abbreviations

AFLP-PCR	amplified fragment length polymorphism PCR
As	*Aphanomyces astaci* genotype group Astacus (genotype A)
DNA	deoxyribonucleic acid
ICS	indigenous crayfish species
ITS	the internal transcribed spacer
$MgCl_2$	magnesium chloride
mRNA	messenger ribonucleic acid
NACS	North American crayfish species
NICS	non-indigenous crayfish species
OIE	World Organization for Animal Health (Office International des Epizooties)
Or	*Aphanomyces astaci* genotype group Orconectes (genotype E)
Pc	*Aphanomyces astaci* genotype group Procambarus (genotype D)
PCR	polymerase chain reaction
proPO	prophenoloxidase
Ps1	*Aphanomyces astaci* genotype group Pacifastacus I, or PsI (genotype B)
Ps2	*Aphanomyces astaci* genotype group Pacifastacus II, or PsII (genotype C)
RAPD	random amplification of polymorphic DNA
RAPD-PCR	random amplification of polymorphic DNA- polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
WCA	water catchment area (according to the Finnish Environment Institute)

1 Introduction

The crayfish plague agent *Aphanomyces astaci* (Schikora,1903) was accidentally introduced into Europe from North America around 1860, and since then it has evoked mass mortalities in all indigenous crayfish species (ICS) of European origin (for reviews see Alderman 1996, Söderhäll and Cerenius 1999, Edgerton et al. 2002). North American crayfish species (NACS) appeared resistant to disease caused by *A. astaci.* This led to the introduction of NACS into Europe to compensate and replace the losses in the European ICS populations. The first introduction of a NACS was the spiny-cheek crayfish *Orconectes limosus* (Rafinesque, 1817), which was imported into Poland in 1890 (reviewed in Souty-Grosset et al. 2006). The noble crayfish *Astacus astacus* (Linnaeus, 1758) populations in Sweden had suffered greatly from the crayfish plague and another NACS was sought as a replacement. The signal crayfish *Pacifastacus leniusculus* (Dana, 1852) was found suitable considering its size and environmental adaptation (Fürst 1977). Large scale introductions of signal crayfish into the Swedish water bodies started in the 1960s, and were soon followed by Finland (Fürst 1977, Nylund and Westman 1995b, Bohman et al. 2006).

Although there was some understanding of the resistance of NACS to the acute disease caused by *A. astaci* (Fürst 1977), little was known about the defence mechanisms of the host animal or the parasitic abilities of the crayfish plague agent, or about its genetic variation in specific hosts. Although the introduction of the signal crayfish has revived the crayfish fisheries in Sweden and in Finland, (Westman 1991, Jussila and Mannonen 2004, Souty-Grosset et al. 2006), its success has complicated the crayfish population management considerably. The management strategies must now shift towards the conservation of the only ICS in Northern Scandinavia, the noble crayfish. Crayfish plague is the main threat for the remaining noble crayfish populations. The key to the successful management of a parasitic disease is the good understanding of the epidemiological features of the causative agent.

In this thesis, the genetic variation of the crayfish plague agent from the Finnish crayfish plague epidemics was studied. Explanation was sought for the variable outcome of the infection in noble crayfish populations, as well as for the reason for the failures in population re-introduction efforts.

2 Review of the literature

2.1 Crayfish plague agent *Aphanomyces astaci*

2.1.1 Taxonomy, morphology and life cycle

Crayfish plague is caused by the oomycete organism *Aphanomyces astaci* (Schikora 1903, Nybelin 1936). The Oomycetes are a group of organisms that were earlier classified as fungi due to the fungal-like growth pattern. Phylogenetic analysis rearranged the *Oomycota* as protists, together with brown algae and diatoms in a group called Stramenopiles (reviewed by Levesque 2011). Oomycetes are generally referred to as water moulds, although several are known as parasites or saprophytes of terrestrial organisms (see review by Kamoun 2003). *Aphanomyces* species belong to the Saprolegniales, a group also including the well-known fish parasitic species *Saprolegnia* spp. (Leclerc et al. 2000). Even the genus *Aphanomyces* is associated with a serious fish disease, the mycotic granulomatosis or EUS (epizootic ulcerative syndrome) caused by *A. invadans* (Lilley et al. 2003). In addition to the aquatic oomycetes associated with pathology of fish or crustaceans, a wide variety of saprophytic species are known to exist in the freshwater environment and most likely there are still numerous of such species to be discovered and described.

It is not possible to define *A. astaci* by species-specific morphological characters and traditionally the species was recognized by challenge tests, which were performed to determine the pathogenicity of the agent towards susceptible crayfish species (Cerenius et al. 1988). Later, the species definition was supported by analysing the internal transcribed spacer (ITS) in the nuclear ribosomal DNA (Diéguez-Uribeondo et al. 2009, Takuma et al. 2010, Makkonen et al. 2011).

The vegetative stage of *A. astaci* comprises a mycelium formed by fungal-like hyphae first described in detail by Rennerfelt (Rennerfelt 1936). The hyphae are aseptate, diffusely branching, uniform 7.5-9.5 µm wide and colourless. The infective stage is a zoospore. Spores are formed in sporangia that are of even width with the hyphae but separated from them by a septum. Inside the sporangium, primary spores are developed from the cytoplasm, and protrude from the tip of the sporangium to form a cluster or spore ball, consisting of 10-40 individual spores encysted as primary cysts. After a resting period, these cysts develop into swimming zoospores, which are 9-11 (8-15) µm in diameter and have two flagella. The zoospore is capable of directing towards nutrients (Cerenius and Söderhäll 1984a). After finding a suitable growth substrate, the zoospore attaches to the surface and sheds its flagella, thus forming a secondary cyst that can germinate to

start new hyphal growth. An exhaustive description of the morphology of the different life stages is given in the OIE Aquatic Manual (OIE 2012).

Although the first authors who described *A. astaci* reported oogonia, these reports were sporadic and inconsistent considering the dimensions, which suggests that other oomycete species might have been involved (Rennerfelt 1936). Later research has never revealed any evidence of sexual propagation of *A. astaci*, and thus it does not support the existence of a long-lived resting stage outside the host. Moreover, no long term existence outside the crustacean host in natural conditions has ever been detected or reported either.

The crayfish plague agent is a highly specialized parasite that found its ecological niche in the crustacean cuticle, where it normally grows restricted by the host immunological defence, but protected from competition by environmental organisms. Transmission between the hosts only occurs through the zoospores (Fig. 1). The zoospore is relatively short-lived but is capable of swimming for a few days (Alderman and Polglase 1986). However, the chance to find a new suitable host is enhanced by repeated zoospore emergence, a mechanism that allows a zoospore to encyst and release a new zoospore in the event that the first growth substrate located appears unsuitable (Cerenius and Söderhäll 1984b). This survival mechanism is typical for parasitic oomycetes (Diéguez-Uribeondo et al. 2009) and can be repeated experimentally at least three times for *A. astaci* (Cerenius and Söderhäll 1985) *in vitro*.

The exact mechanism of how the spore production is triggered is not known, but in general the lack of nutrients seems to trigger the formation of sporangia *in vitro* (Cerenius et al. 1988). In general, the majority of spores are formed when the crayfish host is moulting or dying (Makkonen et al. 2013), but a continuous release of spores has also been demonstrated even from symptom-free carrier crayfish (Strand et al. 2012).

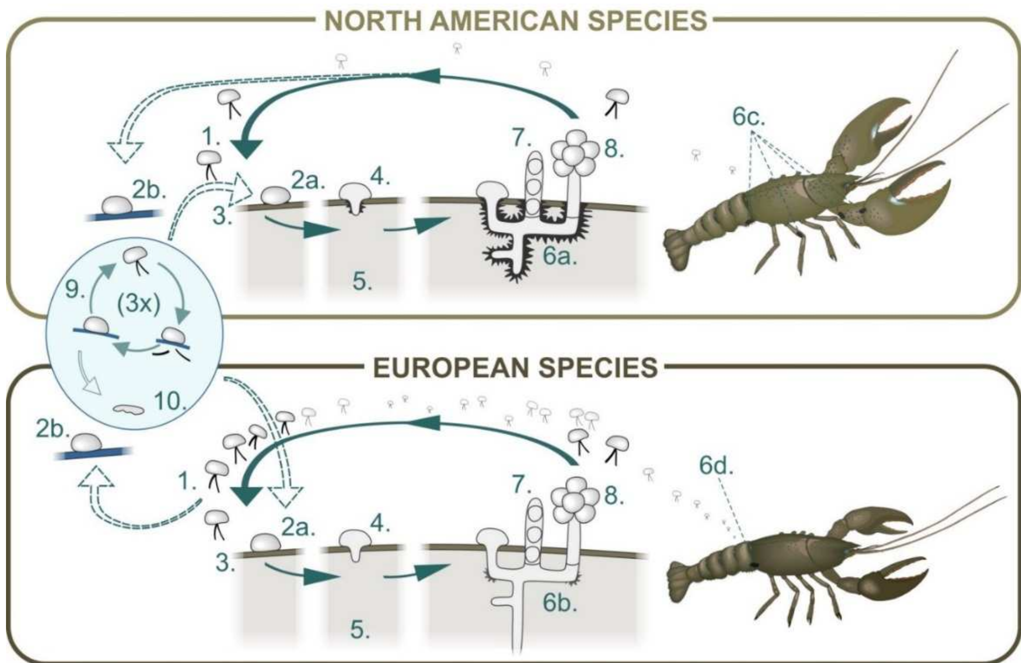

Figure 1. *Aphanomyces astaci* life cycle in the natural North American crayfish host, compared to its life cycle in the novel European crayfish host. The drawing is an imitation of the illustration by Iñaki Diéguez-Uribeondo (Souty-Grosset et al. 2006 'Atlas of crayfish in Europe'), © LUKE

1. infective unit, the secondary zoospore, is released from the primary cyst;
2a. encysting zoospore forms secondary cyst on crayfish;
2b. encysting zoospore on unsuitable surface;
3. crayfish epicuticle;
4. germinating cyst;
5. crayfish cuticle;
6a. melanised hyphae, American crayfish species;
6b. unmelanised or weakly melanised hyphae, European crayfish species;
6c. macroscopic dark melanised spots on American crayfish species;
6d. occasional macroscopic melanised spot on European crayfish;
7. sporangium with primary spores;
8. spore cluster with primary cysts;
9. secondary cyst forms new zoospore (may be repeated three times);
10. unviable dead cyst (no host found).

(Pursiainen and Viljamaa-Dirks 2014)

2.2 Epidemiology and host specificity

2.2.1 *A. astaci* infection in natural and novel hosts

There is a wide consensus about *A. astaci* being a native parasite of North American crayfish (Unestam and Weiss 1970, Unestam 1975, OIE 2012). NACS are relatively resistant to the disease crayfish plague, often carrying *A. astaci* in their cuticle as a latent infection, with mortality occurring only in stress situations (Unestam and Weiss 1970, Unestam et al. 1977, Persson and Söderhäll 1983). Crustacean immunity has been studied in depth mainly due to the need to understand the effect of *A. astaci* on its host (Söderhäll and Cerenius 1999). Crayfish are invertebrates and thus have no immunological memory in the form of antibodies (adaptive immunity). Therefore their immunological defence relies on innate immune response mechanisms (Söderhäll and Cerenius 1992). This involves the activation of the so-called prophenoloxidase-system (proPO) by the pattern recognition of non-self structures such as the β-1,3-glucans in the cell walls of the oomycetes (Söderhäll and Cerenius 1992). The end product from this cascade response is the pigment melanin, which surrounds and restricts the growth of the invading hyphae. An advanced infection by *A. astaci* in NACS can reveal itself by the dark brown melanised spots seen on any part of the exoskeleton (Unestam and Weiss 1970), but an individual or a population can also be infected without any visible sign of the presence of *A. astaci* (Vrålstad et al. 2011).

The response of ICS in Europe after being infected with the crayfish plague agent from 1860s was different to that of NACS. The infection was first noticed as mass mortalities of crayfish populations, and the rapid spread and severity of the phenomenon gave the syndrome its ominous name ”the crayfish plague”. All European ICS appeared highly susceptible to an acute disease by the infection of *A. astaci*, including the southern and western *Austropotamobius* spp., in addition to the eastern and northern *Astacus* spp.

Studies of the pathobiology mostly showed 100% mortality in highly susceptible species under laboratory conditions. The development of the pathology depended on a combination of the infective dose of zoospores and the water temperature (Alderman and Polglase 1986, Alderman et al. 1987, Cerenius et al. 1988).

The basic defence mechanism against invaders relies on the same crustacean immunity mechanism for both the European ICS and the NACS. An experimental challenge by proPO activating polysaccharides in the noble crayfish increased the levels of proPO messenger ribonucleic acid (mRNA) in the haemocytes, which shows the ability of the crayfish to react to an invader (Cerenius et al. 2003). The reaction in signal crayfish is different, in that the proPO transcript was found to be at a permanently high level and could not be elevated further by challenge.

The crayfish plague agent has evolved to cope with this efficient defence mechanism in the natural NACS host, but the European ICS were unprepared for meeting this challenge. The insufficient defence reaction led to the catastrophic imbalance between *A. astaci* and its novel host animals.

The crayfish plague agent was traditionally seen as specialized only to have freshwater crayfish as hosts. Many other crustacean groups that live in freshwater were tested for their susceptibility to *A. astaci* but with negative results (Unestam 1969, Svoboda et al. 2014b). Only the Chinese mitten crab *Eriocheir chinensis* (Milne Edwards, 1853) that lives part of its life cycle in freshwater has been found able to support and transmit *A. astaci* (Schrimpf et al. 2014, Svoboda et al. 2014b), and the freshwater crab *Potamon potamios* (Olivier, 1804) that cohabited a lake with infected signal crayfish was also found to be infected (Svoboda et al. 2014b). Other freshwater crustaceans such as freshwater shrimps have not provided conclusive evidence of having the ability to act as a host for *A. astaci* (Svoboda et al. 2014a).

2.2.2 Genotypes and geographic distribution of *A. astaci*

The amplification of DNA by the polymerase chain reaction (PCR) using arbitrary oligonucleotides as primers is a technique that is used to reveal genetic differences between different isolates of organisms. One variant of this method is called random amplification of polymorphic DNA (RAPD-PCR) (Welsh and McClelland 1990, Williams et al. 1990). The RAPD-PCR was also used to characterise the isolates of *A. astaci* from different sources (Huang et al. 1994). In the original study, two clearly distinct groups and one single strain in addition to these two were recognised. Sexual propagation has not been found in *A. astaci*, thus a high degree of genetic similarity was seen inside those groups, in spite of the large geographical and time span of the isolations. The first main group consisted of isolates from noble crayfish stocks in Sweden and one isolate from the narrow-clawed crayfish *Astacus leptodactylus* (Eschscholtz, 1823) from Turkey. These *A. astaci* strains were present in European waters before the introductions of the signal crayfish, and are called Astacus-strains or group A (hereafter referred to as As). The As genotype strains are therefore generally assumed to represent the first genotype of *A. astaci* accidentally released into Europe about 150 years ago. The original NACS host of this genotype group is unknown. The other main group was formed by isolates from signal crayfish from USA and Sweden, and also from noble crayfish specimens from Sweden after the introductions of signal crayfish. This group is called Pacifastacus strain I or group B (hereafter referred to as Ps1). A third type was represented by a single isolate from signal crayfish, imported into Sweden from Canada; this is called the

Pacifastacus strain II or group C (hereafter referred to as Ps2). Since this original study, a fourth genotype was detected in Southern Europe, carried by the red swamp crayfish *Procambarus clarkii* (Girard, 1852) (group D, hereafter referred to as Pc (Diéguez-Uribeondo et al. 1995). The assumed original continent-wide North American endemic area of *A. astaci* and the numerous NACS inhabiting it has most probably led to more genetic variation yet to be discovered.

The first reported crayfish mass mortalities that were presumably caused by crayfish plague strain As occurred in Europe in 1859, and during the following decades the disease completely destroyed many populations of indigenous crayfish throughout Europe (Alderman 1996). It is unknown how the infection originally was introduced. The first documented intentional introduction of an American crayfish, *Orconectes limosus*, dates from 1890 (Souty-Grosset et al. 2006, Holdich et al. 2009). Although this species has not been stocked in large numbers for aquaculture purposes, it has spread widely in Central Europe (Petrusek et al. 2006, Souty-Grosset et al. 2006) and has been verified as the source of *A. astaci* infection at least in the Czech Republic (Kozubiková et al. 2011, Kozubiková-Balcarová et al. 2014). Large-scale dispersal of the economically more rewarding NACS the signal crayfish (Westman 1991, Gherardi and Holdich 1999, Souty-Grosset et al. 2006, Petrusek and Petrusková 2007, Weinlaender and Fuereder 2009, Skov et al. 2011, Holdich et al. 2014) and the red swamp crayfish (Huner 1977, Souty-Grosset et al. 2006, Loureiro et al. 2015) resulted in new epidemics of *A. astaci* (Bohman et al. 2006). Relatively little is known about the role of different genotypes in earlier epidemics of the crayfish plague. Some studies based on RAPD-PCR have verified the presence of Ps1 genotype causing the disease in ICS in Sweden, Finland, England, Spain and Germany (Huang et al. 1994, Lilley et al. 1997, Vennerström et al. 1998, Diéguez-Uribeondo and Söderhäll 1999, Oidtmann et al. 1999a) and of the Pc genotype in Spain (Rezinciuc et al. 2014). The As genotype was encountered much less often, and its findings were in the first place restricted to Sweden, Finland and Turkey (Huang et al. 1994, Vennerström et al. 1998). Improved molecular methods have only recently started to add more to our understanding of the distribution of the different genotypes throughout Europe (Grandjean et al. 2014). As can be expected, wherever NACS are present or are in the vicinity, disease in nearby ICS seems to be caused by *A. astaci* strains connected with the specific NACS (Kozubiková-Balcarová et al. 2014, Maguire et al. 2016). The assumed spread of the different genotypes of *A. astaci* into Europe is depicted in Fig. 2.

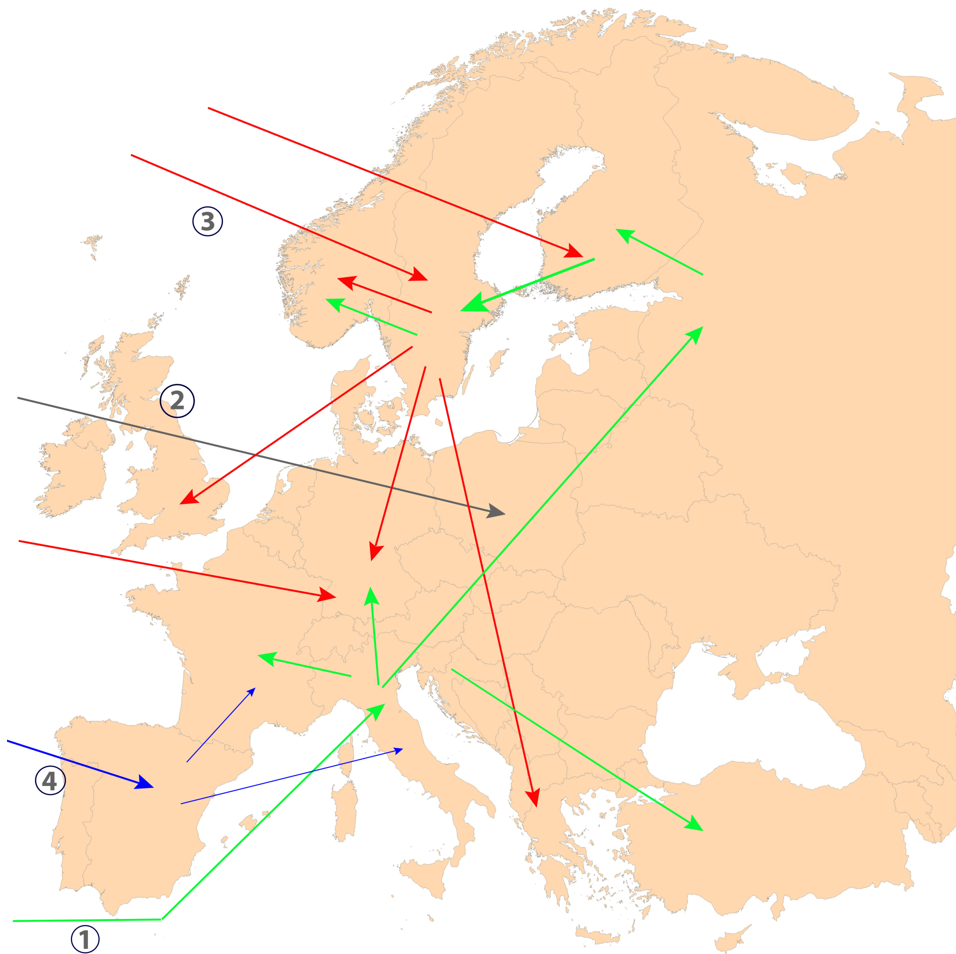

Figure 2. Assumed main introduction paths and spread of the different genotypes of *Aphanomyces astaci* in Europe as based on the verified cases of crayfish plague, and/or knowledge of the introduction of the acknowledged host species (Souty-Grosset et al. 2006). ① green arrows: genotype As (modified from the original drawing of Alderman (1996); ② black arrow: genotype Or (II); ③ red arrows: genotype Ps1; ④ blue arrows: genotype Pc. Map©karttakeskus.fi

Although the first report to describe the genotypes of *A. astaci* was published in the early 1990s (Huang et al. 1994), there have been relatively few attempts at exploring the possible variable features between the genotypes. In general, the lack of sufficient numbers of isolates from each genotype has hampered any comparative studies being made. The Pc genotype was recognised as being able to cope with warmer water temperatures than the other three genotypes known at the time (Diéguez-Uribeondo et al. 1995). Differences in the chitinase genes were detected between the genotypes As and Ps1 (Makkonen et al. 2012a), which possibly has a link to the virulence of the strains: the enzyme chitinase is expressed

by the crayfish plague organism when the hypha of the oomycete grows into the chitin containing cuticle of the host (Andersson and Cerenius 2002). Other possible virulence affecting factors are numerous. The production of zoospores, the ability to locate and attach to the host, germinate and invade the cuticle (Cerenius and Söderhäll 1984a, Cerenius et al. 1988), the production of different enzymes apart from the chitinases (Söderhäll and Unestam 1975, Söderhäll et al. 1978, Persson et al. 1984, Diéguez-Uribeondo and Cerenius 1998, Bangyeekhun et al. 2001) or the ability to repeatedly produce a new zoospore in search for the host (Cerenius and Söderhäll 1984b). Each of these variable features can be subject to evolution. However, variations in the virulence factors between the genotypes have not been studied extensively so far.

2.2.3 Crayfish and crayfish plague in Finland

The noble crayfish *A. astacus* is an indigenous crayfish species to Finland that originally inhabited the southern lakes and rivers, but gradually was introduced throughout the whole of Finland south of the Arctic Circle (Westman 1991). Noble crayfish is an economically important fishery species, whose value is estimated to cover about 10% of the freshwater fisheries in Finland (Savolainen et al. 2012, Pursiainen and Erkamo 2014). In earlier times the noble crayfish was one of the most sought after fishery export items, and between 2 to 15 million individual crayfish were exported to neighbouring countries annually (Westman 1991). Unfortunately, the crayfish plague arrived in Finland in 1893 and it devastated most of the main populations of noble crayfish during the following decades (Järvi 1910, Westman 1991). Currently, a large scale import of crayfish is necessary to cover the domestic demand.

Although the days of catches of noble crayfish that used to number in millions annually are long gone, the crayfish and crayfish-fishing remain a popular recreational and important economic activity (Westman 1999, Jussila and Mannonen 2004). Perhaps due to the very complex structure of the waterways in Finland, the noble crayfish still survives in numerous small lakes and rivers. The annual catch in the 1990s was estimated to be 3-4 million individuals of noble crayfish, compared to the 15-20 million in the beginning of the last century (Pursiainen and Erkamo 2014). However, the catch of the noble crayfish still seems to be declining, the latest estimate being less than a million noble crayfish in 2010, whereas the signal crayfish catch is estimated to be 3.5 to 7 million crayfish annually (Savolainen et al. 2012).

It has been customary in Finland to try to restock the plague-stricken lakes relatively soon after an acute episode of crayfish plague. Since the total mortality of the highly susceptible noble crayfish was assumed, it was considered feasible

to restart with a new plague-free population. In many cases, these re-introductions have failed for no known reason (Westman 1991, Nylund and Westman 1995a). A recent follow-up study (Erkamo et al. 2010) showed that only about one third of the re-stocking produced a thriving or exploitable population. In Sweden the situation with crayfish was comparable to that in Finland after the crayfish plague was brought there in 1903 with the trade of infected animals from Finland (Edsman 2004). Less than one in ten of the analyzed re-introduction programmes in Sweden were reported to be successful (Fürst 1995). Success was mostly associated with small and non-complex lakes that had a uniform crayfish population structure, where the initial infection had a chance for effective spread throughout the entire lake population. In Finland the large and labyrinthine lake systems were suspected of supporting a form of chronic infection of crayfish plague due to the survival of several isolated subpopulations between which the infection could only slowly migrate (Westman and Nylund 1978, Westman 1991, Westman 1999). Distinct subpopulations of crayfish could allow the crayfish plague agent to survive by reaching the next population in the limited time period of the survival of the host animal or the infective zoospores (Westman 1991).

In the hope of reviving the crayfish fisheries to the pre-plague levels, signal crayfish were introduced to Finland thus following the example of Sweden where the strategy seemed to be successful in the first place. Although some introductions of signal crayfish had previously been done into the central, eastern and northern parts of Finland, it was proposed later that signal crayfish stocking should be restricted to a distinct region of southern Finland. This area, with some minor changes, was approved by the fisheries authorities in the first National Crayfish Strategy Agreement (Mannonen and Halonen 2000). Due to many illegal introductions of signal crayfish outside this area, in the latest update of the strategy in 2012 the whole of southern and middle Finland was appointed as the signal crayfish area (Muhonen et al. 2012).

There is no exact information available on crayfish mortality and the prevalence of crayfish plague in Finland. The number of population mortalities has been estimated to be 10-20 annually (Mannonen and Halonen 2000). In many cases the cause of the mortalities cannot be investigated because of the lack of sample material. This is especially true with mortalities that occur during the winter period, when the lakes are covered with ice for several months. The majority of mortalities are suspected to be caused by the crayfish plague; other reasons such as environmental stress are less common (Nylund and Westman 1995a). Both the As and the Ps1 genotypes of *A. astaci* have been detected in Finland (Vennerström et al. 1998), but their prevalence and distribution were unknown.

2.3 Detection and identification of *A. astaci*

2.3.1 Culture based methods

It took more than half a century after the crayfish plague first appeared in Europe, before the oomycete named *Aphanomyces astaci* was accepted as the causal agent in the aetiology of crayfish plague. This long time-gap illustrates the difficulties in the isolation and identification methodologies regarding the organism (Schikora 1903, Nybelin 1936, Schäperclaus 1935, Rennerfelt 1936). Improved isolation methods have since been developed (Alderman and Polglase 1986, Cerenius et al. 1988, Oidtmann et al. 1999b, Viljamaa-Dirks and Heinikainen 2006) but there are only a few laboratories in Europe that have been successfully using them.

Isolation of the crayfish plague agent was considered possible by taking samples from a moribund or freshly dead crayfish specimen (Alderman and Polglase 1986). The crayfish plague agent was mostly found in the soft cuticle parts of the abdomen or the limbs, thus a microscopic study of these sites should lead to the detection of the infection foci (Cerenius et al. 1988). These were then selected for the isolation attempt, by cutting out the cuticle or the walking leg that contained the hyphae and placing it on the growth medium. The inevitable bacterial contamination was restricted by the following measures: extensive cleaning, antibiotics added to the growth medium (Alderman and Polglase 1986), a physical barrier in the form of a ring placed to restrict the bacterial colony growth (Cerenius et al. 1988), or a combination of one or more of these. Although *A. astaci* has a narrow host range, it can readily grow out as axenic culture on a suitable artificial medium containing glucose, peptone and yeast extract in river water (Alderman and Polglase 1986) or a solution of salts (Unestam 1966). However, the isolation is often hampered by contamination of the plague lesions by other aquatic oomycetes or fungi (Kozubiková-Balcarová et al. 2013). Some experience is required in differentiating the hyphae of *A. astaci* from other fungal-like growths, which readily appear in the damaged cuticle areas. When mixed growth does occur, it is usually impossible to achieve a pure culture of *A. astaci*. We had previously developed a culture method that improved the isolation rate from clinical samples (Viljamaa-Dirks and Heinikainen 2006). We abandoned the selection of the seemingly infected spots by microscopy and instead used the whole abdominal cuticle and all walking legs of the crayfish. This novel approach gave us a better opportunity to find an infection focus without interference of competing oomycete growth (Fig. 3), and we obtained an improvement in the isolation rate from 14 to 56% for samples obtained over two successive five year periods. There were even seven cases in which *A. astaci* was isolated from crayfish which had not revealed any suspect fungal-like growth structures upon

microscopic examination. This demonstrates the severe challenges and limitations of exhaustive study of the diseased crayfish by microscope alone.

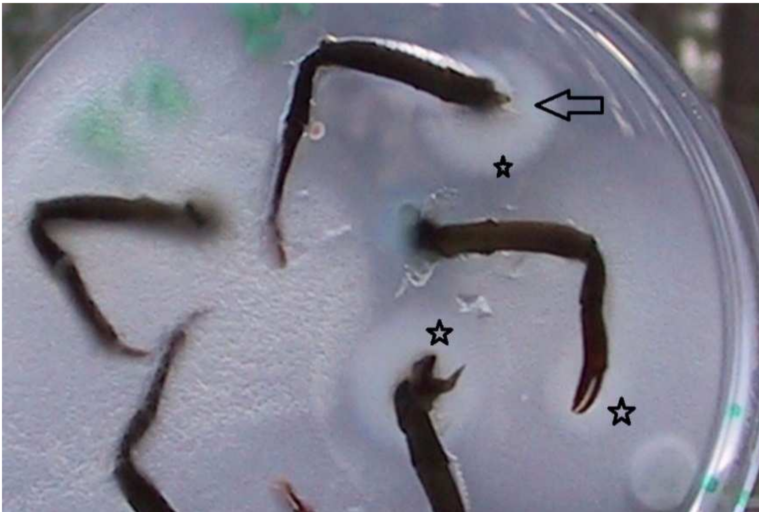

Figure 3. The walking legs (pereiopods) of a crayfish suffering from acute crayfish plague, partly submerged in the PG-1 medium. In addition to unspecific growth of water moulds, there are several joints that present typical hyphal growth of *A. astaci* (asterisks), but only one joint that shows pure growth (arrow). (Viljamaa-Dirks and Heinikainen 2006)

When acute crayfish plague induced mortality is encountered it is usually possible to find individuals that are heavily infected offering a good chance for reliable microscopy and successful isolation. Oidtmann et al. reported an isolation rate of 70% in two cases of acute mortality with an improved isolation method (Oidtmann et al. 1999b). Isolation of the agent from the latent carriers has been incidentally successful, and demanded mostly additional measures such as inducing an acute disease (Persson and Söderhäll 1983).

Identification of an isolate as *A. astaci* in earlier times required the process of zoospore production and test for pathogenicity towards a European ICS (Cerenius et al. 1988), a time consuming and complicated process.

2.3.2 Molecular methods

The development of molecular methods has made a rapid and definitive diagnosis possible. The first polymerase chain reaction (PCR) methods for the identification of *A. astaci* based on the internal transcribed spacer (ITS) region were published by Oidtmann et al. (Oidtmann et al. 2002, Oidtmann et al. 2004). The specificity was less than satisfactory however (Ballesteros et al. 2007), thus an improved method with a more specific amplicon was designed, and the original PCR method

added as a semi nested round to improve the sensitivity (Oidtmann et al. 2006). A TaqMan® (Amersham Biosciences, Buckinghamshire, UK) minor groove binder (MGB) real time PCR targeting an *A. astaci* specific ITS region (Vrålstad et al. 2009) gave the ability to estimate the level of infection in the sample, and appeared highly sensitive and specific (Tuffs and Oidtmann 2011), especially after some minor modification (Strand et al. 2014). Another TaqMan-probe real time PCR method targeted three chitinase encoding genes (Hochwimmer et al. 2009) but as the sensitivity is less compared to the ITS-based methods (Tuffs and Oidtmann 2011) in practice it has been less accepted as a standard diagnostic method.

It has even been possible to use the highly sensitive real time PCR method for detecting and quantifying crayfish plague spores in the environment (Strand et al. 2011, Strand et al. 2014). Using this method, the sporulation rates from infected signal crayfish and also from noble crayfish suffering from experimental or natural plague induced mortality have been successfully studied. However, the analysis of large amounts of water demands special equipment for filtering. To fulfil the purpose of detecting unknown crayfish plague carriers in natural water systems, this method still needs improvement in sensitivity.

Molecular detection methods have enabled the revival of crayfish plague studies all over Europe and further afield. However, the methods outlined above cannot distinguish between the different genotypes of *A. astaci*, and this limits their application in epidemiological studies. The RAPD-PCR based genotyping method requires a pure culture of the organism, and *A. astaci* isolates have certainly been very difficult to obtain especially from latently infected animals such as the NACS. When isolates have been available, the RAPD-PCR used to type *A. astaci* was found to be a reliable and robust method throughout the years (Huang et al. 1994). Moreover, the genotype grouping by RAPD-PCR has also been confirmed by another DNA fingerprinting tool, namely the amplified fragment length polymorphism (AFLP) (Rezinciuc et al. 2014). Recently, co-dominant microsatellite markers were described, which can separate all known RAPD-defined genotypes of *A. astaci* and can be applied to cuticle samples (Grandjean et al. 2014). Although the analysis of low level infected animals does not succeed with this method, its application already started to reveal the distribution of the different genotypes (Vrålstad et al. 2014, Maguire et al. 2016). The microsatellites can even reveal possible subgroups within the genotype groups (Grandjean et al. 2014, Maguire et al. 2016), although care must be taken not to rely upon results obtained solely from crayfish cuticle samples that usually harbour also other oomycetes than the target organism.

3 Aims of the study

The general aim of the study was to improve the understanding of the crayfish plague prevalence and distribution in Finland in order to form a sound basis for implementing and pursuing management strategies for increasing and maintaining exploitable noble crayfish stocks. The specific aims were the following:

1. to gather knowledge of the distribution of the different genotypes of *A. astaci* in Finland.
2. to study the variation of epidemiological features of the *A. astaci* genotypes present in Finland.
3. to ascertain the role of the spiny-cheek crayfish *O. limosus* as the vector for the strain of *A. astaci* that was first introduced into Europe.

4 Materials and methods

4.1 Materials

4.1.1 Crayfish samples

Crayfish samples were received from shareholders of the local Finnish fisheries over the 1996 to 2006 period. Most of the samples received were related to a suspicion of, or actual verified crayfish mortalities. One or more dead crayfish found in the same or adjacent water body during the same summer season were considered as a sign of acute mortality in these studies. One of the criteria for conducting an investigation was a clearly diminished or almost completely disappeared crayfish catch compared with the year before, but without any direct evidence of mortalities. These were categorized as a population decline. Sometimes the sample consisted of a single or a few individuals originating from a water body where there was no known or only a weak crayfish population after the occurrence of a mass mortality event in the past. The weak population in these studies was described as having a verified or suspected history of crayfish plague episodes in the past, but at least two years ago.

Some of the samples consisted of crayfish that had been kept in cages, which were followed for a few weeks to months to study the health status in a water body long after the disappearance or weakening of the population of crayfish. Such 'cage experiments' were mostly performed in preparation for restocking programmes, but sometimes stocking had already been conducted and the success was simply being followed by caging some crayfish. The aim of these experiments was to ascertain the suitability of the water body to support crayfish, since unfavourable water parameters were often suspected as the reason for a low population level. Sometimes acute mortality was recorded in the cages.

Both noble crayfish *A. astacus* and signal crayfish *P. leniusculus* were studied. Signal crayfish were usually received for the purpose of getting a confirmation of their crayfish plague carrier status, and only exceptionally sent for the study of a mortality case. Two samples from the period of the study originated from lakes that had mixed populations of signal and noble crayfish, with signs of mortality in the noble crayfish population. The *A. astaci* isolates from signal crayfish were only used to compare the growth rates between the isolates in the studies summarised in this thesis.

The recommendation was to send only live individuals for investigation, but sometimes the crayfish died during the journey or only dead animals were available in the first place. The crayfish were mostly transported in boxes with moisture holding material such as moss or leaves. Dead or moribund animals were immediately examined upon arrival. Some of the crayfish that exhibited normal behaviour were transferred to small plastic containers containing a small volume of water and kept at 12±2 °C until they showed any behavioural disturbances at which time they were euthanized and examined.

Lake Taulajärvi (WCA 35.311) is a small (56 ha) lake in Southern Finland, which was affected by crayfish plague and was followed for several years. The purpose of the extended study of this lake was to determine the possible time interval needed for the successful re-introduction of noble crayfish. Crayfish fishing continued in spite of the collapse of the stock and the trapped individuals were inspected for their disease status. The noble crayfish samples were received for the first time in 2001 after a reported mortality event. This mortality event was the fourth population crash since the introduction of noble crayfish in Lake Taulajärvi in the 1930s. The mortality was preceded by a sharp increase in the numbers of small-sized crayfish. The mortality of 2001 was diagnosed as crayfish plague, but verification by isolation of the agent was not achieved then. Test trapping was continued yearly until a signal crayfish was discovered in the lake in 2006.

Samples for the study of the *A. astaci* genotype that is carried by the spiny-cheek crayfish *O. limosus* were obtained from a pond in Smečno (Central Bohemia, 50°11.3' N, 14°02.8' E). The vast majority (ca. 95%) of individuals sampled repeatedly from that location were found to be infected by *A. astaci* by PCR analysis (Matasová et al. 2011). Four crayfish were collected in 2010 by manual search and transported to our laboratory in Finland. They were kept at 10 ºC in a small volume of water until examination. Two individuals died after two weeks, and one of these showed signs of paralysis the day before it died. Both individuals were selected for *A. astaci* isolation.

4.1.2 *A. astaci* isolates

Table 1 shows the isolates of *A. astaci* from crayfish specimens that had been collected during these studies. These are arranged by location and were used for further characterization. The table includes information of the isolate identification, the time of the isolation (from the sample identification number), the water body from which the crayfish was caught or kept caged, its water catchment area identification number and co-ordinates, the host species and the reason for the investigation as given by the local shareholders who sent them. In cases where several samples were received from the same or an adjacent area within a short time interval, only one isolate was included assuming that the samples were from the same mortality event.

The isolations referred to in these studies were made during the 1996-2006 period, except the isolations obtained from O. *limosus* which were performed in 2010.

The *A. astaci* reference strains: Da from Swedish noble crayfish (1973) representing the genotype As (Huang et al. 1994); Si from Swedish noble crayfish (1970) representing the genotype Ps1 (Huang et al. 1994); Kv from signal crayfish from Canada (1978) representing the genotype Ps2 (Huang et al. 1994); and Pc from red swamp crayfish from Spain representing the genotype Pc (Diéguez-Uribeondo et al. 1995), were kindly provided by Prof. Söderhäll from Uppsala University and were used to verify the genotypes of the isolates.

All isolates were maintained at 4±2 °C in vials containing PG-1 medium covered with paraffin oil (Unestam 1965).The cultures were refreshed every six months as a rule, with the exception of EviraK047/99 and EviraK086/99; both of which were kept available for reference purposes and maintained on PG-1 Petri dishes by transferring the respective culture to a fresh dish every 2 to 4 weeks.

Table 1. *Aphanomyces astaci* isolates used in this study. Isolate and sample identification number are according to the format of the archive of the Finnish Food Safety Authority. The location of the sample is identified by the name of the lake or river, the Finnish water catchment area number and the co-ordinates of Northern latitude and Eastern longitude (ETRS-TM35FIN). Background information: “Acute mortality” dead or diseased crayfish found in the same or adjacent water body during the same summer; “Population decline” population diminished or nearly completely vanished compared with the preceding fishing season; “Weak population” a weakened population that exists after an earlier population crash; “Cage experiment” sentinel crayfish held in cages. Genotype: RAPD-PCR group, Ps1: Pacifastacus strain I, As: Astacus strain, Or: Orconectes strain. Host: A.a. *Astacus astacus*, P.l. *Pacifastacus leniusculus*, O.l. *Orconectes limosus*. xx information not available.

Isolate identification	Sample identification	Sample location	Water catchment area (3rd level)	Coordinate N/lat	Coordinate E/lon	Host	Background information	Genotype	Publication
Evira1693/00	15.08.2000/46	Lake Tohmajärvi	02.013	6899216	675631	A.a.	Weak population	As	III
Evira4711/06	25.07.2006/64	Lake Kasurinlampi	03.021	6824551	634293	A.a.	Acute mortality	As	III
EviraK086/99	K86/1999	Lake Ihalanjärvi	04.127	6820034	600102	A.a.	Cage experiment	As	III,IV
Evira1704/02	23.07.2002/7	Lake Lieviskäjärvi	04.127	6824870	591674	A.a.	Weak population	As	III,IV
EviraK047/99	K47/1999	Lake Korpijärvi	04.143	6790267	506059	A.a.	Acute mortality	Ps1	III,IV
Evira3625/04	09.08.2004/53	Lake Immalanjärvi	04.192	6788310	603489	A.a.	Acute mortality	As	III,IV
Evira1494/00	25.07.2000/9	Lake Pitkäjärvi	04.199	6801928	623424	A.a.	Acute mortality	As	III,IV
EviraK100/98	K100/1998	Lake Kotkajärvi	04.212	6868029	578706	A.a.	Acute mortality	As	III,IV
Evira1772/02	26.07.2002/15	Lake Sylkky	04.296	6863209	612460	A.a.	Acute mortality	As	III,IV
Evira2193/01	25.10.2001/92	Lake Kuorinkajärvi	04.317	6944588	623329	A.a.	Weak population	As	III,IV
Evira5971/04	08.09.2004/92	River Vuokonjoki	04.411	7028710	607704	A.a.	Population decline	As	III,IV
Evira4426/03	29.07.2003/116	Lake Kelvänjärvi	04.419	6998297	654500	A.a.	Acute mortality	As	III,IV
EviraK071/99	K71/1999	River Lieksanjoki, Pankakoski	04.423	7026507	658260	A.a.	Cage experiment	As	III
EviraK116/98	K116/1998	Lake Pieni-Valtimojärvi	04.462	7057860	592961	A.a.	Population decline	As	III

Evira1580/01	07.08.2001/11	Lake Jännevirta, Pohjanlampi	04.611	6983098	542640	A.a.	Acute mortality	Ps1	III,IV
EviraK121/96	K121/1996	River Vaikkojoki	04.742	7001169	593002	A.a.	Acute mortality	Ps1	III
Evira1781/01	23.08.2001/64	River Koitajoki	04.912	6968426	691717	A.a.	Acute mortality	As	III
Evira2208/99	24.09.1999/34	Lake Issonjärvi	04.922	6952842	707782	A.a.	Acute mortality	As	III
Evira6483/05	10.08.2005/23	River Teutjoki	14.153	6722916	471158	A.a.	Acute mortality	Ps1	III,IV
Evira2010/00	31.08.2000/6	Lake Vehkajärvi	14.177	6794482	476423	A.a.	Acute mortality	Ps1	III,IV
Evira1463/01	25.07.2001/3	Lake Korkeanalanen	14.228	6823669	433850	A.a.	Acute mortality	As	III,IV
EviraK105/98	K105/1998	Lake Päijänne/ Hauhonselkä	14.231	6888665	437468	A.a.	Population decline	As	III
Evira1172/00	30.06.2000/19	Lake Vesijärvi	14.241	6774332	420902	A.a.	Acute mortality	Ps1	III,IV
Evira6458/03	28.08.2003/122	Lake Lievestuoreenjärvi	14.391	6909454	455156	P.l.	Mixed population	Ps1	IV
Evira4774/06	25.07.2006/86	Lake Löytänä	14.438	7011458	431879	A.a.	Population decline	As	III
EviraK136/96	K136/1996	Lake Iso-Suojärvi	14.687	6965713	425094	A.a.	Population decline	As	III
Evira1725/01	20.08.2001/19	Lake Horonjärvi	14.715	6970469	463755	A.a.	Population decline	As	III
Evira2061/00	07.09.2000/36	Lake Iso-Lauas	14.725	6960120	519279	A.a.	Cage experiment	As	III,IV
Evira1705/02	23.07.2002/63	Lake Pieni Tallusjärvi	14.772	6974398	504151	A.a.	Acute mortality	As	III,IV
Evira1859/02	31.07.2002/34	Lake Korosjärvi	14.773	6992535	499885	A.a.	Population decline	As	III,IV
Evira1992/00	29.08.2000/7	Lake Pukarainen	14.812	6820912	442907	A.a.	Acute mortality	Ps1	III
Evira1936/00	28.08.2000/4	Lake Laitjärvi	14.822	6836605	458372	A.a.	Acute mortality	As	III
Evira1588/00	04.08.2000/18	Lake Kilpilampi	14.823	6833412	462168	A.a.	Acute mortality	Ps1	III,IV
Evira0894/01	07.06.2001/82	Lake Iso-Suojärvi	14.823	6965713	425094	A.a.	Acute mortality	As	III,IV
Evira3697/03	11.07.2003/86	Lake Iso-Kuivajärvi	14.824	6816988	458616	A.a.	Acute mortality	Ps1	III,IV
EviraK110/98	K110/1998	Lake Rautavesi	14.831	6835224	445689	P.l.	Symptomatic signal crayfish	Ps1	IV
EviraHki/48/01	25.07.2001/48	Lake Saarijärvi	14.911	6769858	482554	A.a.	Acute mortality	Ps1	III

Evira1609/02	16.07.2002/97	Lake Hirvijärvi	14.924	6852732	467544	A.a.	Acute mortality	Ps1	III
Evira1595/00	04.08.2000/42	Lake Harjujärvi	14.939	6862364	505607	A.a.	Acute mortality	Ps1	III
Evira7442/03	07.08.2003/80	Lake Valkjärvi	23.074	6694308	324138	P.l.	Population decline	Ps1	IV
Evira1140/00	29.06.2000/8	Lake Ylistenjärvi	35.138	6797375	281117	A.a.	Acute mortality	Ps1	III,IV
Evira1219/02	05.06.2002/135	Lake Iso-Arajärvi	35.290	6791599	315814	A.a.	Acute mortality	Ps1	III,IV
Evira2807/04	06.07.2004/27	Lake Taulajärvi	35.311	6839755	329698	A.a.	Weak population	As	I,III,IV
Evira6672/05	16.08.2005/17	Lake Taulajärvi	35.311	6839755	329698	A.a.	Weak population	As	I,III,IV
Evira6540/06	25.08.2006/66	Lake Taulajärvi	35.311	6839755	329698	A.a.	Mixed population	Ps1	I,III
Evira3234/02	02.10.2002/45	Lake Pulesjärvi	35.318	6835117	341382	A.a.	Mixed population	Ps1	III,IV
Evira4011/06	29.06.2006/13	River Koronjoki	35.441	6902008	324595	A.a.	Acute mortality	Ps1	III
Evira6207/05	27.07.2005/116	Lake Valkiajärvi	35.546	6863944	262367	A.a.	Acute mortality	As	III,IV
Evira7901/06	04.10.2006/110	Lake Valkiajärvi	35.546	6863944	262367	A.a.	Cage experiment	As	III
Evira2557/02	26.08.2002/11	Lake Mallasvesi	35.711	6799786	347428	P.l.	Symptomatic signal crayfish	Ps1	IV
EviraK104/98	K104/1998	Lake Konaanjärvi	35.773	6793622	367925	A.a.	Acute mortality	Ps1	III
Evira7203/03	11.09.2003/70	Lake Kukkia	35.781	6801172	377743	P.l.	Symptomatic signal crayfish	Ps1	IV
Evira2947/04	14.07.2004/26	Lake Ormajärvi	35.792	6775535	390118	P.l.	Symptomatic signal crayfish	Ps1	IV
Evira1908/02	02.08.2002/5	River Jokilanjoki	35.892	6756430	361619	P.l.	Symptomatic signal crayfish	Ps1	IV
Evira5158/06	04.08.2006/1	River Karviajoki	36.022	6855221	248143	A.a.	Population decline	As	III
Evira2128/99	13.08.1999/42	River Kyrönjoki	42.022	6989218	268595	A.a.	Acute mortality	Ps1	III
Evira3583/04	06.08.2004/73	River Ähtävänjoki/ Vitsjö	47.014	7045745	317008	A.a.	Cage experiment	As	III,IV
Evira0577/04	02.03.2004/22	River Välijoki	47.023	7024646	328911	A.a.	Cage experiment	As	III
Evira7005/04	29.09.2004/91	Lake Lappajärvi	47.031	7006424	332266	A.a.	Cage experiment	As	III,IV

Evira8951/03	07.11.2003/45	River Perhonjoki	49.023	7041263	339272	A.a.	Cage experiment	As	III,IV
Evira8065/04	10.11.2004/113	River Perhonjoki	49.023	7041263	339272	A.a.	Cage experiment	As	III,IV
Evira5339/06	09.08.2006/44	River Lestijoki, Toholampi	51.023	7074426	365682	A.a.	Acute mortality	Ps1	III
Evira7614/06	27.09.2006/89	River Lestijoki, Sykäräinen	51.031	7056806	371742	A.a.	Acute mortality	Ps1	III
Evira8224/06	01.11.2006/20	River Vääräjoki	53.093	7102722	363351	A.a.	Cage experiment	Ps1	III
Evira7512/03	18.09.2003/53	River Pyhäjoki, Helaakoski	54.011	7143480	371096	A.a.	Cage experiment	Ps1	III,IV
Evira7862/03	07.10.2003/5	River Pyhäjoki, Oulaistenkoski	54.012	7128473	394519	A.a.	Cage experiment	Ps1	III,IV
Evira5041/04	24.08.2004/16	River Pyhäjoki, Venetpalo	54.041	7085698	440197	A.a.	Cage experiment	As	III,IV
Evira5721/04	03.09.2004/42	River Pyhäjoki, Mieluskoski	54.022	7117350	410608	A.a.	Cage experiment	Ps1	III,IV
Evira5727/04	03.09.2004/53	River Pyhäjoki, Joutenniva	54.032	7103521	428120	A.a.	Weak population	As	III,IV
Evira7246/03	10.10.2003/92	Lake Pyhäjärvi	54.051	7051553	447165	A.a.	Cage experiment	As	III,IV
Evira7948/06	09.10.2006/64	Lake Pyhäjärvi	54.051	7051553	447165	A.a.	Cage experiment	As	III
Evira10789/05	02.12.2005/67	Lake Kivesjärvi	59.351	7146068	521040	A.a.	Acute mortality	As	III,IV
Evira6462/06	25.08.2006/5	Lake Kivesjärvi	59.351	7146068	521040	A.a.	Cage experiment	As	III
Evira10278/05	10.11.2005/28	Lake Kivesjärvi, farm	59.xx	xx	xx	A.a.	Mortality	As	IV
Evira0418/06	31.01.2006/65	Lake Kivesjärvi, farm	59.xx	xx	xx	A.a.	Mortality	As	IV
Evira2680/06	10.05.2006/42	Lake Kivesjärvi, farm	59.xx	xx	xx	A.a.	Mortality	As	IV
Evira3514/04	03.08.2004/135	Lake Jokijärvi	61.312	7266888	573011	A.a.	Weak population	As	III,IV
Evira6128/06	21.08.2006/54	River Kemijoki	65.112	7331320	400388	A.a.	Population decline	As	III
Evira6443/06	24.08.2006/100	Lake Ottojärvi	86.003	6739066	548520	A.a.	Acute mortality	Ps1	III

Evira2859/03	05.06.2003/117	farm Eurajoki	xx	xx	xx	A.a.	Mortality	Ps1	IV
Evira6696/04	16.09.2004/13	farm Ruovesi	xx	xx	xx	A.a.	Mortality	Ps1	IV
Evira9623/05	18.10.2005/37	farm Hämeenlinna, Evo	xx	xx	xx	A.a.	Mortality	Ps1	IV
Evira0384/06	30.01.2006/4	farm Hämeenlinna, Evo	xx	xx	xx	P.l.	Mortality	Ps1	IV
Evira10399/05	15.11.2005/13	farm Uusikaarlepyy	xx	xx	xx	A.a.	Mortality	Ps1	IV
Evira3092/06	23.05.2006/120	farm Lappeenranta	xx	xx	xx	A.a.	Mortality in mixed population	Ps1	IV
Evira4805a/10	6.7.2010/37	Pond Smečno, Czech Republic	xx	xx	xx	O.l.	Symptomatic spiny-cheek crayfish	Or	II
Evira4806a/10	6.7.2010/37	Pond Smečno, Czech Republic	xx	xx	xx	O.l.	Symptomatic spiny-cheek crayfish	Or	II

4.2 Methods

4.2.1 Isolation of *A. astaci* (I-IV)

During the 1996-1998 interval the method described by Cerenius et al. (1988) was followed, with a few exceptions. The crayfish were studied macroscopically and microscopically. For practical reasons, only the ventral abdominal soft cuticle was examined by light microscopy (100x). The cuticle was extensively cleaned with a cotton swab using sterile water and additionally 70% ethanol before excision. When hyphae were detected, the infected part of the cuticle was cut into pieces for cultivation. A cylinder made of plastic was used, submerged in the growth medium into which the cut piece of the cuticle was placed. No potassium tellurite was used in the cylinder. Instead, the antibiotics ampicillin and oxolinic acid at a concentration of 10 mg/l as suggested by Alderman and Polglase (1986) were added to the peptone-glucose-salt agar PG-1. The plates were incubated at 20±2 °C.

Some modifications were made to the method from 1999 onwards (Viljamaa-Dirks and Heinikainen 2006). The abdominal cuticle was examined by light microscopy as earlier in order to reach a preliminary diagnosis. Regardless of the outcome of the preliminary diagnosis, the abdominal cuticle in one piece was soaked in 70% ethanol for 10-30 seconds to diminish the bacterial contamination and then rinsed with sterile water. Then the whole cuticle was plated on PG-1 agar, which contained antibiotics in order to reduce bacterial contamination. All the walking legs (pereiopods) were cut off at the most proximal joint and treated in a similar manner to the cuticle samples, except that a longer treatment with ethanol (30-60 sec.) was applied. When plated, the legs were partly inserted into the matrix of the agar to allow direct contact between the soft cuticle of the joints and the growth medium (Fig. 3). The incubation temperature was 15±2 °C. Inoculated dishes were examined daily using a microscope and any oomycete that had features consistent with *A. astaci* (i.e., frequently branching, non-septate hyphae of a diameter about 9 µm) was transferred to a new dish for further study.

For analyzing the Lake Taulajärvi samples this method was used only from 2004 onwards.

4.2.2 Identification and the genotype determination of *A. astaci* (I-IV)

Spore production tests and infection challenge experiments were performed according to the method described by Cerenius et al. (1988) on all isolates in 1998-2000 and the isolate Evira4806a/10 from the spiny-cheek crayfish. Challenge experiments were performed with farmed noble crayfish, using three to five crayfish in each test. Briefly,

the oomycete to be tested was cultured in PG-1 broth, after which the zoospore production was initiated by replacing the broth with sterilized lake water. The presence of zoospores was verified with the microscope and the zoospores were added to the test tank containing the susceptible crayfish. Mortality in the challenged crayfish, combined with the detection of typical hyphae in the cuticle of the dead crayfish was considered as evidence of the pathogenicity of the isolate.

All isolates collected after 1996 were also subjected to the RAPD-PCR method with the Operon B01 primer according to Huang et al. (1994) with some minor modifications. Briefly, PCR reactions were carried out in a 50 µl volume that contained 2.5 units of HotStarTaq DNA polymerase (Qiagen), 1.5 mM $MgCl_2$, 200 µM of each dNTP, and 0.5 µM primer in standard buffer for the enzyme. Amplified DNA was resolved in 1.5% agarose that contained ethidium bromide and photographed under UV light. The obtained RAPD-PCR profiles were compared visually against those of the reference strains Da (As) and Si (Ps1). The spiny-cheek crayfish isolates were also compared against the reference strains Kv (Ps2) and Pc (Pc). The infection challenge experiment from 2001 onwards was only performed in cases where the RAPD-PCR profile of the isolate showed any variation in the profiles of the As and Ps1 genotypes, which were the two genotypes that were recognized as causal agents for crayfish plague in Finland at the time (Vennerström et al. 1998).

When a specific PCR-method i.e. the method described by Oidtmann et al. (2006) later became available, all isolates of the collection were tested using that method as a single round PCR detection assay. The mycelia were grown in PG-1 medium and DNA was isolated by DNeasy Plant Mini kit (Qiagen) after the grinding of the mycelia with ceramic beads in a Magna Lyser instrument (Roche) for the PCR based methods.

A 1354 bp fragment of the ribosomal DNA (rDNA) region (GenBank accession number JF827153) from one of the isolates (Evira4805b/10) was amplified using primers NS5 and ITS4 (White et al. 1990). Both strands of purified PCR products were directly sequenced on a capillary sequencer.

4.2.3 Radial growth rate (IV)

A total of 28 isolates belonging to the genotype group As, and also 25 isolates of the genotype group Ps1 were studied. The selected isolates were all separated in terms of their origin either temporally or by location. Each isolate was tested for growth rate by first being inoculated onto a PG-1 medium plate, and then incubated at 20 °C for 6 days. Then standard pieces, 6 mm in diameter, were stenciled out from the outer edge of the mycelial mat and then placed into the middle of a fresh PG-1 medium plate. The cultures

were incubated at 20 °C for 14 days and the maximal linear extension of the mycelial mat was measured at 24 hour intervals. The cultures were followed until they filled the plate or for up to 14 days. The growth rates at 15 °C of 11 of the isolates (As n=5, Ps1 n=6) were studied as well.

The daily radial growth of the hyphae was determined by the difference between the diameters of the mycelial mat, as the mean of the 3 separate cultures divided by 2. The overall radial growth rate of an isolate was calculated from the daily values during the exponential growth phase in days 2 to 7 using MS Excel.

4.2.4 Infection trial (IV)

The infection trial was performed during the winter of 2006-2007. A slow growing and a fast growing representative of *A. astaci* were chosen from both As and Ps1 genotype groups to test the virulence of the pathogen in noble crayfish. The mean growth rate for the fast growing As isolate Evira4426/03 (AsFast) was 4.5 mm day^{-1} and for the slow growing As isolate Evira6672/05 (AsSlow) 2.0 mm day^{-1}. The mean growth rate for the fast growing Ps1 isolate Evira3697/03 (Ps1Fast) was 4.2 mm day^{-1} and for the slow growing Ps1 isolate Evira7862/03 (Ps1Slow) 2.8 mm day^{-1}.

Twelve intermoult farmed crayfish (N=180) were placed into each of 15 separate tanks. Each tank contained 15 L lake water with constant aeration and plastic tubes for hides. The temperature of the tanks throughout the trial was maintained at 20±2 ºC.

The selected *A. astaci* isolates were incubated in PG-1 broth at 20 ºC for 9 days, after which the zoospore production was initiated by replacing the broth with sterilized lake water. The zoospore density was determined for each of the strains by the Bürker chamber counting method and the counts varied between 4000-12600 spores mL^{-1}. The final density of the zoospores for the test tanks was adjusted to approximately 100 zoospores mL^{-1} by adding 120-400 mL spore suspension per tank, except for the 3 control tanks. Each test strain was used to infect 3 tanks (AsFast/1-3, AsSlow/1-3, Ps1Fast/1-3 and Ps1Slow/1-3).

The crayfish were monitored daily and dead individuals were collected and immediately examined microscopically for signs of crayfish plague infection. The re-isolation of the crayfish plague agent from at least one of the animals from each test tank was performed to confirm the success of the infection method.

4.2.5 Statistical methods (IV)

Comparisons of the growth rates of the crayfish plague isolates between the genotype groups As and Ps1 were made by comparing the measured diameters of the cultures at 20 °C on day 7. The Mann Whitney U test (SOFA Statistics 1.4.3, Paton-Simpson & Associates Ltd, Auckland, New Zealand) was used as a non-parametric test to compare the distributions of the growth rates of these two genotype groups.

The mortality rates in the infection trial were compared by counting the day on which the last crayfish in the test group had died. The statistical comparisons between the infection types, genotypes and fish tanks were made separately, and used Kaplan-Meier survival analysis (Kaplan and Meier 1958). Pairwise log rank comparisons were conducted to determine which groups had different survival distributions. In order to keep the overall confidence at a 95% level, a Bonferroni correction was made with statistical significance accepted at the $p < .005$ level for isolate types, $p < .0167$ level for genotypes and $p < .0004716$ for tanks since there were 10, 3 and 105 comparisons, respectively. The Log rank test was used since censoring patterns were rather similar. Statistical analyses were carried out with IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA).

5 Results

5.1 Genotypes and distribution of *A. astaci* in Finland (III)

Between 1996 and 2006 *A. astaci* was isolated from 69 batches of noble crayfish and seven batches of signal crayfish sent for examination (Table 1). All isolates fulfilled the morphological criteria of *Aphanomyces* species, and 18 isolates tested were all pathogenic towards the noble crayfish. All isolates produced the expected PCR product. All Finnish isolates of *A. astaci* had RAPD-PCR profiles belonging to one of the two genotypes As or Ps1 (Table 1). The reference strains gave identical profiles with the two genotypes detected in our study. Of the total number of 69 isolates from noble crayfish, 43 represented the As genotype and 26 the Ps1 genotype. There was a high homology between the RAPD-PCR profiles inside the groups. All As-genotype profiles were characterized by a strong 1300 bp band, and Ps1-genotype profiles by 1200 and 800 bp bands. Outside these conserved bands, minor variations were detected among the weaker bands in both genotype groups. All signal crayfish isolates belonged to the genotype Ps1.

The majority of the noble crayfish samples were obtained from water bodies in central and eastern Finland, and accordingly most of the isolates (48) also originate from those areas (Fig. 4). In the eastern part of Finland, all but two of the noble crayfish isolates were of the As-genotype, whereas 15 from 22 isolates from the southern part of Finland

belonged to the Ps1 group. The geographical distribution of the Ps1-isolates corresponded with the area where the signal crayfish has been introduced extensively into the Finnish water bodies. Both genotypes were present in the areas adjacent to the signal crayfish territory. Occasional isolations of Ps1-strains were made from four water catchment areas in the western part of the country, where As-strains are also common. In the samples from the northern Finland, only As-strains were detected.

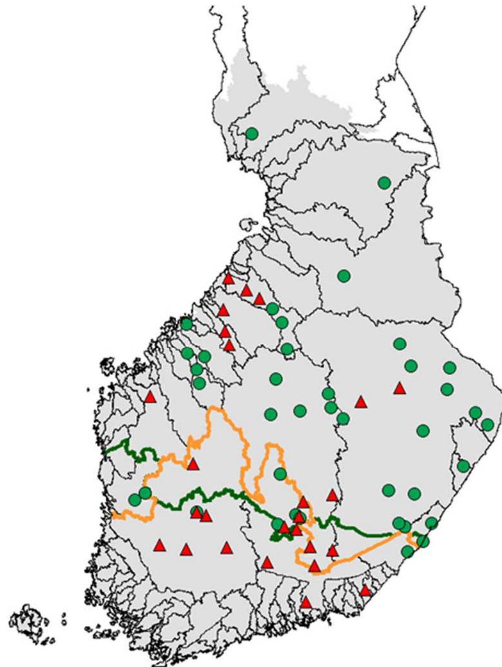

Figure 4. Crayfish plague identified in noble crayfish in 1996-2006, placed on the map of the main water catchment areas of Finland. Green circle: *A. astaci* genotype As, Red triangle: *A. astaci* genotype Ps1. The solid orange line demarcates the northern border of the signal crayfish stocking area (Mannonen and Halonen 2000), the solid green line demarcates the northern border of the original distribution area of noble crayfish in Finland (Järvi 1910). The light grey background shows the present distribution of noble crayfish. (III)

5.2 Detection of persistent crayfish plague infections in noble crayfish populations (I, III)

The noble crayfish population of Lake Taulajärvi was affected by an acute mortality event in 2001 that drastically reduced the catch from a mean of 6.2 individuals per trap per night in the season of 2000 down to a few individuals from the entire lake in 2001. Although hyphal growth that suggested crayfish plague as the culprit was indeed

detected in the abdominal cuticle of diseased crayfish, isolation of the crayfish plague agent was not achieved due to contamination by nonspecific water moulds. In the following years, test trapping revealed a weak population remaining after the main mortality event. In 2002 crayfish samples were judged to be negative for crayfish plague, since no typical hyphal growth was detected. In 2003, however, two crayfish showed melanised areas in the abdominal cuticle, with hyphae typical for *A. astaci*. Isolation attempts to determine the pathogen failed again due to nonspecific growth of water moulds. In the summer of 2004, one out of five individuals had a melanised area in the abdominal cuticle with hyphae typical of *A. astaci* (Fig. 5). The crayfish plague agent was isolated and confirmed as *A. astaci* of the genotype As. In 2005, two out of six crayfish exhibited macroscopic and microscopic signs of crayfish plague. Again, *A. astaci* genotype As was isolated.

Figure 5. The distal segment of the abdominal cuticle of a noble crayfish that had been caught in Lake Taulajärvi in July 2004, showing the melanised area with hyphae (arrow), caused by an infection by a low-virulent *A. astaci* Evira2807/04. Stereomicroscopic view, magnification 10X. (I)

In 2006, signal crayfish were detected in Lake Taulajärvi. Nine noble crayfish individuals were caught at the same time, and two of them had signs of an acute crayfish

plague infection. This time *A. astaci* belonging to the Ps1 genotype associated with the signal crayfish was detected. The appearance of signal crayfish in Lake Taulajärvi discouraged the aim to re-populate the lake with noble crayfish and thus the follow up of the original crayfish plague epizootic was ceased. However, verification of a persistent infection by isolation of the agent was obtained for two successive years 2004 and 2005, and by microscopy based evidence for four years (from 2001 to 2005).

There were some more examples of situations where the crayfish plague agent seemed to remain for long periods in the same water body. The As-genotype crayfish plague agent was isolated in successive years in samples taken from Lake Valkiajärvi (WCA 35.546). Most probably it was being maintained by the weak population that survived an earlier crayfish plague outbreak. The spread of the disease in Lake Kivesjärvi (WCA 59.351) was also extremely slow judged by the isolations in December 2005 and again in August 2006. The later isolation was made from caged crayfish, about 6 km from the original infection site where the crayfish had already disappeared. Caged crayfish were followed for longer than a year in a cage experiment in the River Perhonjoki (WCA 49.023), and isolates of an As-strain were cultured from samples taken at the beginning and at the end of this period.

The long persistence of the infection that had earlier been recognised as a chronic crayfish plague in large lakes such as Lake Pyhäjärvi (WCA 54.051) was also confirmed, by isolates cultured from samples taken in 2003 and 2006. Both of these isolates belonged to the genotype As.

5.3 Comparison of virulence between the genotypes As and Ps1 (III, IV)

The background of the *A. astaci* isolates from clinical noble crayfish samples suggested a difference in virulence between the As and Ps1 isolates. The information gained about the epidemiological status of the affected water bodies (III) revealed that in the majority of the cases (21 samples out of 24) Ps1 isolates were associated with acute mortality in the noble crayfish population. As-type isolates were predominantly (29 out of 43) associated with population declines (9 samples), weak populations (7 samples) or cage experiments (13 samples). Fourteen cases of acute crayfish mortality were caused by As isolates, accounting only for 33% of all As isolates. There was a significant difference between the genotypes Ps1 and As regarding the frequency of acute mortality events, even when the population declines and cage experiments involved with mortality are included in acute mortality (Fisher's P, df1, P=0.007).

There seemed to be a substantial difference between the isolates with regard to the growth rate in the PG-1 medium, thus the radial growth rate was compared between the two genotypes (IV). Most (n=18) of the Ps1 isolates included in the study (n=25)

colonized the total agar surface within 9-14 days at 20 °C , but the colonization by 7 isolates was less extensive indicating a more modest growth rate. This was different in the As group from which only 7 isolates colonized the total agar surface in 9-14 days at 20 °C, whereas most (n=21) of the tested isolates (n=28) showed a more restricted growth. The radial growth rate at 20 °C in the group of As isolates ranged between 1.8-4.5 mm day^{-1}, the mean being 2.9 mm day^{-1} with a standard deviation (SD) of 0.8, (n=23). The radial growth rate at the same temperature in the group of Ps1 isolates was between 2.8-4.7 mm day^{-1}, the mean (n=24) was 3.8 mm day^{-1} (SD 0.54). If there were more than one isolate from the same location, only one of them was included in the comparison. EviraK086/99 (As) and EviraK047/99 (Ps1), were used as control strains for diagnostic purposes and therefore transferred considerably more often than the stock cultures of other strains. Both control strains differed substantially from the others in their respective groups, and they were therefore excluded from these comparisons. Fig. 6 illustrates the variation of growth rates inside and between the genotype groups.

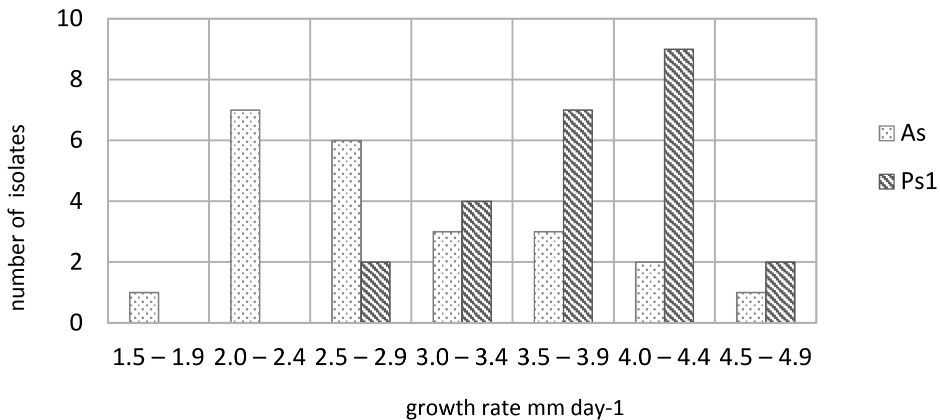

Figure 6. The radial growth rate of *Aphanomyces astaci* isolates belonging to the genotypes As and Ps1 in PG-1 medium at 20 ºC. (IV)

The challenge test towards noble crayfish with a fast and a slow growing representative of the As and Ps1 genotypes revealed differences in virulence between the strains tested. The first crayfish plague induced mortalities in the Ps1Fast groups were seen as early as on day 5 and total mortality was reached on day 10. Microscopic examination showed a heavy growth of typical hyphae in the abdominal cuticle. The development of mortality was slower in the Ps1Slow groups, and there was more variation between the three tanks.

Typical hyphal growth was, nevertheless, seen in the abdominal cuticle in almost all of the individuals. In addition, four individuals of the Ps1Slow/1 group showed some melanisation, as did one individual in each of the other two groups.

The mortality associated with AsSlow was significantly slower than with both Ps1 strains (log rank $p < 0.001$). Mortalities were recorded during days 11 to 128. The microscopic outcome was variable, as it ranged from heavy growth of typical hyphae to a few melanised foci. Melanisation was a common feature, as 7 individuals in AsSlow/1 and 9 in AsSlow/2 in addition to 9 in AsSlow/3 showed melanised areas in the abdominal cuticle and/or joints. Melanisation had already been noticed at the time when the first deaths occurred.

The AsFast groups did not differ from the uninfected control groups. Both showed a steady development of mortality throughout the experiment that lasted until the last crayfish in the infected groups perished on day 244. Mortality in the AsFast/1 tank started on day 69 with the last crayfish dying on day 244. The mortalities for AsFast/2 and AsFast/3 tanks started from day 1 and from day 48 and the last occurred on days 219 and 161, respectively. A microscopic study of these groups revealed a few foci of typical hyphal growth that was melanised in the majority of the individuals.

The combined cumulative mortality of the test groups is shown in Fig. 7. The presence of viable *A. astaci* was confirmed from each of the challenged groups by re-isolation of the agent.

With the exception of AsFast, all of the tested strains caused elevated mortality in comparison with the control group (log rank $p<0.001$). There was a statistically significant difference (log rank, $p<0.001$) in the development of mortality between As and Ps1 infected genotypes, with the Ps1 groups having a higher mortality. Within the Ps1 genotype, Ps1Slow induced mortality slower than Ps1Fast (log rank $p<0.001$). Surprisingly, AsFast did not differ from the uninfected control group (log rank $p=0.924$).

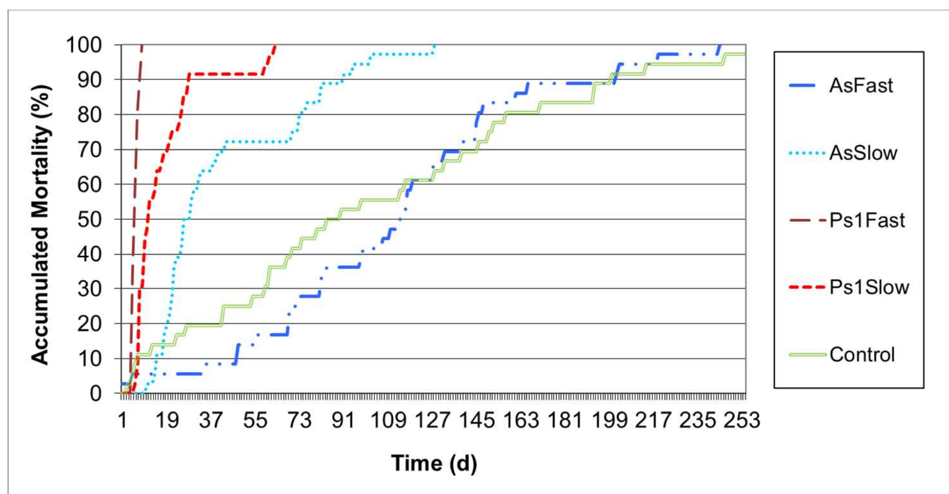

Figure 7. Cumulative mortality in the infection trial with *Aphanomyces astaci*, combined from 3 separate test tanks with 12 noble crayfish in each tank. Ps1Fast: test groups Ps1Fast/1-3, infected with Evira3697/03; Ps1Slow: test groups Ps1Slow/1-3, infected with Evira7862/03; AsFast: test groups AsFast/1-3 infected with Evira4426/03; AsSlow: test groups AsSlow/1-3 infected with Evira6672/05. Control: control groups 1-3. (IV)

5.4 Detection of a novel genotype of *A. astaci* in *Orconectes limosus* (II)

The examined spiny-cheek crayfish *O. limosus* showed a few barely observable areas of macroscopic melanisation, especially in the joints of the walking legs. Microscopic examination, however, revealed that the abdominal cuticles and joints of two crayfish had several foci of short, partly melanised hyphae (Fig. 8). Hyphae suspected to be *A. astaci* emerged from the crayfish cuticle in the cultures incubated at 15 ºC after five to six days. Subcultured isolates fulfilled the morphological criteria for *Aphanomyces* sp. Specific PCR, sequencing and the infection trial confirmed the isolates as *A. astaci*. However, the RAPD-PCR profiles of the four isolates obtained from the two crayfish individuals were identical to each other but clearly different from the profiles of the four known genotype groups of *A. astaci* described earlier (Fig. 9). These isolates from the spiny-cheek crayfish thus belong to a hitherto unknown genotype, which was named Or (or group E) after the host species *Orconectes limosus*.

Figure 8. Melanised hyphae of *A. astaci* in the cuticle of the spiny-cheek crayfish *Orconectes limosus* from Pond Smečno (light microscopy image, magnification 100X). (II)

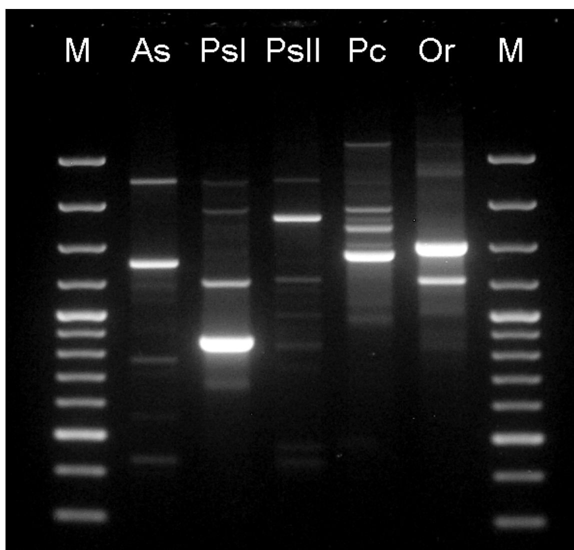

Figure 9. Agarose gel with RAPD-PCR patterns of the new genotype of *A. astaci*, genotype group Orconectes (genotype E) (Or), second from the right, compared to all four previously known genotypes (As, PsI, PsII, Pc); results of amplification using the primer B01 after Huang et al. (1994). Abbreviations: M = DNA marker (300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000, 3000 bp bands; 500 and 1000 bp bands are more intensive), As = strain Da from *Astacus astacus* (group A), PsI = strain Si from *Pacifastacus leniusculus* (group B), PsII = strain Kv from *Pacifastacus leniusculus* (group C), Pc = strain Pc from *Procambarus clarkii* (group D), and Or = the new strain Evira4805a/10 from *Orconectes limosus* (group E). (II)

6 Discussion

6.1 Genetic diversity of *A. astaci*

Identification of the pathogen's genotype can assist in tracing sources of infection (Williams et al. 1990). The epidemiological study of crayfish plague has been complicated by problems in diagnostic methods and difficulties in the isolation of the causative agent in pure culture, which is necessary to achieve the genotyping by the RAPD-PCR method. Improved isolation rates achieved by a modification of the suggested methods (Viljamaa-Dirks and Heinikainen 2006) supported the use of the RAPD-PCR method and frequently we could identify the genotype groups of *A. astaci* isolates. The results have been very consistent and in our studies the genotypes As and Ps1 have given nearly identical profiles for their respective group over the years. The consistency of the profiles in the different genotype groups was already noted in the original study by Huang et al. (1994) and was explained by the lack of sexual propagation in *A. astaci*, which is still the case in our findings.

Although the spiny-cheek crayfish *O. limosus* has been recognised as a vector of *A. astaci* (Vey et al. 1983), the pathogen had never been isolated from this species. We showed that *O. limosus* does indeed carry a genotype of *A. astaci* not described before. This novel genotype was named *Aphanomyces astaci* genotype group Orconectes (genotype E) and designated with the abbreviation Or according to its host species. Other *Aphanomyces* sp. resembling *A. astaci* in morphology have been isolated from crayfish (Royo et al. 2004, Kozubiková-Balcarová et al. 2013). Therefore the determination of our new isolates required meeting strict species confirmation criteria. Our isolates fulfilled morphological characteristics of *A. astaci*, were pathogenic to noble crayfish, and the ITS sequence obtained from one of them was consistent with that of the other genotypes of the species, thus confirming the correct species identification.

It seems that each of the NICS that originate from North America carry their own genotype or genotypes of *A. astaci*. The signal crayfish has been recognized as the host of two different genotypes, Ps1 and Ps2, with a different geographic origin (Huang et al. 1994). Although the RAPD-PCR method does not reveal differences between the Pc isolates from *Procambarus* sp., differences have been detected by the microsatellite method (Viljamaa-Dirks, unpublished results). There have already been several procambarids introduced into Central Europe that possibly carry their own variant of the crayfish plague agent. Crayfish plague has so far not been a conservational or economic problem in North America and thus there is a lack of studies describing this parasite in its native distribution area. Several hundred indigenous crayfish species live in North America. Each of them potentially harbours its own type of *A. astaci*, with variable

features including virulence and the ability for physiological adaptation. Only the crayfish species that were introduced into Europe have been studied more closely for their carrier status. Even after our studies many gaps in the knowledge still remain. The original host of the genotype As has not been identified. Although *O. limosus* was known to have been introduced after the first crayfish mortalities were reported, its possible role in the spread of the crayfish plague needed to be investigated. Our study clearly shows that the spiny-cheek crayfish is not carrying the same As strain as was implicated in the original crayfish plague. Further analysis of crayfish plague affected noble crayfish in the vicinity of *O. limosus* did confirm *A. astaci* genotype Or from them (Kozubiková-Balcarová et al. 2014). The origin and the introduction route of the first *A. astaci* infection into Europe remains thus unsolved.

6.2 Variable virulence of *A. astaci*

The expected outcome of the infection with *A. astaci* was a total mortality in the populations of European ICS (Alderman et al. 1987, Söderhäll and Cerenius 1999). This assumption was supported by the results of clinical experience and laboratory experiments that were conducted during the time when there was no knowledge of the existence of different genotypes of *A. astaci* (Unestam and Weiss 1970, Alderman et al. 1987). It appeared later that the English isolates belonged to the genotype group Ps1. Our study (IV) and later studies (Makkonen et al. 2012b, Becking et al. 2015) showed that Ps1 was associated with the high virulence traditionally connected with the crayfish plague agent. On the other hand, the disappointing results of Swedish re-stocking efforts suggested that one of the explanations for the failure to re-establish viable crayfish populations could be that the old type (i.e. As genotype) crayfish plague agent could have the capability to survive in a weak and reduced population of noble crayfish i.e. the weakened population acted as a reservoir for the pathogen (Fürst 1995). Although there was no direct evidence to support this theory, it was recognized that the development of the experimentally induced mortality due to crayfish plague infection was influenced by the density of zoospores and by the water temperature (Alderman et al. 1987). It was also recognized that there was a susceptibility difference between the European ICS, as the narrow clawed crayfish *Astacus leptodactylus* showed some degree of resistance to the acute disease (Fürst 1995).

Lake Taulajärvi lies in southern Finland and appeared to be a perfect location for searching for the crayfish plague agent that could possibly survive in infected noble crayfish. After a long history of repeating episodes of crayfish plague, crayfish trapping in Lake Taulajärvi was still continued by dedicated local shareholders. After the acute mortality phase in 2001 a small number of crayfish was found yearly. This indicated the

existence of a weak population even after the plague had induced widespread mortality. In earlier years this phenomenon might have been explained by these crayfish individuals having been able to avoid the infection by living in refuges at the time of the epidemic. Surprisingly, individuals that manifested signs of crayfish plague were found over several years. However, isolation of the agent in successive years 2004 and 2005 only succeeded long after the acute disease episode in 2001. These successful isolations were achieved by using the improved isolation method (Viljamaa-Dirks and Heinikainen 2006). Crayfish plague agent genotype As was isolated at both times. Unfortunately, Lake Taulajärvi is situated in the signal crayfish area and the large adjacent lake, Lake Näsijärvi, harbours a signal crayfish population that is infected with the Ps1 strain of *A. astaci* (Viljamaa-Dirks, unpublished results). In 2006, signal crayfish were detected in Lake Taulajärvi resulting in yet another acute plague episode in the remaining noble crayfish population, but this time the crayfish plague was caused by a different strain belonging to the genotype Ps1. The best explanation for the recurrent finding of the As genotype strain infected crayfish during several years is a persistent infection in the remnants of the noble crayfish population.

A sharp increase in the numbers of individuals in the smaller size classes of the noble crayfish caught annually was recorded in Lake Taulajärvi before the acute phase of the crayfish plague in 2001. It can be speculated that the increased number of host animals offered the crayfish plague agent the opportunity to spread more efficiently. At a certain point the amounts of zoospores might have been enough to cause a new acute phase of the disease in the population. Recurrent episodes of crayfish plague have been recognized in a large number of Swedish and Finnish lakes (Fürst 1995, Erkamo et al. 2010, Pursiainen and Viljamaa-Dirks 2014). Increased population density and the shareholders decision to allow the crayfish trapping again has often preceded the crayfish plague occurrence, making it easy to blame the trappers with contaminated equipment for re-introducing the disease. However, these infections could have remained in the lake from earlier outbreaks, and the increased density of the crayfish population could simply have triggered the acute phase of the disease again.

New molecular methods that are suitable for carrier detection (Oidtmann et al. 2006, Vrålstad et al. 2009) have revealed other European ICS populations and individuals infected with *A. astaci* (Kokko et al. 2012, Svoboda et al. 2012, Kušar et al. 2013, Maguire et al. 2016). The isolation of the crayfish plague agent from asymptomatic carriers is difficult and has therefore rarely been attempted. As the result the genotype involved has often remained unclear. Our study of the crayfish plague incidence and the genotypes involved (III) is one of the few attempts to unveil the epidemiology of the different genotypes in a geographical area so far. After the publication of the

microsatellite method that can be used without the isolation of the agent being required (Grandjean et al. 2014), new insights have already started to appear (Vrålstad et al. 2014, Maguire et al. 2016). The knowledge of the epidemiology of crayfish plague can therefore be expected to improve significantly.

It was strikingly clear that genotype Ps1 isolates detected in our study were the cause of the acute mortalities traditionally associated with crayfish plague infection in susceptible European ICS. This high virulence was verified in the challenge trial. We had chosen conditions for our experimental set-up that were expected to favour the crayfish plague agent, but in an environment that was less favourable to the crayfish. Accordingly both tested Ps1 isolates appeared to be highly virulent. In all experiments with Ps1 isolates, the infection has always resulted in swift and total mortality of the challenged European crayfish species (Alderman et al. 1987, Makkonen et al. 2012b, Jussila et al. 2013, Makkonen et al. 2014) Genotype As of *A. astaci* on the other hand, was in our study often found in situations where a chronic infection was suspected. This detection of the lower virulence of the As strains was also verified in the challenge trial. The experimental infection with the As genotype isolates showed clearly a slower development of the mortality in the test groups, even to the extent that there was no difference between some of the groups with the unchallenged control animals. The overall lower virulence of the As genotype was confirmed in other experimental challenges using other *A. astaci* genotype As isolates (Makkonen et al. 2012b, Makkonen et al. 2014, Becking et al. 2015). Recently, further evidence from several European ICS populations harbouring asymptomatic crayfish plague infection has been published. The crayfish plague still exists in Lake Eğirdir in Turkey (Svoboda et al. 2012, Kokko et al. 2012), which was infected in the mid-1980s (Baran et al. 1989). The hitherto flourishing Turkish narrow clawed crayfish trade has not fully recovered since the introduction of the crayfish plague in the mid-1980s (Harlioğlu 2008). This situation is similar to that of the Finnish crayfish fisheries related to the noble crayfish. At least one isolate from Turkey has been recognised as genotype As (Huang et al. 1994). Genotype As has also been detected in the ICS populations in the Czech Republic (Kozubiková-Balcarová et al. 2014) and in Croatia (Maguire et al. 2016). In all of those cases the crayfish plague agent seems to have been able to survive supported only by the European host which adds to the evidence of the lower virulence of these strains.

Due to the slow growth of *A. astaci* as compared with saprophytic water moulds (Lilley and Roberts 1997, Diéguez-Uribeondo et al. 2009), isolation of the agent is challenging from crayfish with only low levels of infection (OIE 2012, Kozubiková-Balcarová et al. 2013). The improved isolation method enabled us to detect isolates of *A. astaci* that exhibit a remarkably slow growth on artificial media. They all belonged to the genotype

As (IV). The growth rate of the pathogen is temperature dependent: *A. astaci* grows slower at lower temperatures (Alderman and Polglase 1986, Diéguez-Uribeondo et al. 1995). A lower temperature has also delayed the development of mortality in experimental infections (Alderman et al. 1987). The reduced growth rate could be a survival strategy of *A. astaci* in the highly susceptible European ICS, as the host defensive melanisation in these novel host animals seems to be activated slowly compared to that in the more resistant NACS (Cerenius et al. 2003). Although it is not directly comparable with the growth in the crayfish cuticle, the growth of *A. astaci* isolates in artificial medium can reflect the overall potential for growth. This association led us to study the variable virulence between the different *A. astaci* isolates by comparing their differences in radial growth rate. The two Lake Taulajärvi isolates were good examples of a slowly growing low-virulence strain, as these were the slowest growers in our collection. The As isolates in the comparative study had higher variation in radial growth rates but in general grew slowly in comparison to the Ps1 isolates. We tested slow and fast growing isolates of As and Ps1 genotypes in the infection trial to explore the eventual differences in virulence. The results including the highly virulent nature of the Ps1 genotype compared to the As genotype explain the former misconception about the total mortality in all crayfish plague cases. It also explains the recurrent nature of crayfish plague infections in Finland, all of which have been connected to the As genotype up to the present time. However, it was not possible to link the virulence directly with the growth rate due to the unexpected lack of virulence of the AsFast strain. With the other 3 tested strains, Ps1Fast, Ps1Slow and AsSlow, the mortality developed in the same order as the diminishing growth capacity of the strain. The precise mechanism that explains the lower virulence of the As genotype isolates thus could not be clarified in this study. The relatively long history of the As strain with the European ICS, and the wide geographical distribution could have led to a variety of survival strategies, where the diminished virulence towards the novel host was essential for the survival of the parasite. It seems likely that the parasite and the host would seek a balance to survive in the surroundings where they co-exist regularly. A reduced growth rate could be one strategy, but on the other hand some As isolates show a growth rate comparable with the Ps1 isolates. Several other possibilities for the parasite to reduce the negative effect on the host population have to be explored in further studies.

There is a fundamental difference between the As genotype group and the other genotype groups of *A. astaci* present in Europe. Only the As strain appears and spreads without a recognized NACS host being present. All other genotypes can be supported by their original hosts and therefore we might not see the development of a reduced virulence in them. The anecdotal evidence of the effect of the first waves of the crayfish plague seems to indicate an originally highly virulent causative agent. However, without the

knowledge about the natural host and the association with more naturally supported As genotype strains, the lower virulence mechanism in small weakened populations of crayfish remains speculative.

It is now more than 100 years since the introduction of the As-type crayfish plague into Finland and some adaptation in the host is also a possibility. On the other hand, the active stocking policies may not have favoured effective selection for better resistance.

6.3 Distribution of *A. astaci* genotypes in Finland

The first reports of crayfish mortalities in Finland are from 1893, and during the following decades the epidemic spread to all main water catchment areas containing natural noble crayfish stocks (Järvi 1910). At that time the cause of the trouble was not clear, but in the light of current knowledge the culprit was *A. astaci* genotype As. The greater variations of the growth rate and virulence among the strains of the As genotype group that we see today could reflect the long history of the genotype As in Finland, during which the pathogen has had to cope with the low resistance of the novel host and was thus subjected to harsh selection pressure. Nevertheless, it must be kept in mind that during the first 70 years, the pathogen managed to survive in Finland supported only by the highly susceptible European host. Even the introduction of the signal crayfish may not have offered more choices of host for the pathogen, since the As genotype has never been recovered from signal crayfish in natural conditions (Viljamaa-Dirks, unpublished results). In an experimental challenge an As genotype isolate seemed to cause elevated mortality in signal crayfish (Aydin et al. 2014), but the amount of spores used for the challenge was far beyond the level of what has been estimated in a natural outbreak (Strand et al. 2014). Additionally, the challenged signal crayfish were already infected with crayfish plague, which leaves the question open of the cumulative effect of two different strains in one host animal.

There have been 10 to 20 cases of crayfish plague estimated in Finland annually during the last decades (Mannonen et al. 2006), and roughly the same numbers of submissions of crayfish specimens for investigation. Although minor modifications in the isolation process resulted in improved yields of *A. astaci* in clinical cases (Viljamaa-Dirks and Heinikainen 2006), a culture based method is likely to give negative results in mildly infected animals. The adoption of sensitive molecular methods later made it possible to reach a reliable diagnosis in samples taken from carriers, and even in samples not suitable for culturing such as deteriorated or preserved samples. However, it can be difficult to obtain sample material in suspected epizootics, because sudden mortality may occur unobserved in wild populations. Thus, the verified cases of crayfish plague

probably represent only a part of the true incidence, even with the sensitive molecular detection methods available today.

During an acute mortality period, crayfish are usually heavily infected, thus improving the chance of successful isolation of the plague agent. The Ps1-genotype of *A. astaci* seems to be more often involved with acute mortality in noble crayfish, and therefore this genotype might be more readily isolated than the As-strains. Nevertheless, we found strains of the Ps1 genotype less frequently than strains of the As genotype. Southern Finland must *a priori* be considered as being now endemic for the Ps1 genotype of *A. astaci*, since infected populations of signal crayfish, the original host of this genotype, are widely established there. This was also confirmed by isolating representatives of the Ps1 genotype from the signal crayfish. Noble crayfish samples from the signal crayfish stocking area were obtained mainly from 2003 onwards, and were thus unevenly represented in our sample collection. Therefore it is impossible to compare the incidence of the genotypes in Finland accurately. However, it is evident from our study that the As genotype isolates are only rarely found from the signal crayfish area. It seems that in the areas where NACS are found in the wild, crayfish plague is caused by the genotypes carried by those species (Lilley et al. 1997, Oidtmann et al. 1999a, Grandjean et al. 2014, Kozubiková-Balcarová et al. 2014). The As-genotype crayfish plague devastated the main noble crayfish populations in the southern part of Finland for decades before the introduction of signal crayfish carrying the Ps1 genotype of *A. astaci*. It is therefore reasonable to assume that there were weak noble crayfish populations carrying As genotype crayfish plague in these water bodies, but that they eventually vanished in response to the introduction of signal crayfish infected with Ps1 genotype crayfish plague. Today, only scattered harvestable populations of noble crayfish still exist in the smaller lakes in this area. In addition to the low numbers of the highly susceptible noble crayfish populations currently present, low detections of As genotype *A. astaci* could be explained by a general choice of the shareholders to introduce signal crayfish in that area, if the noble crayfish population is not productive. This choice has seemed to be more relevant than trying to study the crayfish plague status by sampling a weak population or by organizing cage experiments. Both of these are the current prevailing situations whereby the majority of As-strains are detected in the rest of Finland. The newly implemented European invasive species regulation that prohibits new introductions of signal crayfish may change this attitude in the future.

In regions close to the signal crayfish territory, noble crayfish populations are continuously at risk of becoming infected by the *A. astaci*- carrying signal crayfish, as illustrated by the high number of crayfish disease outbreaks in immediately adjacent

areas. This might encourage stakeholders to undertake unauthorised introductions of signal crayfish, and thus further diminish the natural habitat for the noble crayfish.

It is noteworthy that the Ps1 genotype of crayfish plague was only incidentally isolated from areas not directly connected with the signal crayfish territory in this study. The general awareness of the public about the risks involved in transfers of crayfish or crayfish trapping equipment might have been the reason for the limited spread of the Ps1 genotype crayfish plague to central, eastern and northern Finland. However, in later years Ps1 genotype induced crayfish plague mortalities have been increasingly encountered in the noble crayfish area (Viljamaa-Dirks et al. 2011). Crayfish trade is extensive and crayfish of both species are transported widely over the country. The live crayfish trade may also spread the plague, although it has been illegal to place the crayfish even temporarily in waters other than where they were caught. As a preventive method it would therefore be worth considering a ban on the transport and selling of live signal crayfish in the areas dedicated to the noble crayfish.

It is not clear how many times *A. astaci* has been transferred to Finland from different locations. The origins of the plague could be limited to a few sources: Russian trade is implicated in the spread of the As genotype and imports from the USA Lake Tahoe and Lake Hennessy are implicated in the spread of the Ps1 genotype (Westman 1991, Kirjavainen and Westman 1999). The lack of variation was seen in the analysis of chitinase genes in the group of the Ps1 genotype isolates, whereas there was clearly some diversity among the As genotype isolates (Makkonen et al. 2012a). In our study, the Ps1 genotype isolates showed little variation in their growth patterns, which also may reflect a genetic uniformity by limited number of transfers to Finland, in addition to the presence of the natural host signal crayfish. It is likely that the presence of the As genotype in Europe resulted from one accidental release in Italy that spread and manifested as population mortalities from 1859 onwards (Alderman 1996). The remarkable variation of the growth rate and other variable features in the As genotype representatives could result from development of subtypes in separated water catchments during its long history in Europe and in Finland.

The survival of the crayfish plague agent for prolonged periods in noble crayfish populations has become evident in our studies. It also explains the phenomenon of chronic crayfish plague in the main waterways. The limited availability of new host animals in weak populations, and generally low water temperatures in the northern waters may create favourable circumstances for low virulent *A. astaci* strains to maintain their parasitic life cycles even in highly susceptible hosts. Unfortunately, the result of an infection with the As genotype of *A. astaci* in most cases can be expected to be a permanently lost productivity of the noble crayfish stock. Even seemingly recovered

populations are likely to crash shortly after revival, which makes the expensive and time-consuming re-stocking efforts ultimately unprofitable. The possibility of a latent *A. astaci* infection must be considered whenever planning management of the crayfish stocks, and the donating and receiving water bodies must be carefully studied for their crayfish plague status. It is also necessary to define the genotype of any crayfish plague caused mortality event: the chance for a complete wipe out of the population is much higher for an infection with a Ps1 genotype strain, and paradoxically, it makes the successful re-introduction of noble crayfish more likely. A highly virulent Ps1 strain of *A. astaci* being deliberately artificially introduced into a water body that is threatened by a permanent loss of the productive noble crayfish stock by a low virulent crayfish plague infection could be considered. Naturally the presence of signal crayfish would rule out this strategy.

All successful management demands knowledge of the basic factors that influence the outcome of management decisions. Such knowledge has been insufficient for the crayfish plague until recently. One of the main obstacles in earlier times was the laborious and highly uncertain and unreliable detection methods used for detecting the presence of *A. astaci* in the crayfish. Since this obstacle was finally overcome, new insights into the world of this much feared but fascinating crayfish parasite *A. astaci* opened up.

7 Conclusions

1. There are two genotypes of the crayfish plague agent *Aphanomyces astaci*, As and Ps1, present in Finland.

2. The As genotype strains of *A. astaci* show a lower virulence towards the noble crayfish than the Ps1 genotype strains. The persistent nature of the crayfish plague in the Finnish noble crayfish water bodies is due to the reservoir of low virulent As genotype *A. astaci* strains that can be carried by the weakened populations of noble crayfish for extended periods.

3. The detection of the persistent infection of the genotype As of *A. astaci* in weak populations of noble crayfish suggests that the number of verified cases of As-genotype strains might represent only a very small proportion of the actual prevalence of the infection.

4. Noble crayfish population management should be based on investigations of the genotype of the *A. astaci* strain in any crayfish plague event, and determination of the disease status in both the target water body and the donating population before re-introduction programs.

5. The signal crayfish spreads a highly virulent Ps1 genotype of *A. astaci* and every effort should be taken to prevent illegal introductions of signal crayfish into noble crayfish areas.

6. The spiny-cheek crayfish *O. limosus* is not the carrier of the strain of *A. astaci* that was first introduced to Europe, the genotype As, but it is the host for a previously undescribed genotype named Orconectes genotype, which is given the designation Or (or genotype E)

7. Although molecular methods are more sensitive for the detection of *A. astaci* isolation attempts should be continued because pure cultures are a necessity for the study of variable features inside and between the genotype groups.

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Persistent infection by crayfish plague *Aphanomyces astaci* in a noble crayfish population – a case report

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Abstract

Crayfish plague infection, caused by the oomycete *Aphanomyces astaci*, is believed to lead to a total mortality of the populations of highly susceptible freshwater crayfish species like the noble crayfish *Astacus astacus*. It has therefore been customary in Finland to restock affected water bodies relatively soon after the population has been wiped out in an acute episode of crayfish plague. In many cases, these re-introductions have failed without any explanation. During a follow-up study of a small lake, no restocking was undertaken after an outbreak had struck the population in 2001. The crayfish plague agent was isolated three years after the outbreak and again a year later in 2005. This capability of *A. astaci* to survive supported by a weak noble crayfish population may explain the often recorded failures of repopulation attempts, and recurrent episodes of acute crayfish plague in certain lakes.

Introduction

The oomycete organism *Aphanomyces astaci*, a parasite of the North-American freshwater crayfish, appeared in Europe about one and a half centuries ago, causing a devastating disease, crayfish plague, in all freshwater crayfish species of European origin (reviewed by Alderman, 1996; Söderhäll and Cerenius, 1999; Edgerton et al., 2002). The noble crayfish *Astacus astacus* is an indigenous crayfish species in Finland, originally inhabiting the southern lakes and rivers, but gradually introduced throughout the whole Finland south of the Arctic Circle. The crayfish plague arrived in Finland about 120 years ago when there was a lively crayfish trade with Russia, and during the following decades it devastated most of the main populations of noble crayfish (reviewed by Westman, 1991). It has been customary in Finland to try to restock plague-stricken lakes relatively soon after an acute episode of crayfish plague. In many cases, these re-introductions have failed without any known reason. A recent follow-up study showed that only about one third of restockings produced a thriving or exploitable population (Erkamo et al., 2010).In Sweden, where the situation with crayfish was compa-

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rable to Finland, less than 10% of the analyzed re-introduction programs have been successful (Fűrst, 1995). Success was mostly connected with a small and non-complex form of the lake with a uniform crayfish population structure, where the infection had the chance for effective spread. In Finland, the large and labyrinthine lake systems are suspected of supporting a form of chronic infection of crayfish plague due to several scattered subpopulations, where the infection could slowly migrate (Westman and Nylund, 1978; Westman, 1991). Distinct subpopulations could allow the crayfish plague agent to survive by reaching the next population in the limited time period of the survival of the host animal or the infective zoospores, which has been estimated to be no more than a few weeks (Edgerton et al., 2002).

Based on randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), four distinct genotypes of *A. astaci* have been recognized: the groups A, B, C and D (Huang et al., 1994; Diėguez-Uribeondo et al., 1995). The originally introduced group A (later referred to as As-genotype) has managed to survive until today and still is responsible for mortalities in noble crayfish stocks in Finland (Vennerström et al., 1998; Jussila et al., 2008). In an attempt to improve the fisheries, the plague resistant signal crayfish (*Pacifastacus leniusculus*) was introduced in the 1960's. The signal crayfish brought group B (later PsI-genotype) crayfish plague to Finland (Vennerström et al., 1998), complicating the crayfish plague situation even further.

In this present study, a small lake affected by crayfish plague was followed for several years, in order to determine the possible time schedule needed for successful re-introduction of noble crayfish. Crayfish fishing was continued in spite of the collapse of the stock and the trapped individuals were inspected for their disease status in the laboratories of Evira (the Finnish Food Safety Authority Evira).

Materials and methods

Lake Taulajärvi, the target locality of this study, is a small lake of 56 hectares in southern Finland (Figure 1). It is connected by a 500 meter long river to a large lake system Näsijärvi. These two lakes are separated by a dam, which keeps the water level constant in Taulajärvi. In exceptional cases the water level may be so high as to allow water to flow backwards over the dam. The shape of the lake is simple and the depth is on average about 5 m. Water quality is good (monitored by the Water Protection Association of the River Kokemäenjoki) and there are several species of fish inhabiting the lake.

Figure 1. Schematic map of Lake Taulajärvi (6839772N/329577E) situated in the municipality of Tampere.

The first introductions of noble crayfish in Lake Taulajärvi were done at the beginning of the last century. Since then, the lake has experienced four episodes of population crashes, believed to be due to crayfish plague. The first time was recorded in 1936. The latest re-introduction of crayfish was in 1971, after a putative crayfish plague in 1969. Even when the neighboring lake Näsijärvi was inhabited by a crayfish plague carrying population of signal crayfish (Evira, unpublished results from 1997), Lake Taulajärvi managed to support a productive stock of noble crayfish, until the plague struck again in 2001. The acute epidemic was preceded by increasing of the population, especially the growth of the number of smaller size classes, as judged by the bookkeeping of one local fisherman (Figure 2). The fishing effort was on average 376 (255-500) trap-nights (number of traps X number of nights) yearly, except for the year 1995, when no statistics were kept.

Crayfish samples were sent for the first time to Evira during an acute mortality event observed in May 2001. During 2001, two more trapping trials were made, the first one yielding 28 specimens in July and the second only five individuals in August. During 2002-2006, test trappings were conducted mostly once a year, consisting of 60 to100 trap-nights. All crayfish caught were sent to be examined in Evira. After the discovery of signal crayfish, systematic trapping was discontinued.

The crayfish was examined according to the method of Cerenius et al. (1988) until year 2004, after that the same method was used modified as described by Viljamaa-Dirks and Heinikainen (2006). In brief, the crayfish were inspected macroscopically for any signs of infection (damaged or melanized areas in the exoskeleton). The abdominal cuticle was examined for the hyphal growth typical for crayfish plague by light microscopy (100X). During the first years of the study period, culturing the crayfish plague agent was attempted mainly when microscopy was positive. From 2004, the abdominal cuticle and walking legs were used for culture regard-

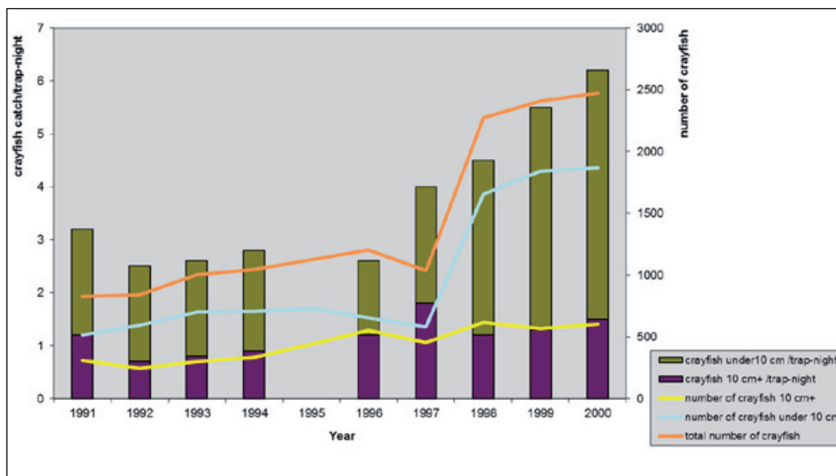

Figure 2. Crayfish catch from Lake Taulajärvi in 1991-2000 by one fisherman. Market size crayfish with total length of 10 cm or longer were counted separately.

less of the results of microscopy.

Isolates that fulfilled the morphological description of *Aphanomyces* sp. were studied by the RAPD PCR method described by Huang et al. (1994). Isolates were also confirmed as *Aphanomyces astaci* by the PCR methods of Oidtmann et al. (2004 and 2006).

Results

The acute mortality event in 2001 diminished the catch of noble crayfish in Lake Taulajärvi from an average of 6.2 individuals /trap-night in the season of 2000 down to a few individuals from the whole lake after the summer of 2001.

In May 2001, only dead individuals were available for inspection. One was badly deteriorated; the other three had hyphal growth in the abdominal cuticle, consistent with acute crayfish plague infection. None had any melanized areas in the cuticle. Isolation of the crayfish plague agent failed due to contamination with non-specific water molds.

In 2002, crayfish samples were judged to be negative for crayfish plague, since no typical hyphal growth was seen. In the late summer sample of 2003, two crayfish showed melanized areas in the abdominal cuticle, with hyphae typical of *A. astaci*. Isolation of the agent as pure culture failed again due to nonspecific growth of water molds. In the summer of 2004, one out of five individuals had a melanized area in the abdominal cuticle, with typical hyphae (Figure 3). The crayfish plague agent was isolated and confirmed to be *A. astaci* belonging to the genotype As. In 2005, two out of six crayfish exhibited macroscopic and microscopic signs of crayfish plague. Again, *A. astaci* genotype As was isolated. During 2003-2005, the crayfish catch from the Lake Taulajärvi remained low being between 0.05 -0.15 individuals/trap-night.

In 2006, signal crayfish were detected in the lake. Seven individuals of signal crayfish were caught. These showed no visible signs of infection and were not studied further. At the same time, nine noble crayfish were caught. None of them showed macroscopic signs of crayfish plague but two had hyphal growth without melanization in the abdominal cuticle, a sign of an acute infection. Crayfish plague agent *A. astaci* representing the genogroup of signal crayfish strains (PsI) was isolated. A summary of the results of the laboratory examinations is presented in Table 1.

Discussion

The total elimination of populations of highly susceptible crayfish species has been the expected outcome of infection with the crayfish plague agent *A. astaci* (Alderman et al., 1987;

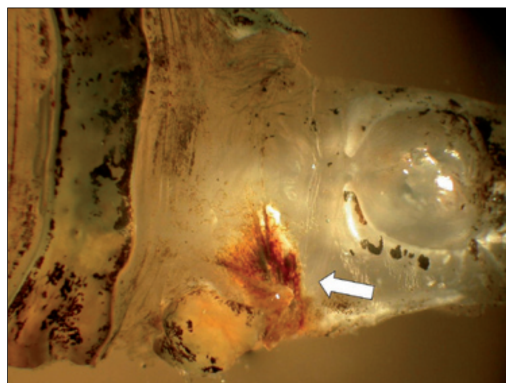

Figure 3. The distal segment of the abdominal cuticle of a noble crayfish caught from Lake Taulajärvi in July 2004, showing the melanised area with hyphae (arrow). Stereomicroscopic view 10X.

Table 1. Noble crayfish samples from Lake Taulajärvi, analyzed in Evira 2001-2006. Diagnosis of crayfish plague based on microscopy was made in 2001 and 2003, and based on isolation of the agent in 2004, 2005 and 2006. nd: Isolation was not attempted.

Date	Number of crayfish	Melanization in exoskeleton	Microscopic detection of typical hyphae	Culture
29.5.2001	4	0	3	-
17.7.2001	1	1	0	nd
28.8.2001	5	1	1	-
11.9.2002	2	0	0	nd
17.6.2003	3	0	0	nd
6.8.2003	6	2	2	(+)
6.7.2004	5	1	1	+ (As)
2.9.2004	2	0	0	-
16.8.2005	6	2	2	+ (As)
25.8.2006	9	0	2	+ (PsI)
Total number	43	7	11	

- Negative
(+) Isolation in pure culture failed
+ (As) *Aphanomyces astaci*, belonging to genogroup As was isolated.
+ (PsI) *Aphanomyces astaci*, belonging to genogroup PsI was isolated.

Söderhäll and Cerenius, 1999).The proposal that the crayfish plague has the capability to stay alive supported by a weak population of noble crayfish was put forward already after the analysis of the poor results of Swedish restocking efforts (Fürst, 1995), but there has been no direct evidence to support this theory.

After a long history of episodes of crayfish plague in Lake Taulajärvi, the situation was followed by test trapping after the acute mortality phase. A small number of crayfish did remain alive in the lake. Crayfish plague infected individuals were found many years after the acute episode. It seems unlikely that the infection would have been introduced several times in this small lake, especially when there was no actual crayfish fishery after the acute episode in 2001. No other cases of plague epizootics in noble crayfish were reported in the vicinity of Lake Taulajärvi during the study period 2001-2006. Furthermore, in the linked water system, Lake Näsijärvi, a plague carrying signal crayfish population exists, but the plague strain carried by this species belongs to a different genogroup. As a result of the appearance of signal crayfish in Lake Taulajärvi, there was an acute plague episode, caused by this different strain of crayfish plague. Afterwards, no systematic follow-up was conducted, but the signal crayfish population has now developed to an exploitable level. One noble crayfish sized 12 cm was caught in 2010 (Aarre Siro, personal communication). Most probably this individual had been surviving in the lake from 2006 and managed to avoid the infection.

The origin of the crayfish plague infection in 2001 in Lake Taulajärvi remains unclear. The outbreak followed after a sharp increase in the numbers of smaller size classes of crayfish. It is not uncommon that a lake has recurrent episodes of crayfish plague (Fürst, 1995; Erkamo et al., 2010). If they are preceded by an increased population density, a crayfish fishery has often been started, and not surprisingly, the trappers are blamed for having re-introduced the plague infection. However, one could argue that these infections have remained in the lake from the earlier outbreaks, and the increased density of the crayfish population triggers the acute phase of the disease.

Earlier experimental studies have shown that the time of the survival of the host is dependent on the spore density as well as the water temperature (Alderman et al., 1987). In a sparse crayfish population and considering the relative brevity of the warm water period in northern countries, the survival of the plague organism for prolonged periods in noble crayfish populations seems possible. It would also seem likely that the parasite and the host would seek a balance to survive in the surroundings, where their co-existence is no longer purely accidental. Since it is now more than one hundred years after the introduction of As-type crayfish plague into Finland, this may have led to adaptation towards less virulent strains. Adaptation in the host is also a possibility, although the active stocking policies may not have favoured effective selection for better resistance.

The ability of some strains of crayfish plague to survive supported by a sparse population of noble crayfish could explain the phenomenon of the chronic plague in the main waterways with part of the recurrent episodes of acute epidemics in smaller lakes. This possibility needs to be considered in restocking programs as well. Further studies will be necessary to compare the virulence of crayfish plague strains and genotypes. New molecular methods which are suitable for carrier detection (Oidtmann et al., 2006; Vrålstad et al., 2009), can nowadays be included in surveys in order to provide a better understanding of the epidemiology of crayfish plague.

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Short Communication

Spiny-cheek crayfish *Orconectes limosus* carry a novel genotype of the crayfish plague pathogen *Aphanomyces astaci*

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A B S T R A C T

The oomycete *Aphanomyces astaci* causes mass mortalities of European crayfish. Different species of North American crayfish, original hosts of this parasite, seem to carry different strains of *A. astaci*. So far, four distinct genotype groups have been recognised using Random Amplification of Polymorphic DNA (RAPD-PCR). We succeeded in isolating *A. astaci* from the spiny-cheek crayfish *Orconectes limosus*, a widespread invader in Europe, and confirmed that this species carries a novel *A. astaci* genotype. Improving knowledge on the diversity of this parasite may facilitate identification of genotypes in mass mortalities of European crayfish, thus tracing the sources of infection.

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1. Introduction

The fungal-like parasite *Aphanomyces astaci* (Oomycetes) is the causative agent of mass mortalities of European crayfish, called the crayfish plague. This disease severely endangers wild populations of indigenous crayfish in Europe and causes economic losses in their aquaculture (Souty-Grosset et al., 2006). *A. astaci* apparently originates from North America; North American crayfish species are much less susceptible to crayfish plague than those indigenous to other parts of the world (Unestam, 1969). However, American crayfish may carry chronic infections and their populations are often sources of the pathogen (Cerenius et al., 1988).

The first crayfish mass mortalities presumably caused by crayfish plague occurred in Europe in 1859, and during the following decades the disease wiped out many populations of indigenous crayfish throughout Europe (Alderman, 1996). It is unknown how the infection originally reached the continent; the first documented intentional introduction of an American crayfish, the spiny-cheek crayfish *Orconectes limosus*, is only from 1890 (Holdich et al., 2009). Although this species was not stocked in large numbers for aquaculture purposes, it colonised vast areas, particularly in Central Europe (Filipová et al., 2011). Large-scale imports and dispersal during the 20th century of economically more important American crayfish, the signal crayfish *Pacifastacus leniusculus* and the red swamp crayfish *Procambarus clarkii*, has resulted in new waves of the spread of *A. astaci* in recent decades.

A. astaci has no species-specific morphological characters; it is therefore traditionally defined by its pathogenicity to crayfish (Johnson et al., 2002; Cerenius et al., 1988). Although virulence may apparently vary among different *A. astaci* strains (Viljamaa-Dirks and Torssonen, 2008), the species definition has been supported by molecular analyses. *Aphanomyces* strains highly pathogenic to European crayfish share very similar sequences of the internal transcribed spacer (ITS) in the nuclear ribosomal DNA (Makkonen et al., 2011), which are distinct from those of related congeneric species (Diéguez-Uribeondo et al., 2009; Takuma et al., 2010). On the contrary, whole-genome analysis based on Random Amplification of Polymorphic DNA (RAPD-PCR) showed substantial genetic differences among groups of *A. astaci* isolates (Huang et al., 1994).

To differentiate among *A. astaci* genotypes using RAPD-PCR, it is necessary to obtain pure cultures of the parasite (Huang et al., 1994). Unfortunately, isolation of *A. astaci* is complicated, especially from American species that are usually much less infected than European crayfish dying from the crayfish plague (Vrålstad et al., 2009). Therefore, the diversity of *A. astaci* strains is still not well known. Four groups of isolates have been identified so far (Huang et al., 1994; Diéguez-Uribeondo et al., 1995). Group A (genotype As) is known only from European crayfish. It is probably the pathogen that caused the first wave of crayfish mass mortalities in the 19th century and has persisted in Europe since then (Huang et al., 1994). Groups B (genotype PsI) and C (PsII) are associated with *P. leniusculus* and group D (type Pc) was isolated from *P. clarkii*. Although *O. limosus* is a confirmed vector of *A. astaci*

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(Vey et al., 1983), the pathogen has not yet been isolated from this species and genetically characterised.

In cases of indigenous crayfish mass mortalities, identification of the pathogen genotype may assist in tracing sources of infection (Lilley et al., 1997; Vennerström et al., 1998; Oidtmann et al., 1999). Thus, the better our knowledge of crayfish plague pathogen genetic diversity, the more precisely we can identify the pathways by which it is spread.

2. Methods

We sampled presumably infected *O. limosus* to obtain laboratory cultures of *A. astaci* from this species for further characterisation. Selection of a suitable source of crayfish for parasite isolation was based on data of its prevalence in Czech populations of *O. limosus* (Kozubíková et al., 2009). We chose a pond in Smečno (Central Bohemia, 50°11.3′N, 14°02.8′E), as the vast majority (ca 95%) of individuals sampled repeatedly from this locality were found to be infected by *A. astaci* (Matasová et al., 2011). Furthermore, the infection dose in some local crayfish individuals was high (up to agent level A5 after Vrålstad et al., 2009).

Four crayfish were collected by manual searching and transported to the Evira Kuopio laboratory. They were kept at about 10 °C in a small volume of water until examination. Two individuals died after two weeks, one of them showing signs of paralysis the day before. These individuals were selected for *A. astaci* isolation by the method of Cerenius et al. (1988) modified by Viljamaa-Dirks and Heinikainen (2006). Crayfish were examined macroscopically and microscopically. The whole abdominal cuticle and all walking legs were cleaned with sterile water and a quick bath in 70% ethanol, submerged slightly in peptone-glucose-salt agar (PG-1) with antibiotics, and incubated at 15 °C. Inoculated plates were examined daily by microscope, and any oomycete having features consistent with *A. astaci* (i.e., frequently branching, non-septate hyphae about 9 μm in diameter with rounded tips; Cerenius et al., 1988) was transferred to a new plate for further study.

DNA was isolated by a DNeasy Plant Mini kit (Qiagen) after grounding of the mycelium with ceramic beads in a Magna Lyser instrument (Roche). To confirm the isolates as *A. astaci*, we applied a single round PCR detection assay after Oidtmann et al. (2006), and tested the pathogenicity of one of the isolates (Evira4806a/10) in an infection trial towards noble crayfish *Astacus astacus* (as described in Cerenius et al., 1988). From another isolate (Evira4805b/10), a 1354 bp fragment of the rDNA region was amplified using primers NS5 and ITS4 (White et al., 1990). This fragment was cloned into a pCR®2.1-TOPO plasmid (Invitrogen) and sequenced with universal primers M13R and T7 in a commercial sequencing facility. The resulting sequence is deposited in GenBank under accession number JF827153. The genotype grouping of the isolates was based on RAPD-PCR after Huang et al. (1994).

3. Results

The examined crayfish showed a few barely-observable areas of macroscopic melanisation, especially in the joints of the walking legs. Under microscopic examination, the abdominal cuticles and joints of the two crayfish had several foci of short, partly melanised hyphae (Fig. 1). Hyphae suspected to be *A. astaci* emerged from the crayfish cuticle in the cultures after 5–6 days. We obtained four isolates (Evira4805a/10, Evira4805b/10, Evira4806a/10 and Evira4806b/10), two from each crayfish individual used for pathogen isolation. Subcultured isolates fulfilled the morphological criteria for *Aphanomyces* sp. in hyphal growth and sporangia morphology. Specific PCR suggested these isolates to be *A. astaci*, which was confirmed both by infection trial and sequencing. The ITS sequence obtained from one of the isolates was identical to other genotypes of this species. However, the RAPD profiles of all the isolates were identical to each other but clearly different from the profiles of the four *A. astaci* genotype groups described so far (Fig. 2).

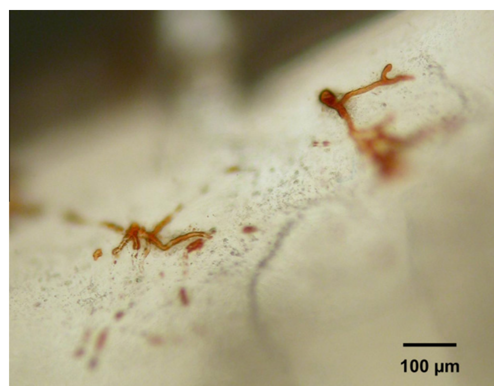

Fig. 1. Melanised hyphae in the cuticle of the spiny-cheek crayfish *Orconectes limosus* from Smečno.

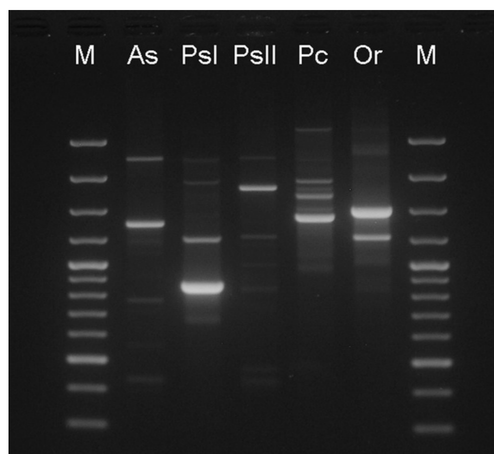

Fig. 2. Agarose gel with RAPD-PCR patterns of the new genotype of *A. astaci* in comparison to all four previously known genotype groups; results of amplification using the primer B01 after Huang et al. (1994). Abbreviations: M = DNA marker (300, 400, 500, 600, 700, 800, 900, 1000, 1200, 1500, 2000, 3000 bp bands; 500 and 1000 bp bands are more intense), As = strain Da from *Astacus astacus* (group A), PsI = strain Si from *Pacifastacus leniusculus* (group B), PsII = strain Kv from *Pacifastacus leniusculus* (group C), Pc = strain Pc from *Procambarus clarkii* (group D), and Or = the new strain Evira4805a/10 from *Orconectes limosus* (group E).

4. Discussion

Our study shows that *O. limosus* carries a novel genotype of *A. astaci* (Or, group E). As the RAPD pattern of our isolates was different from all other so far tested, special attention was given to the species identification. *Aphanomyces* similar to *A. astaci* but genetically divergent and not pathogenic to crayfish has been recently described from crayfish (Royo et al., 2004), and an apparently different though related lineage was detected by ITS sequencing in signal crayfish by Kozubíková et al. (2009). However, the isolates obtained by us fulfilled the morphological characteristics

of *A. astaci*, the ITS sequence corresponded to this species, and the infection trial confirmed pathogenicity to a susceptible crayfish host. The correct identification of the isolates as *A. astaci* is therefore unambiguous.

Previous knowledge of the prevalence and intensity of *A. astaci* infection in *O. limosus* populations was crucial for the selection of source material, as many populations of North American crayfish seem to be very lightly infected (Kozubíková et al., 2009). The population in Smečno is one of the most infected in Czechia, and some crayfish there reached agent levels comparable with European crayfish dying from crayfish plague (Kozubíková, 2011). Following the stress caused by collection and transport, the infection could have thrived in the animals, probably leading to their death. This might also have facilitated our successful isolation of the pathogen.

O. limosus may host more than one genotype of *A. astaci*, especially in its original range. However, we presume that only a limited pathogen diversity was imported to Europe with this species, as it seems that all European populations of *O. limosus* descend from a single batch of only 90 individuals (Filipová et al., 2011). Nevertheless, we cannot exclude that different *A. astaci* genotypes could have been transferred to *O. limosus* in Europe from other American species.

It seems that many North American crayfish species carry their own genotypes of the crayfish plague agent. As these genotypes may vary in physiological features and virulence (Diéguez-Uribeondo et al., 1995; Viljamaa-Dirks and Torssonen, 2008), further studies should evaluate their abilities to infect and cause mortality in hosts other than the original ones. If cross-infection reduced the fitness of other American crayfish, some aggressive strains could be potentially used in invasive crayfish control not only in Europe but also North America, where crayfish invasions are widespread and biocontrol agents are being sought (Davidson et al., 2010). Therefore, attempts to isolate and characterise new *A. astaci* genotypes should continue. Furthermore, markers should be developed that would bypass the need to culture the pathogen for routine genotyping, and allow identification of *A. astaci* strains directly from the DNA isolates of infected hosts.

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Distribution and epidemiology of genotypes of the crayfish plague agent *Aphanomyces astaci* from noble crayfish *Astacus astacus* in Finland

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ABSTRACT: The crayfish plague agent *Aphanomyces astaci* was isolated from 69 noble crayfish *Astacus astacus* samples in Finland between 1996 and 2006. All isolates were genotyped using randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR). Altogether, 43 isolates belonged to the genotype group of Astacus strains (As), which is assumed to represent the genotype originally introduced into Europe around 1860 and into Finland in 1893. There were 26 crayfish plague isolates belonging to the group of Pacifastacus strain I (Ps1), which appeared in Europe after the stocking of the North American species signal crayfish *Pacifastacus leniusculus*. The geographical distribution of the 2 genotypes in Finland corresponded with the stocking strategies of signal crayfish. The majority of Ps1-strains (83%) were associated with a classical crayfish plague episode involving acute mortality, compared with only 33% of the As-strains. As-strains were found more often by searching for reasons for population declines or permanently weak populations, or through cage experiments in connection with reintroduction programmes. In some water bodies, isolations of the As-strains were made in successive years. This study shows that persistent crayfish plague infection is not uncommon in noble crayfish populations. The described epidemiological features suggest a difference in virulence between these 2 genotypes.

KEY WORDS: Crayfish plague · *Aphanomyces astaci* · RAPD-PCR · Oomycete · Virulence

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INTRODUCTION

Crayfish plague, caused by the oomycete *Aphanomyces astaci* (Schikora 1903), is the most serious disease threatening European freshwater crayfish populations. Its first appearance in Finland in 1893 was followed by a severe decline of the native noble crayfish *Astacus astacus* (Linnaeus) populations during the subsequent decades (Järvi 1910, Westman et al. 1973). In 2 Scandinavian countries, Sweden and Finland, where the noble crayfish is an economically significant species, attempts to reintroduce noble crayfish in main water courses were mostly unsuccessful (Westman 1991, Fürst 1995, Nylund & Westman 1995a). Therefore, a North American species, signal crayfish *Pacifastacus leniusculus* (Dana), was introduced first in Sweden, soon followed by Finland (Fürst 1995, Nylund & Westman 1995b, Bohman et al. 2006). North American crayfish species are relatively resistant to the crayfish plague, often carrying *A. astaci* in their cuticle as a latent infection, with mortality occurring only in stress situations (Unestam & Weiss 1970, Unestam et al. 1977, Persson & Söderhäll 1983). The signal crayfish is now widely spread throughout many European countries including Finland (Westman 1991, Gherardi & Holdich 1999).

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Although some introductions of signal crayfish were made previously in the middle, eastern and northern parts of Finland, there was later a proposal (Kirjavainen 1989) that signal crayfish stocking should be restricted to a distinct region of southern Finland. This area, with some minor changes, was approved by the fisheries authorities in a national crayfish strategy agreement (Mannonen & Halonen 2000). As the carrier of crayfish plague, the signal crayfish has been shown or suspected to be the source of native crayfish mortalities in numerous reports. There are also numerous reports that invading North American crayfish species have been shown or suspected to be the source of native crayfish mortalities (e.g. Huang et al. 1994, Vennerström et al. 1998, Lilley et al. 1997, Oidtmann et al. 1999a, Pöckl & Pekny 2002, Bohman et al. 2006).

In spite of the long history of crayfish plague in Europe, relatively little is known about the behaviour of *Aphanomyces astaci* in natural epidemics involving the highly susceptible European species. It took over 50 yr before the oomycete was accepted as the etiological agent for crayfish plague, illustrating the difficulties in the isolation and identification of the organism (Schäperclaus 1935, Nybelin 1936, Rennerfelt 1936). Subsequent research has provided improved methods for isolation (Alderman & Polglase 1986, Cerenius et al. 1988, Oidtmann et al. 1999b, Viljamaa-Dirks & Heinikainen 2006), and the development of molecular methods has made possible a both rapid and definitive diagnosis (Oidtmann et al. 2004, Oidtmann et al. 2006, Vrålstad et al. 2009).

The studies concerning pathobiology have mostly shown 100% mortality in susceptible species under laboratory circumstances, with the development of the pathology depending on the infective dose and water temperature (Alderman et al. 1987, Cerenius et al. 1988). No long-term existence outside the crayfish host has ever been detected. The controversial phenomenon of re-appearing epizootics in the main Finnish waterways was postulated to be attributable to the existence of scattered crayfish subpopulations, allowing the crayfish plague infection to slowly move around from one subpopulation to another, thus keeping the parasite alive in the complex water body systems (Westman 1991). The persistent problems caused by crayfish plague have also been described in Sweden, with the continuous presence of *Aphanomyces astaci* in a very weak population of noble crayfish being proposed as the culprit (Fürst 1995), although there was no direct evidence other than systematic failure of the reintroduction attempts.

Recently, a follow-up study in a small Finnish lake showed that crayfish plague could persist in a weak noble crayfish population for several years following the acute phase of the disease (Viljamaa-Dirks et al. 2011). Persistent infection has also recently been described in Turkey (Svoboda et al. 2012).

Amplification of DNA by PCR using arbitrary oligonucleotides as primers is a technique to reveal genetic differences between different isolates of organisms, and this method was applied by Huang et al. (1994) to characterise isolates of *Aphanomyces astaci* from different sources. In the original study, 2 clearly distinct groups and a single strain outside of these groups were recognised. Sexual propagation is not a known feature of *A. astaci*; accordingly, a high degree of genetic similarity was seen inside the groups, in spite of the large geographical and time span of the isolations. The first main group consisted of isolates from noble crayfish in Sweden and one isolate from the narrow-clawed crayfish *Astacus leptodactylus* (Eschscholtz) from Turkey. These *A. astaci* strains were present in Europe before the introductions of the signal crayfish, and are called Astacus strains or group A (hereafter referred to as As). The As-type strains are therefore generally assumed to represent the first genotype of *A. astaci* introduced to Europe approximately 150 yr ago, the original North American crayfish host of this genotype group remaining unknown. The other main group was formed by isolates from signal crayfish from USA and Sweden, as well as from noble crayfish from Sweden after the introductions of signal crayfish. This group is called Pacifastacus strain I or group B (hereafter referred to as Ps1). In later studies, isolates from European crayfish species in Finland, England and Germany were also found to be a member of this group (Vennerström et al. 1998, Lilley et al. 1997, Oidtmann et al. 1999a). A third type was represented by a single isolate from signal crayfish imported into Sweden from Canada; this is called Pacifastacus strain II or group C (hereafter referred to as Ps2). Since this original study, 2 novel genotypes have been recognized from invasive North American species in Southern and Central Europe, one from red swamp crayfish *Procambarus clarkii* (Girard) (group D, hereafter referred to as Pc), and one from spiny cheek crayfish *Orconectes limosus* (Rafinesque) (group E, hereafter referred to as Or) (Diéguez-Uribeondo et al. 1995, Kozubíková et al. 2011).

The present study describes the genotypes of *Aphanomyces astaci* isolates from Finnish noble crayfish, their geographical distribution and the differences concerning their epidemiological features.

MATERIALS AND METHODS

Sample material

The sample material consists of 69 samples of noble crayfish specimens that were sent to the Finnish Food Safety Authority during the years 1996 to 2006 and were found to be positive for the presence of *Aphanomyces astaci* through a successful isolation of the organism. There were from 1 to 29 living, moribund or dead specimens of noble crayfish in each sample. Samples were sent to the laboratory mostly by shareholders of the local fisheries, mainly to determine the reason for a decline in a crayfish population (9 samples) or acute crayfish mortality (34 samples). Dead crayfish found in the same or adjacent water body during the same summer season were considered as a sign of acute mortality in this study. In 2 cases, the crayfish came from cage experiments connected with an acute episode. When the crayfish catch was clearly diminished or almost completely lost compared with the year before, but without evidence of mortalities, we categorized this phenomenon as a population decline.

A weak population had a verified or suspected history of crayfish plague episodes in the past, but at least 2 yr had passed before sampling. Some samples consisted of those remaining crayfish individuals (7 samples).

Several samples (15) were crayfish from cages that were followed for a few weeks to months to study the situation in a water body long after the disappearance or weakening of the population of crayfish. Such cage experiments were mostly performed in preparation for restocking programmes, but sometimes stocking had already been conducted and the success was being monitored by caging some individuals. The aim of these experiments was to ascertain the suitability of the water body to support crayfish, since unfavourable water parameters were often suspected as the reason for a low population level.

Two samples originated from lakes with a mixed population of signal and noble crayfish, with signs of mortality concerning the noble crayfish.

Table 1 shows the isolates of *Aphanomyces astaci* arranged by location and including information on the time of isolation (the sample identification number), the water body where the crayfish were caught and its water catchment area identification number and coordinates, the sample size, and the reason for the investigation, as informed by the local stakeholders. In those occasions where isolations were made from the same or adjacent area within a short time interval, only one isolate was included. Neither farmed crayfish nor isolates from signal crayfish were included in this study.

The isolates were placed on the map of the Finnish main water catchment areas, using the coordinates of the exact sample site. If the exact site was not known, the midpoint of the lake or the stretch of the river was used (Fig. 1). The map also shows the original distribution of the noble crayfish (Järvi 1910) and the area of the signal crayfish introduction strategy (Mannonen & Halonen 2000).

Isolation and identification of *Aphanomyces astaci*

The diagnostic method used in 1996–1998 was modified from that of Cerenius et al. (1988), i.e. 2 antibiotics, oxolinic acid and ampicillin (Alderman & Polglase 1986), were added to the growth medium PG-1, instead of potassium tellurite drops on the sample. After 1998, the method was modified to always include the whole abdominal cuticle as well as all pereiopods as the cultivation material (Viljamaa-Dirks & Heinikainen 2006). Challenge experiments as described by Cerenius et al. (1988) were performed with all isolates in 1998–2000. Tests were performed with farmed noble crayfish, using 3 to 5 animals in each test. The isolates were refrigerated at +4 ± 2°C in vials containing PG1-medium and covered with mineral oil, and the mycelium was transferred to a fresh growth medium every 6 mo. A specific PCR-method developed by Oidtmann et al. (2006) was used to verify the correct identification of the strains, except for the 2 earlier characterised strains K121/1996 and K136/1996 (Vennerström et al. 1998), which were no longer available.

Randomly amplified polymorphic DNA-PCR

Mycelium was grown in PG-1 medium, and ground with ceramic beads in a Magna Lyser instrument (Roche). DNA was isolated according to the manufacturer's instructions using the DNeasy Plant Mini kit (Qiagen). DNA was subjected to randomly amplified polymorphic DNA (RADP)-PCR with Operon B01 primer as described by Huang et al. (1994), with minor modifications. Briefly, PCR reactions were carried out in 50 µl volume containing 2.5 units of HotStarTaq DNA polymerase (Qiagen), 1.5 mM $MgCl_2$, 200 µM of each dNTP, and 0.5 µM primer in standard buffer for the enzyme. Amplified DNA was resolved in 1.5% agarose containing ethidium bromide and

Table 1. Noble crayfish samples positive for *Aphanomyces astaci* between 1996 and 2006. Sample identification number is according to the archive of the Finnish Food Safety Authority. Sample location is identified by the name of the lake or river, the water catchment area number and the geographical coordinates (given using the Finnish Uniform Coordinate System). Background information is based on anamnestic information available for each sample. Acute mortality: dead or diseased crayfish found in the same or adjacent water body during the same summer; population decline: population diminished or nearly vanished compared with the preceding fishing season; weak population: a weak population existing after a population crash in the past; cage experiment 1: connected with acute mortality; cage experiment 2: no mortality involved. Genotype (RAPD-PCR group): Ps1, Pasifastacus strain I; As, Astacus strain

Sample no.	Identification	Sample location	Water catchment area (3rd level)	N/lat	E/lon	Sample size	Background information	Genotype
1	15.08.2000/46	Lake Tohmajärvi	02.013	6902108	3675874	5	Weak population	As
2	25.07.2006/64	Lake Kasurinlampi	03.021	6827413	3634518	3	Acute mortality	As
3	K86/1999	Lake Ihalanjärvi	04.127	6822895	3600313	8	Cage experiment 2	As
4	23.07.2002/7	Lake Lieviskäjärvi	04.127	6827733	3591882	1	Weak population	As
5	K47/1999	Lake Korpijärvi	04.143	6793116	3506231	6	Acute mortality	Ps1
6	09.08.2004/53	Lake Immalanjärvi	04.192	6791158	3603701	5	Acute mortality	As
7	25.07.2000/9	Lake Pitkäjärvi	04.199	6804781	3623645	5	Acute mortality	As
8	K100/1998	Lake Kotkajärvi	04.212	6870909	3578908	20	Acute mortality	As
9	26.07.2002/15	Lake Sylkky	04.296	6866087	3612677	2	Acute mortality	As
10	25.10.2001/92	Lake Kuorinkajärvi	04.317	6947499	3623551	1	Weak population	As
11	08.09.2004/92	River Vuokonjoki	04.411	7031655	3607920	1	Population decline	As
12	29.07.2003/116	Lake Kelvänjärvi	04.419	7001229	3654734	19	Acute mortality	As
13	K71/1999	River Lieksanjoki, Pankakoski	04.423	7029451	3658496	3	Cage experiment 2	As
14	K116/1998	Lake Pieni-Valtimojärvi	04.462	7060817	3593171	8	Population decline	As
15	07.08.2001/11	Lake Jännevirta, Pohjanlampi	04.611	6986024	3542829	6	Acute mortality	Ps1
16	K121/1996	River Vaikkojoki	04.742	7004102	3593211	5	Acute mortality	Ps1
17	23.08.2001/64	River Koitajoki	04.912	6971346	3691966	7	Acute mortality	As
18	24.09.1999/34	Lake Issonjärvi	04.922	6955756	3708038	1	Acute mortality	As
19	10.08.2005/23	River Teutjoki	14.153	6725738	3471316	1	Acute mortality	Ps1
20	31.08.2000/6	Lake Vehkajärvi	14.177	6797333	3476583	5	Acute mortality	Ps1
21	25.07.2001/3	Lake Korkeanalanen	14.228	6826532	3433994	8	Acute mortality	As
22	K105/1998	Lake Päijänne/ Hauhonselkä	14.231	6891554	3437614	5	Population decline	As
23	30.06.2000/19	Lake Vesijärvi	14.241	6777175	3421040	3	Acute mortality	Ps1
24	25.07.2006/86	Lake Löytänä	14.438	7014397	3432024	1	Population decline	As
25	K136/1996	Lake Iso-Suojärvi	14.687	6968634	3425235	4	Population decline	As
26	20.08.2001/19	Lake Horonjärvi	14.715	6973391	3463912	1	Population decline	As
27	07.09.2000/36	Lake Iso-Lauas	14.725	6963038	3519459	7	Cage experiment 2	As
28	23.07.2002/63	Lake Pieni Tallusjärvi	14.772	6977322	3504325	1	Acute mortality	As
29	31.07.2002/34	Lake Korosjärvi	14.773	6995466	3500056	2	Population decline	As
30	29.08.2000/7	Lake Pukarainen	14.812	6823774	3443054	7	Acute mortality	Ps1
31	28.08.2000/4	Lake Laitjärvi	14.822	6839473	3458526	10	Acute mortality	As
32	04.08.2000/18	Lake Kilpilampi	14.823	6836278	3462323	7	Acute mortality	Ps1
33	07.06.2001/82	Lake Iso-Suojärvi	14.823	6834372	3463713	1	Acute mortality	As
34	11.07.2003/86	Lake Iso-Kuivajärvi	14.824	6819848	3458769	8	Acute mortality	Ps1
35	25.07.2001/48	Lake Saarijärvi	14.911	6772699	3482717	17	Acute mortality	Ps1
36	16.07.2002/97	Lake Hirvijärvi	14.924	6855607	3467702	3	Acute mortality	Ps1
37	04.08.2000/42	Lake Harjujärvi	14.939	6865242	3505780	3	Acute mortality	Ps1
38	29.06.2000/8	Lake Ylistenjärvi	35.138	6800228	3281198	4	Acute mortality	Ps1
39	05.06.2002/135	Lake Iso-Arajärvi	35.290	6794450	3315909	9	Acute mortality	Ps1
40	06.07.2004/27	Lake Taulajärvi	35.311	6842626	3329800	5	Weak population	As
41	16.08.2005/17	Lake Taulajärvi	35.311	6842626	3329800	6	Weak population	As
42	25.08.2006/66	Lake Taulajärvi	35.311	6842626	3329800	9	Mixed population	Ps1
43	02.10.2002/45	Lake Pulesjärvi	35.318	6837985	3341488	4	Mixed population	Ps1
44	29.06.2006/13	River Koronjoki	35.441	6904904	3324695	1	Acute mortality	Ps1
45	27.07.2005/116	Lake Valkiajärvi	35.546	6866825	3262442	8	Acute mortality	As
46	04.10.2006/110	Lake Valkiajärvi	35.546	6866825	3262442	7	Cage experiment 2	As
47	K104/1998	Lake Konaanjärvi	35.773	6796474	3368042	3	Acute mortality	Ps1
48	04.08.2006/1	River Karviajoki	36.022	6858098	3248212	2	Population decline	As
49	13.08.1999/42	River Kyrönjoki	42.022	6992149	3268673	7	Acute mortality	Ps1

Table 1 (continued)

Sample no.	Identification	Sample location	Water catchment area (3rd level)	N/lat	E/lon	Sample size	Background information	Genotype
50	06.08.2004/73	River Ähtävänjoki	47.014	7048699	3317106	6	Cage experiment 2	As
51	02.03.2004/22	River Välijoki	47.023	7027591	3329014	8	Cage experiment 2	As
52	29.09.2004/91	Lake Lappajärvi	47.031	7009361	3332370	6	Cage experiment 2	As
53	07.11.2003/45	River Perhonjoki	49.023	7044215	3339379	2	Cage experiment 2	As
54	10.11.2004/113	River Perhonjoki	49.023	7044215	3339379	4	Cage experiment 2	As
55	09.08.2006/44	River Lestijoki, Toholampi	51.023	7077391	3365800	5	Acute mortality	Ps1
56	27.09.2006/89	River Lestijoki, Sykäräinen	51.031	7059764	3371862	1	Acute mortality	Ps1
57	01.11.2006/20	River Vääräjoki	53.093	7105698	3363468	10	Cage experiment 1	Ps1
58	18.09.2003/53	River Pyhäjoki, Helaakoski	54.011	7146472	3371216	6	Cage experiment 2	Ps1
59	07.10.2003/5	River Pyhäjoki, Oulaistenkoski	54.012	7131459	3394649	7	Cage experiment 2	Ps1
60	03.09.2004/42	River Pyhäjoki, Mieluskoski	54.022	7120332	3410744	4	Cage experiment 2	Ps1
61	03.09.2004/53	River Pyhäjoki, Joutenniva	54.032	7106497	3428263	4	Weak population	As
62	24.08.2004/16	River Pyhäjoki, Venetpalo	54.041	7088667	3440345	5	Cage experiment 2	As
63	10.10.2003/92	Lake Pyhäjärvi	54.051	7054504	3447317	29	Cage experiment 2	As
64	09.10.2006/64	Lake Pyhäjärvi	54.051	7054504	3447317	2	Cage experiment 2	As
65	02.12.2005/67	Lake Kivesjärvi	59.351	7149060	3521221	3	Acute mortality	As
66	25.08.2006/5	Lake Kivesjärvi	59.351	7149060	3521221	13	Cage experiment 1	As
67	03.08.2004/135	Lake Jokijärvi	61.312	7269929	3573213	3	Weak population	As
68	21.08.2006/54	River Kemijoki	65.112	7334387	3400521	6	Population decline	As
69	24.08.2006/100	Lake Ottojärvi	86.003	6741894	3548710	9	Acute mortality	Ps1

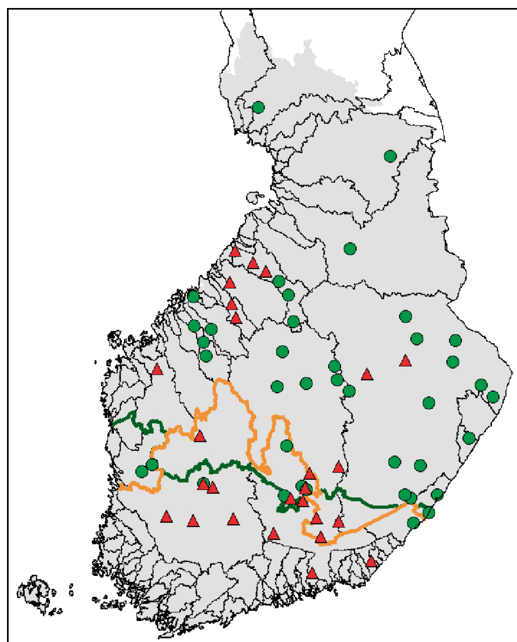

Fig. 1. Locations of crayfish plague *Aphanomyces astaci* identified in noble crayfish in 1996–2006, overlaid on the map of the main water catchment areas of Finland. Green circles: *A. astaci* genotype As; red triangles: *A. astaci* genotype Ps1; orange line: northern border of the signal crayfish stocking area; green line: northern border of the original distribution area of noble crayfish in Finland (Järvi 1910). The light grey background shows the present distribution of noble crayfish

photographed under UV light. The obtained RAPD profiles were compared visually with each other and the reference strains Da, Si, Kv, Pc and Or (Huang et al. 1994, Diéguez-Uribeondo et al. 1995, Kozubíková et al. 2011), representing the 5 currently known genotype groups of *Aphanomyces astaci*.

RESULTS

Genotypes of Finnish *Aphanomyces astaci* isolates

Between 1996 and 2006, *Aphanomyces astaci* was isolated from 69 batches of noble crayfish sent for examination from 2 to 11 cases each year,

except for 1997, when no isolations were made. All selected isolates fulfilled the morphological criteria of *Aphanomyces* species, including aseptate, profusely branching hyphae of 5 to 10 µm width. The 18 isolates tested were all pathogenic towards noble crayfish. All isolates produced the expected PCR product by the method of Oidtmann et al. (2006). All Finnish isolates of *A. astaci* had RAPD-PCR profiles belonging to one of the 2 genotypes, As or Ps1 (Table 1). The reference strains gave identical profiles with the 2 genotypes recognised in our study (Fig. 2). From the total number of 69 isolates, 43 were As-strains and 26 were Ps1-strains. There was a high homology between the RAPD-PCR profiles inside the groups. All As-genotype profiles were characterised by a strong 1300 bp band, and all Ps1-genotype profiles by 1200 and 800 bp bands. Outside these conserved bands, minor variations were detected among the weaker bands in both genogroups.

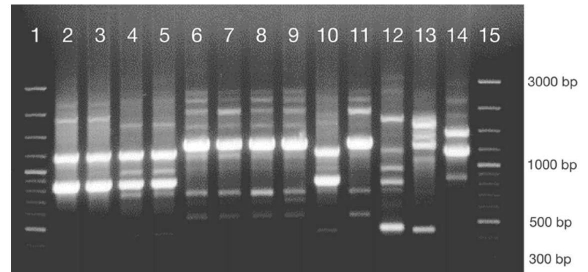

Fig. 2. RAPD-PCR analysis of some Finnish *Aphanomyces astaci* isolates. Lanes 1 and 15: molecular weight marker 100+ bp. Lanes 2–5: isolates representing Pasifastacus strain I type (Ps1). Lanes 6–9: isolates representing Astacus type (As). Lanes 10–14: reference strains Si (Ps1), Da (As), Kv (Ps2), Pc and Or. Note the genetic stability between the isolates due to asexual propagation

Geographical distribution of the genotypes

The majority of the crayfish samples were obtained from middle and eastern Finland, and accordingly most of the isolates (48) also originate from these areas (Fig. 1).

In the eastern part of Finland, all but 2 (samples 15 and 16) of the isolates were of the As-genotype, while 15 from 22 isolates from the southern part of Finland belonged to the Ps1 group. The geographical distribution of the Ps1-strains corresponded with the area where the signal crayfish has been introduced extensively into the Finnish water bodies. Both genotypes were present in the border areas of the signal crayfish territory. Occasional isolations of Ps1-strains were made from 4 water catchment areas in the western part of the country, where As-strains are also common. In the samples from the northern Finland, only As-strains were detected.

Epidemiological features of the genotypes

Only strains that were isolated from noble crayfish populations are considered in the numerical comparison, since population dynamics may affect the cause of the epidemic in mixed populations; thus samples 42 and 43 are excluded. The information gained about the epidemiological status of the affected water bodies shows that in the majority of the cases (21 samples out of 24) Ps1-strains were associated with acute mortality. Twenty strains (83% of all Ps1-strains) were isolated from mortality episodes, while one strain originated from a cage experiment in connection with a disease episode (sample 57). The other 3 cases of Ps1 strains isolated from cage experiments (samples 58, 59, and 60) were all connected with the same large-scale reintroduction programme of noble crayfish in River Pyhäjoki in western Finland.

As-strains were predominantly (29 out of 43) found in connection with population declines (9 samples), weak populations (7 samples), or cage experiments (13 samples). One cage experiment involved acute mortality during the same season (sample 66). Acute crayfish mortality occurred in 14 samples with As-type strains, i.e. only 33% of all samples with As-strains. There was a significant difference between the genotypes Ps1 and As concerning the frequency of acute mortality events, even when population declines and cage experiments involved with mortality are included (Fisher's P, df = 1, p = 0.007).

In some cases the crayfish plague agent seemed to remain for long periods in the same population, in addition to the earlier reported case of Lake Taulajärvi (samples 40 and 41; Viljamaa-Dirks et al. 2011). In Lake Valkiajärvi, the As-genotype crayfish plague agent was isolated in successive years, most probably being maintained by the weak populations that survived an earlier crayfish plague outbreak (samples 45 and 46). Also in Lake Kivesjärvi, the spread of the disease was extremely slow (samples 65 and 66). In a cage experiment in River Perhonjoki, the caged crayfish were monitored for longer than a year, and isolations of an As-strain were made at the beginning and the end of this period (samples 53 and 54). The

long persistence of the infection as recognised earlier in large lakes such as Lake Pyhäjärvi was confirmed (samples 63 and 64).

The epidemiological information for each isolate is presented in Table 1.

DISCUSSION

The epidemiological study concerning crayfish plague is complicated by problems in diagnostic methods and difficulties in the isolation of the causative agent in pure culture, which is necessary to achieve genotyping by the RAPD-PCR method. Isolation methods were reported to give positive results at best in 70% of infected crayfish in the acute disease period (Oidtmann et al. 1999b). Although minor modifications in the process have resulted in improved yield in clinical cases (Viljamaa-Dirks & Heinikainen 2006), cultivation is likely to give negative results in mildly infected individuals. It can even be difficult to obtain sample material in suspected epizootics, because high and sudden mortality can occur unobserved in wild populations. Thus the verified cases of crayfish plague probably represent only a part of the true incidence, even when sensitive molecular detection methods are in use.

Because of the laborious method of isolation and identification, only one isolate was purified and studied further from each sample of crayfish. Due to the anticipated rapid development of the mortality, it was not expected to find representatives of different genotypes in the same sample. Accounting present knowledge, this would be possible, but would probably be highly exceptional and difficult to verify.

The results obtained with RAPD-PCR concerning *Aphanomyces astaci* isolates were very consistent, giving nearly identical profiles inside each group. The lack of sexual propagation would explain the uniformity of the genotypes, as already noted by Huang et al. (1994).

When we compare the case reports between the Ps1 and As strains, it seems that there is a difference in the consequences of the infection at the population level. We categorised the background information concerning the samples into 3 groups according to the time when actual mortality had taken place. Acute mortality has generally been assumed to occur within a few weeks after the infection, at least during the warm water period in the summer (Alderman et al. 1987). It is sometimes difficult to verify the peak of the mortality in wild crayfish populations. In order to ensure inclusion of all cases, we included all samples connected with dead crayfish found during the same summer season in the acute mortality group. In cases of population decline, mortality presumably took place during the winter or late in the autumn, since the population level in the preceding summer was reported as being good (i.e. enough for fishing). It is possible that some cases categorised as population declines were actually acute mortalities that had passed undetected during the first 2 summer months. In Finland, the crayfish fishing season starts on 21 July. Prior to this date there is hardly any surveillance of the crayfish stocks. However, even if we count the population declines as acute outbreaks, Ps1 and As strains were significantly different in their behaviour. In fact, in our samples, Ps1-strains were only isolated in connection with mortalities. Even the 3 Ps1-strains from River Pyhäjoki, categorized as cage experiments, were associated with a failure of the reintroduction project in the main river (Jussila et al. 2008). In addition, the noble crayfish developed acute disease in mixed crayfish populations. Thus this genotype acts as predicted in crayfish plague infections.

One major concern, however, is the large number of atypical cases connected with the As-genotype. Our results show that infection by this genotype can exist in successive years and is not uncommon in weak noble crayfish populations. This possibility was already discussed by Fürst (1995) in his follow-up study of the Swedish noble crayfish reintroduction attempts. Unfortunately his proposal lacked scientific evidence and was not taken into account in stocking strategies, resulting in recurrent failures of a considerable number of restocking attempts (Erkamo et al. 2010).

Recent evidence from Turkey shows that crayfish plague still exists in Lake Eğirdir (Svoboda et al. 2012), which was infected in the mid-1980s (Baran et al. 1989). The flourishing Turkish crayfish trade has not recovered from the introduction of crayfish plague (Harhoglu 2004), very similar to the Finnish crayfish fisheries of the noble crayfish. An isolate from Turkey has been recognised as genotype As (Huang et al. 1994). It thus seems that there too the As-genotype shows lower virulence than expected, leading to persistent infection.

During an acute mortality period, crayfish are usually heavily infected, thus improving the chance for successful isolation of the organism. Since the Ps1-genotype seems to be more often involved with acute mortality, this type might be more readily isolated than the As-strains. Nevertheless, we have found strains of the Ps1-group less frequently than

strains of the As-group. Southern Finland must be considered as being endemic for the Ps1-type crayfish plague, since populations of signal crayfish, the original host of this genotype, are widely established here. Representatives of the same group have also been isolated from the signal crayfish in Finland (data not shown). Noble crayfish samples from the signal crayfish stocking area have been obtained mainly from 2003 and later, and are thus unevenly represented in our clinical material. These differences make it impossible to compare the incidence of the genotypes in Finland. However, it is evident from our study that As-strains are only rarely recovered from the signal crayfish territory. It seems that in the areas where North American species are found in the wild, crayfish plague is caused by the genotypes carried by those species (Lilley et al. 1997, Oidtmann et al. 1999a). The As-genotype plague devastated the main noble crayfish populations in the southern part of Finland for decades before the introduction of signal crayfish carrying the Ps1-genotype of *Aphanomyces astaci*. It is therefore reasonable to assume that there were weak noble crayfish populations carrying As-type plague in these water bodies, but that they eventually vanished in response to the introduction of signal crayfish infected with Ps1-type plague. Nowadays only scattered harvestable populations of noble crayfish still exist in the smaller lakes in this area. In addition to the limited number of the highly susceptible noble crayfish populations at present, low incidence of As-strains could be explained by a general tendency to introduce signal crayfish in that area if the noble crayfish population is not productive. This choice seems more appropriate than trying to study the crayfish plague status by sampling a weak population or organising cage experiments, methods by which the majority of As-strains are identified in the rest of Finland.

In regions close to the signal crayfish territory, noble crayfish populations are continuously at risk of becoming infected by the plague-carrying signal crayfish, as illustrated by the high number of disease outbreaks in the border areas. This might encourage stakeholders to undertake unauthorised introductions of signal crayfish, thus further diminishing the natural habitat for the noble crayfish.

It is noteworthy that Ps1-type crayfish plague was only incidentally isolated from areas not directly connected with the signal crayfish territory. The general public awareness concerning the risks involved in transfers of crayfish or crayfish fishing equipment might have been the reason for limited spread of the Ps1-type crayfish plague to Mid, Eastern and Northern Finland. Outside of the signal crayfish territory, most of the incidence of Ps1-type plague was connected with known or suspected introductions of crayfish, although no firm evidence of disease introductions could be found. The River Pyhäjoki repopulation programme is an example of a large-scale transfer of noble crayfish from other parts of the country to this area in West Finland, resulting in a co-infection of 2 crayfish plague genotypes in the same river. This highlights the need for a careful study of the donating population whenever transfers of crayfish are being considered, especially concerning the highly susceptible species.

This study did not take into account the locations of crayfish aquaculture, which may, if signal crayfish is being farmed, act as a source of crayfish plague infection. Fortunately, crayfish farming is concentrated in the southern part of Finland, with only a few small-scale farms outside the signal crayfish area. In contrast, crayfish trade and marketing is extensive and crayfish of both species are transported over much of the country. The live crayfish trade can also act as a source of the plague, although it is illegal to place the crayfish even temporarily in waters other than where they were caught.

While isolation methods have been improved, there are still epizootics where isolation fails, making further characterisation of the agent impossible. Low-level infections are virtually impossible to detect by culture methods. Molecular methods offer a better chance for a diagnosis, but differentiation between the genotypes still demands an isolation procedure. Until molecular strain differentiation methods are developed, isolation will remain necessary in order to expand our knowledge of the epidemiology of crayfish plague. The results of the present study suggest that differences between the genotypes exist, and that more detailed work should be carried out to study their virulence and prevalence. Our results are based mainly on clinical material received from suspected disease outbreaks, and only partly on systematic follow-up studies through cage experiments. Given the number of water bodies known to suffer repeated episodes of crayfish plague in Finland, the number of isolations of As-genotype might represent only a very small proportion of the actual prevalence. Taking into consideration the present knowledge of the possibility of persistent infection, active surveillance programmes in noble crayfish populations need to be developed in order to efficiently prevent the spreading of this economically serious disease.

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IV

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Variation in the hyphal growth rate and the virulence of two genotypes of the crayfish plague organism *Aphanomyces astaci*

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Abstract

Crayfish plague, a devastating disease of freshwater crayfish, is caused by an oomycete organism, *Aphanomyces astaci*. Currently five genotypes of *A. astaci* are known, but variable features between the strains or genotypes have not been studied extensively. This study analysed 28 isolates of the As genotype and 25 isolates of the Ps1 genotype and reveals that the radial growth rate is significantly ($P < 0.001$) different between these two genotypes, although highly variable inside the genotype As. Two Ps1 genotype isolates and two As genotype isolates with different radial growth rates were tested in an infection trial. Clear differences were detected in the development of mortality in the test groups. The representatives of the Ps1 genotype caused total mortality within a short time span. The As genotype isolates were much less virulent. The slow-growing As isolate showed higher virulence than the As isolate with a high growth capacity. Although slow growth could be one survival strategy of the pathogen, several other mechanisms are involved in the pathogenicity and warrant further studies.

Keywords: Aphanomyces astaci, epidemiology, genotype, growth rates, infection experiments.

Introduction

Crayfish plague is the most serious crustacean disease in Europe (Söderhäll & Cerenius 1999; Edgerton *et al.* 2002). The disease is caused by the oomycete organism *Aphanomyces astaci* (Schikora) that is native to North America and which was accidentally transferred to Europe in the second half of the 19th century (Alderman 1996). European crayfish species appeared to be highly susceptible and infection was reported to lead to extinction of entire populations (Westman 1991; Alderman 1996; Söderhäll & Cerenius 1999). The original host species for this first invasive strain, genotype group As (also referred to as A) (Huang, Cerenius & Söderhäll 1994), remains unknown. Later, resistant North American crayfish species were introduced into Europe to improve crayfish fisheries, and with these introductions, new genotypes of *A. astaci* were imported: genotype groups Ps1 (B) and Ps2 (C) from the signal crayfish, *Pacifastacus leniusculus* (Dana), (Huang *et al.* 1994), group Pc (D) from the red swamp crayfish, *Procambarus clarkii* (Girard), (Diéguez-Uribeondo *et al.* 1995) and group Or (E) from the spiny cheek crayfish, *Orconectes limosus* (Rafinesque) (Kozubíková *et al.* 2011). In Finland, a country with a vigorous crayfish fishery and a relatively long history with crayfish plague (Westman 1991), two genotypes have been found at present: the signal crayfish genotype Ps1 carried by the widely spread signal crayfish, and the originally encountered noble crayfish genotype As which is still causing epidemics in noble crayfish, *Astacus astacus* (L.), populations in some parts of Finland (Vennerström, Söderhäll & Cerenius 1998; Viljamaa-Dirks *et al.* 2013). The expected total mortality of the noble crayfish population after the crayfish plague infection led to the common practice of

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restocking a plague-stricken water body soon after the assumed disappearance of the native crayfish. However, the results often proved to be less than satisfactory (Erkamo *et al.* 2010). Also in Sweden, the re-introductions of noble crayfish were reported mainly to fail due to new outbreaks of crayfish plague (Fürst 1995). Scattered individual crayfish and crayfish populations in the large and geographically complicated lake areas in Finland seem to provide endless survival niches for the crayfish plague organism (Westman 1991; Viljamaa-Dirks *et al.* 2013) and the persistent presence of an As-type *A. astaci* even in a small lake has been verified (Viljamaa-Dirks *et al.* 2011). Crayfish plague strains belonging to the genotype group As have been reported in cases of recurrent or persistent infections in noble crayfish unlike the genotype group Ps1 (Viljamaa-Dirks *et al.* 2013). Laboratory trials have also indicated that there are differences in virulence between strains (Makkonen *et al.* 2012b, 2014). In this study, we compared the radial growth rate of a number of crayfish plague isolates from the genotype groups As and Ps1. Additionally, one slow- and one fast-growing representative from both groups were used in the comparison of their virulence in noble crayfish.

Materials and methods

Isolates of *Aphanomyces astaci*

Aphanomyces astaci isolates from Finnish noble crayfish and signal crayfish were used in this study. The isolations were made during 1998–2006, using the isolation process described by Viljamaa-Dirks & Heinikainen (2006). The verification of the isolates as *A. astaci* was performed by the PCR method of Oidtmann *et al.* (2006), and the genotype determination by randomly amplified polymorphic DNA (RAPD)-PCR (Huang *et al.* 1994) with minor modifications as described by Viljamaa-Dirks *et al.* (2013).

A total of 28 isolates were studied from the genotype group As, with 25 isolates from the genotype group Ps1. The selected isolates were all separated in terms of their origin either temporally or by location. Isolate identification number, sample date, host animal, location by name as well as coordinates and sample background are listed in Table 1.

Radial growth rate

The cultures were maintained refrigerated at 4 °C on PG-1 medium (Cerenius, Söderhäll & Fuller 1987) in vials covered with mineral oil (Unestam 1965). The cultures were transferred to a fresh medium every 6 months with the exception of two isolates, EviraK047/99 and EviraK086/99; these were kept available for reference purposes and maintained on PG-1 plates by transferring to a fresh plate every 2–4 weeks. In the tests of the growth rate, the isolate was first inoculated onto a PG-1 medium plate, and after an incubation period of 6 days at 20 °C, standard pieces with a 6-mm diameter were stenciled out from the outer edge of the mycelial mat and replaced in the middle of a fresh PG-1 medium plate with a diameter of 85 mm and thickness of 6 mm. Three plates were prepared from every isolate. The cultures were incubated at 20 °C, and the maximal linear extension of the mycelial mat was measured at 24-hour intervals. The cultures were followed until they filled the plate or for up to 2 weeks. For a number of the isolates (As n = 5, Ps1 n = 6), the growth rate at 15 °C was studied as well.

The radial growth was determined by the difference between the diameters of the mycelial mat, as the mean of the three separate cultures divided by 2. The radial growth rate of an isolate was calculated during the exponential growth phase in days 2–7, using MS Excel.

Infection trial

The infection trial was performed during the winter 2006–07. A slow-growing and a fast-growing representatives were chosen from both genotype groups As and Ps1 to test the virulence against noble crayfish. The selected isolates had all been isolated from populations of noble crayfish 3 years or less prior to the trial. The average growth rate for the fast-growing As isolate Evira4426/03 (AsFast) was 4.5 mm day^{-1} and for the slow-growing As isolate Evira6672/05 (AsSlow) 2.0 mm day^{-1}. The average growth rate for the fast-growing Ps1 isolate Evira3697/03 (Ps1Fast) was 4.2 mm day^{-1} and for the slow-growing Ps1 isolate Evira7862/03 (Ps1Slow) 2.8 mm day^{-1}.

Intermolt noble crayfish with a carapace length of 65–92 mm originating from a crayfish farm

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Table 1 Finnish *Aphanomyces astaci* isolates and their radial growth rates

Genotype[a]	Isolate[b]	Sample identification[b]	Host[c]	Sample location[d]	Water catchment (3rd level)[e]	N/lat[f]	E/lon[f]	Background[g]	Growth rate[h]
As	EviraK086/99	K86/1999	Noble	Lake Ihalanjärvi	04.127	6820034	600102	Cage experiment	5.6
As	Evira1704/02	23.07.2002/7	Noble	Lake Lieviskäjärvi	04.127	6824870	591674	Weak population	3.2
As	Evira3625/04	09.08.2004/53	Noble	Lake Immalanjärvi	04.192	6788310	603489	Mortality	4.1
As	Evira1494/00	25.07.2000/9	Noble	Lake Pitkäjärvi	04.199	6801928	623424	Mortality	2.3
As	EviraK100/98	K100/1998	Noble	Lake Kotkanjärvi	04.212	6868029	578706	Mortality	1.9
As	Evira1772/02	26.07.2002/15	Noble	Lake Sylkky	04.296	6863209	612460	Mortality	3.3
As	Evira2193/01	25.10.2001/92	Noble	Lake Kuorinka	04.317	6944588	623329	Weak population	3.3
As	Evira5971/04	08.09.2004/92	Noble	River Vuokonjoki	04.411	7028710	607704	Population decline	2.4
As	Evira4426/03	29.07.2003/116	Noble	Lake Kelvänjärvi	04.419	6998297	654500	Mortality	4.5
As	Evira1463/01	25.07.2001/3	Noble	Lake Korkeanalanen	14.228	6823669	433850	Mortality	3.8
As	Evira2061/00	07.09.2000/36	Noble	Lake Iso-Lauas	14.725	6960120	519279	Cage experiment	2.6
As	Evira1705/02	23.07.2002/63	Noble	Lake Pieni Tallusjärvi	14.772	6974398	504151	Mortality	4.2
As	Evira1859/02	31.07.2002/34	Noble	Lake Korosjärvi	14.773	6992535	499885	Population decline	2.1
As	Evira0894/01	07.06.2001/82	Noble	Lake Iso-Suojärvi	14.823	6965713	425094	Mortality	3.5
As	Evira2807/04	06.07.2004/27	Noble	Lake Taulajärvi	35.311	6839755	329698	Weak population	1.8
As	Evira6672/05	16.08.2005/17	Noble	Lake Taulajärvi	35.311	6839755	329698	Weak population	2
As	Evira6207/05	27.07.2005/116	Noble	Lake Valkiajärvi	35.546	6863944	262367	Mortality	2.7
As	Evira3583/04	06.08.2004/73	Noble	River Ähtävänjoki	47.014	7045746	317008	Cage experiment	2.8
As	Evira7005/04	29.09.2004/91	Noble	Lake Lappajärvi	47.031	7006424	332266	Cage experiment	2.6
As	Evira8951/03	07.11.2003/45	Noble	River Perhonjoki	49.023	7041263	339272	Cage experiment	2.3
As	Evira8065/04	10.11.2004/113	Noble	River Perhonjoki	49.023	7041263	339272	Cage experiment	2.8
As	Evira5727/04	03.09.2004/53	Noble	River Pyhäjoki, Joutenniva	54.032	7103521	428120	Weak population	2.5
As	Evira5041/04	24.08.2004/16	Noble	River Pyhäjoki, Venetpalo	54.041	7085698	440197	Cage experiment	2
As	Evira7246/03	10.10.2003/92	Noble	Lake Pyhäjärvi	54.051	7051553	447165	Cage experiment	2
As	Evira10278/05	10.11.2005/28	Noble	Lake Kivesjärvi, farm	59.351	–	–	Mortality	3.5
As	Evira10789/05	02.12.2005/67	Noble	Lake Kivesjärvi	59.351	7146068	521040	Mortality	3
As	Evira0418/06	31.01.2006/65	Noble	Lake Kivesjärvi, farm	59.351	–	–	Mortality	3.3
As	Evira2680/06	10.05.2006/42	Noble	Lake Kivesjärvi, farm	59.351	–	–	Mortality	2.6
As	Evira3514/04	03.08.2004/135	Noble	Lake Jokijärvi	61.312	7266888	573011	Weak population	2.9
Ps1	EviraK047/99	K47/1999	Noble	Lake Korpijärvi	4.143	6790267	506059	Mortality	5
Ps1	Evira1580/01	7.8.2001/11	Noble	Lake Jännevirta, Pohjanlampi	4.611	6983098	542640	Mortality	3
Ps1	Evira6483/05	10.08.2005/23	Noble	River Teutjoki	14.153	6722916	471158	Mortality	3.3
Ps1	Evira1172/00	30.6.2000/19	Noble	Lake Vesijärvi	14.241	6774332	420902	Mortality	3.5
Ps1	Evira6458/03	28.08.2003/122	Signal	Lake Lievestuoreenjärvi	14.391	6909454	455156	Mixed population	3.8
Ps1	Evira1588/00	04.08.2000/18	Noble	Lake Kilpilampi	14.823	6833412	462168	Mortality	4.3
Ps1	Evira3697/03	11.07.2003/86	Noble	Lake Iso-Kuivajärvi	14.824	6816988	458616	Mortality	4.2
Ps1	EviraK110/98	K110/1998	Signal	Lake Rautavesi	14.831	6835224	445689	Symptomatic signal crayfish	3.8
Ps1	Evira2010/00	31.08.2000/6	Noble	Lake Vehkajärvi	14.932	6794482	476423	Mortality	3.2
Ps1	Evira7442/03	07.08.2003/80	Signal	Lake Valkjärvi	23.074	6694308	324138	Population decline	4.2

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Table 1 *Continued*

Genotype[a]	Isolate[b]	Sample identification[b]	Host[c]	Sample location[d]	Water catchment (3rd level)[e]	N/lat[f]	E/lon[f]	Background[g]	Growth rate[h]
Ps1	Evira1140/00	29.06.2000/8	Noble	Lake Ylistenjärvi	35.138	6797375	281117	Mortality	3.5
Ps1	Evira1219/02	05.06.2002/135	Noble	Lake Iso-Arajärvi	35.290	6791599	315814	Mortality	3.7
Ps1	Evira3234/02	02.10.2002/45	Noble	Lake Pulesjärvi	35.318	6835117	341382	Mortality in mixed population	4.3
Ps1	Evira2557/02	26.08.2002/11	Signal	Lake Mallasvesi	35.711	6799786	347428	Symptomatic signal crayfish	4.7
Ps1	Evira7203/03	11.09.2003/70	Signal	Lake Kukkia	35.781	6801172	377743	Symptomatic signal crayfish	4.3
Ps1	Evira2947/04	14.07.2004/26	Signal	Lake Ormajärvi	35.792	6775535	390118	Symptomatic signal crayfish	4.6
Ps1	Evira1908/02	02.08.2002/5	Signal	River Jokilanjoki	35.892	6756430	361619	Symptomatic signal crayfish	4.2
Ps1	Evira7512/03	18.09.2003/53	Noble	River Pyhäjoki, Helaakoski	54.011	7143480	371096	Cage experiment	3.6
Ps1	Evira7862/03	07.10.2003/5	Noble	River Pyhäjoki, Oulaistenkoski	54.012	7128473	394519	Cage experiment	2.8
Ps1	Evira5721/04	03.09.2004/42	Noble	River Pyhäjoki, Mieluskoski	54.022	7117350	410608	Cage experiment	3.3
Ps1	Evira2859/03	05.06.2003/117	Noble	Farm Eurajoki	–	–	–	Mortality	4.3
Ps1	Evira6696/04	16.09.2004/13	Noble	Farm Ruovesi	–	–	–	Mortality	4.3
Ps1	Evira9623/05	18.10.2005/37	Noble	Farm Hämeenlinna, Evo	–	–	–	Mortality	4.4
Ps1	Evira0384/06	30.01.2006/4	Signal	Farm Hämeenlinna, Evo	–	–	–	Mortality	4.5
Ps1	Evira10399/05	15.11.2005/13	Noble	Farm Uusikaarlepyy	–	–	–	Mortality	2.9
Ps1	Evira3092/06	23.05.2006/120	Noble	Farm Lappeenranta	–	–	–	Mortality in mixed population	3.7

[a]Genotype: As Astacus genotype, Ps1 Pacifastacus genotype group I.
[b]Isolate identification number and sample identification according to the archive of the Finnish Food Safety Authority.
[c]Host: Noble *Astacus astacus*, Signal *Pacifastacus leniusculus*.
[d]Sample location by the name of the river or lake.
[e]The water catchment area number (third level).
[f]The geographical coordinates (ETRS-TM35FIN), except for farms not directly connected with a lake or river (-).
[g]Background: the background information available for each sample.
[h]Growth rate: average radial growth rate during the exponential growth phase, mm day^{-1}.
Dotted background: diagnostic reference strains. Grey background: selected for infection trial.

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were used in the trial. They had been kept in the experimental fish tank facilities of Evira Kuopio 4 months before the start of the experiments. In the infection trial, the crayfish were placed into 15 separate tanks containing 15 L lake water with constant aeration and plastic tubes for hides. During the acclimation period and the trial, lake water from Lake Kallavesi that had been standing in containers for at least 3 weeks to avoid any contamination with viable crayfish plague spores from outside was used. The temperature throughout the trial was 20 ± 2 °C. Six male and six female randomly selected crayfish were placed into each tank. The mean carapace length for each group varied from 77 to 82 mm. The crayfish were fed twice a week with green peas or pieces of carrot.

The selected crayfish plague strains were incubated in PG-1 broth at 20 °C for 9 days with a few times fragmenting the mycelium after filtration with a cheese cloth with a scalpel and adding fresh broth. Zoospore production was initiated as described in Cerenius *et al.* (1988) by replacing the broth with sterilized lake water. The zoospore density was determined for each of the strains by the Bürker chamber counting method and varied between 4000 and 12 600 spores mL^{-1}. The final density of the zoospores for the test tanks was adjusted to approximately 100 zoospores mL^{-1} by adding 120–400 mL spore suspension per tank, except for the three control tanks. Every test strain was used to infect three tanks (AsFast/1-3, AsSlow/1-3, Ps1Fast/1-3 and Ps1Slow/1-3). The hides were moved from the tanks before adding the zoospores and replaced on the next day to avoid cannibalism. At the same time, the water was changed for a temporal limitation of the initial infection period.

The crayfish were monitored daily, and dead individuals were collected and examined microscopically for signs of crayfish plague infection. One-third of the water was replaced after feeding twice a week. From every test tank, the re-isolation of the crayfish plague agent was performed to confirm the successful infection.

Statistical methods

The comparisons of the growth rates of the crayfish plague isolates between the genotype groups As and Ps1 were made by the diameter of the cultures on day 7 at 20 °C. Isolates that were from the same location although separated temporally were included in the comparison only once (locations 35.311, 49.023, 59.351, farm Evo). The comparison was made using Mann–Whitney *U*-test (SOFA Statistics 1.4.3, Paton-Simpson & Associates Ltd).

The mortality rate in the infection trial was compared by counting the day on which the last crayfish in the test group had died. The statistical comparisons between the infection types, genotypes and fish tanks, separately, were made using Kaplan–Meier survival analysis (Kaplan & Meier 1958). Pairwise log rank comparisons were conducted to determine which groups had different survival distributions. To keep the overall confidence at a 95% level, a Bonferroni correction was made with statistical significance accepted at the $P < 0.005$ level for infection types, $P < 0.0167$ level for genotypes and $P < 0.0004716$ for tanks as there were 10, 3 and 105 comparisons, respectively. Log rank was used as censoring patterns were rather similar. Statistical analyses were carried out with IBM SPSS Statistics version 22 (IBM Corp.).

Results

Radial growth rate

During the first day, the hyphal growth rate was always slower than during the following days. The exponential growth phase started from day 2. At 20 °C, most of the Ps1 isolates ($n = 18$) colonized the total agar surface in 9–14 days, while seven isolates remained smaller. This was in contrast to the As group where only seven isolates colonized the total agar surface in 9–14 days, while most of the isolates ($n = 21$) showed more restricted growth. At 15 °C, none of the isolates reached the edge of the agar plate in 14 days. There were two exceptions: EviraK086/99 (As) and EviraK047/99 (Ps1), used as control strains for diagnostic purposes and therefore transferred considerably more often than the stock cultures of other strains, both colonized the whole surface of the plates in 13 days at 15 °C. The growth behaviour of these two strains differed substantially from the others in their respective groups, and they were excluded from the subsequent comparisons.

The radial growth rate at 20 °C in the group of As isolates was between 1.8 and 4.5 mm day^{-1},

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the mean being 2.9 mm day^{-1} (standard deviation [SD] 0.8, n = 23). The radial growth rate at the same temperature in the group of Ps1 isolates was between 2.8 and 4.7 mm day^{-1}, the mean being 3.8 mm day^{-1} (SD 0.54, n = 24). Table 1 lists the growth rate at 20 °C for each of the isolates, and Fig. 1 shows the variation of growth rates inside the genotype groups.

As expected, at 15 °C, the growth rate was lower, being between 1.5 and 3.1 mm day^{-1} for the As isolates (mean 2.2 mm day^{-1}, SD 0.71, n = 5) and between 2.3 and 3.5 mm day^{-1} for the Ps1 isolates (mean 2.9 mm day^{-1}, SD 0.48, n = 6). The overall growth pattern at 15 °C was comparable with the growth at 20 °C for each of the tested isolates. The difference in the growth rates between the As genotype and the Ps1 genotype was statistically significant at 20 °C (P < 0.001), but not at 15 °C (P < 0.142).

Figure 2 shows the average growth curves of the genotype groups, as well as the growth curves of the strains used in the infection trial. Figure 3 presents the comparison of the genotype groups at the growth temperatures 15 °C and 20 °C as the diameter of the mycelium on day 7.

Infection trial

The first crayfish plague-induced mortalities in the Ps1Fast groups were seen already on day 5, and total mortality was reached on day 10. Microscopic examination showed a heavy growth of typical hyphae in the abdominal cuticle, and crayfish plague agent was re-isolated from every test tank. The development of mortality was slower in the Ps1Slow groups, and there was more variation between the three tanks. Mortality started in Ps1Slow tanks 1, 2 and 3 on day 8, 7 and 6,

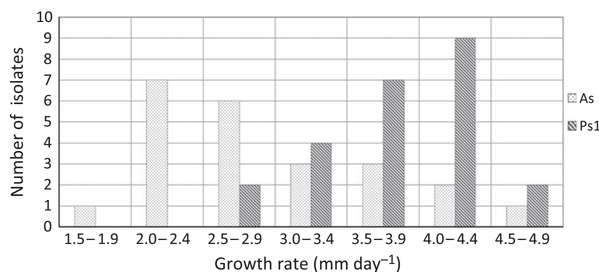

Figure 1 The radial growth rate of *Aphanomyces astaci* isolates in the genotype groups As and Ps1.

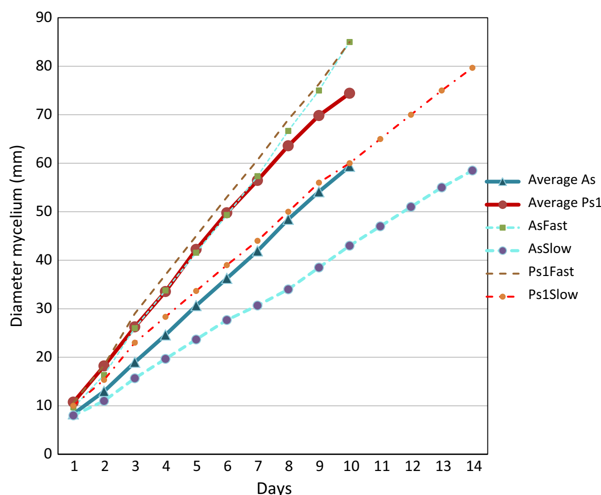

Figure 2 Hyphal growth curves at 20 °C of the genotype groups As and Ps1 of *Aphanomyces astaci*. Average As: average value for 23 As isolates, Average Ps1: average value for 24 Ps1 isolates. AsFast, AsSlow, Ps1Fast, Ps1Slow: fast- and slow-growing isolates from the groups As and Ps1, selected for the infection trial. Growth curves are presented as the mean of the daily measured diameters of the mycelial mat of three cultures.

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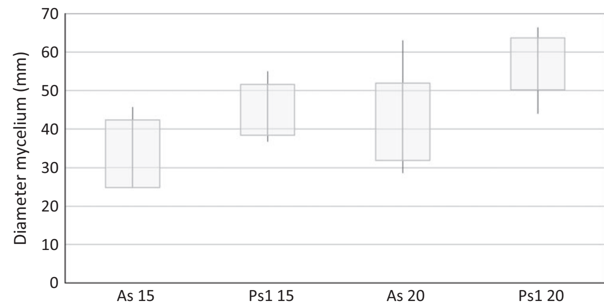

Figure 3 The growth comparison of isolates of *Aphanomyces astaci* of the genotype groups As and Ps1, as box plot comparison at two temperatures, 15 and 20 °C on day 7. As 15 (n = 5), Ps1 15 (n = 6), As 20 (n = 20), Ps1 20 (n = 23). The box denotes the first and third quartiles; the whiskers represent the highest and lowest measurements.

respectively. The last crayfish died on day 24 in Ps1Slow/2 and on day 14 in Ps1Slow/3. In tank Ps1Slow/1, three individuals remained alive for a longer period, succumbing on days 60–63. Typical hyphal growth was seen in the abdominal cuticle in almost all of the individuals in all three test groups. In addition, four individuals in Ps1Slow/1 showed some melanization, as well as one individual in both of the other two groups. Crayfish plague agent was also successfully isolated from all Ps1Slow tanks.

The mortality associated with AsSlow was significantly slower than with both Ps1 strains (log rank $P < 0.001$). Mortalities were recorded during days 20–128 for AsSlow/1, days 11–103 for AsSlow/2 and days 18–91 for AsSlow/3. The microscopic picture was variable, from heavy growth of typical hyphae to a few melanized foci. Melanization was a common feature, as seven individuals in AsSlow/1 and nine in AsSlow/2 as well as AsSlow/3 showed melanized areas in the abdominal cuticle and joints. Melanization was seen already from the time when the first deaths occurred. *Aphanomyces astaci* was re-isolated from all AsSlow tanks.

AsFast groups did not differ from the control groups that showed also a steady development of mortality during the experiment that lasted until the last crayfish in the infected groups perished on day 244. Mortality in AsFast/1 started on day 69 with the last crayfish dying on day 244. For AsFast/2 and AsFast/3, the mortalities occurred from day 1 and day 48 to day 219 and 161, respectively. The first two dead individuals in AsFast/2 were most probably victims of cannibalism and could not be studied further. A microscopic study of these groups revealed a few foci of typical hyphal growth that was mostly melanized in the majority of individuals (Fig. 4). The isolation of *A. astaci* did not succeed as easily as from the other groups, but was finally achieved from all the AsFast tanks as well.

The three control groups experienced low mortality from day 1, most probably due to cannibalism and the suboptimal environment. In none of the control groups was there any evidence of crayfish with cuticular hyphal growth resembling crayfish plague. A few melanized areas were recorded once, this being connected with an atypical fungal growth. Most of the control crayfish were cultured for the presence of *A. astaci*, all with negative results.

The combined cumulative mortality of the test groups is shown in Fig. 5.

With the exception of AsFast, all of the tested strains caused elevated mortality in comparison with the control group (log rank $P < 0.001$). There was a statistically significant difference in the development of mortality between the test groups infected with genotype As and genotype

Figure 4 Melanized hyphae in the abdominal cuticle of a noble crayfish *Astacus astacus* infected with a fast-growing As genotype crayfish plague *Aphanomyces astaci*, 5 months post-infection. Light microscopy image, scale bar: 100 μm.

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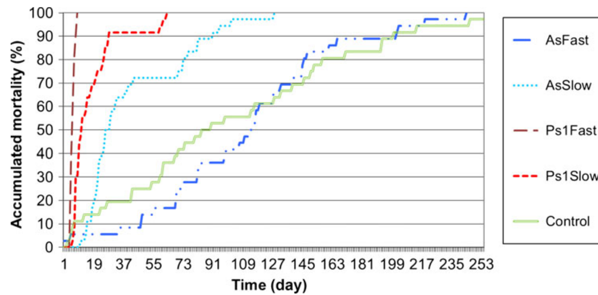

Figure 5 The cumulative mortality in the infection trial with *Aphanomyces astaci*, combined from three separate test tanks with 12 noble crayfish each. Ps1Fast: test groups Ps1Fast/1-3, infected with Evira3697/03; Ps1Slow: test groups Ps1Slow/1-3, infected with Evira7862/03; AsFast: test groups AsFast/1-3 infected with Evira4426/03; AsSlow: test groups AsSlow/1-3 infected with Evira6672/05. Control: control groups 1-3.

Ps1 (log rank $P < 0.001$), the latter having higher mortality. Additionally, Ps1Slow induced mortality slower than Ps1Fast (log rank $P < 0.001$). Strikingly, AsFast did not differ from the control group (log rank $P = 0.924$).

There were no significant differences (Table S1) between the tanks infected with the same strain, except with Ps1Slow/1 that differed from the two other Ps1Slow tanks ($P = 0.000263$ and 0.000004).

Discussion

Aphanomyces astaci has been a challenging object for study due to the demanding isolation process which has been reported to be successful in only about 70% of acutely ill animals (Alderman & Polglase 1986; Oidtmann *et al.* 1999). Due to the slow growth of *A. astaci* as compared with saprophytic water moulds (Lilley & Roberts 1997), isolation is especially difficult from crayfish with only low levels of infection such as the North American carrier species (Kozubíková-Balcarová *et al.* 2013). Using improved isolation processes (Viljamaa-Dirks & Heinikainen 2006), we have isolated crayfish plague strains that exhibit a remarkably slow growth on artificial media. These belong to the genotype group As that was presumably the strain that already had a long history, being present for more than a hundred years in Finland; that is, this strain seems to possess the ability to persist in Finnish waterways (Viljamaa-Dirks *et al.* 2011, 2013). For another aquatic parasitic oomycete, *Saprolegnia parasitica* (Coker), temperature-dependent growth factors seem to differ depending on the host animal (Willoughby & Copland 1984) and the same property has been shown between some genotypes of *A. astaci* (Diėguez-Uribeondo *et al.* 1995). *Aphanomyces astaci* grows slowly in lower temperatures (Alderman & Polglase 1986; Diėguez-Uribeondo *et al.* 1995), and a reduced temperature has also delayed the appearance of mortality in infection trials (Alderman, Polglase & Frayling 1987). Compared with saprophytic Aphanomyces species, the growth rate in animal pathogens *A. astaci* and *Aphanomyces invadans* (David & Kirk) is extremely low (Lilley & Roberts 1997; Diėguez-Uribeondo *et al.* 2009). The reduced growth rate could promote the survival of *A. astaci* in the highly susceptible European crayfish species, as a difference in the host resistance seems to be the slow trigger of the defensive melanization in these novel host animals compared with the more resistant North American crayfish species (Cerenius *et al.* 2003). Although the growth in artificial medium cannot be compared directly with the growth in the crayfish cuticle, it can reflect the overall potential for growth. Culturing *A. astaci* is relatively easy on artificial media containing glucose (Unestam 1965). To study the radial growth rates, we chose the PG-1 medium because of its standardized formula, compared with media containing river water. Most of the isolates of the Evira culture collection in 2006 were compared with each other at a growth temperature 20 °C. Part of the collection was studied also at 15 °C, but because the relationships between the isolates remained the same for both temperatures, the higher temperature, which was also used in the infection trial, was chosen for the overall comparison. Measuring the daily diameters of the triple cultures per isolate led to some minor variation due to some unequal growth rates of individual hyphae, but this tended to be compensated in the following measurements.

The growth rate pattern seems a fairly consistent feature, as judged by the repeating pattern of the isolates from the same host population. On

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the other hand, often repeated selection of the fastest growing part of the mycelium, which normally happens when refreshing the stock culture, seems to affect the growth rate. Unfortunately, the initial growth rate was not studied in our two reference strains, EviraK086/1999 and EviraK047/1999, that showed exceptionally high growth capacities. However, an estimation of the radial growth was made during the initial isolation process for EviraK047/1999; it was found to be approximately 3 mm day^{-1}. The measured growth rate after 7 years and countless transfers was 5.2 mm day^{-1}. This shows once again that care must be taken in studying organisms that have been intensely manipulated under laboratory conditions. With the exception of these two strains, the other isolates had been subject to far less re-culturing and thus should exhibit the same or nearly the same growth capacity than when initially isolated.

Aphanomyces astaci is not known to undergo sexual propagation (Dièguez-Uribeondo *et al.* 2009), which is also reflected in the uniformity of the RAPD-PCR profiles inside the genotype groups across time and space (Huang *et al.* 1994; Viljamaa-Dirks *et al.* 2013). It is therefore surprising that such a large variation of growth rates is seen in the phenotype of the As genotype isolates. It is not clear how many times *A. astaci* has been transferred to Finland from different locations. The origins of the infections could be limited to a few sources: Russian trade for the genotype As and Lake Tahoe and Lake Hennessy (USA) for the genotype Ps1 (Westman 1991; Nylund & Westman 1995). The Ps1 genotype isolates show little variation in their growth patterns, which may reflect a limited number of transfers to Finland, as well as the presence of the natural host signal crayfish. The lack of variation was also seen in the analysis of the chitinase genes in the Ps1 genogroup, while there was clearly a diversity inside the As genotype group (Makkonen, Jussila & Kokko 2012a). It is likely that the presence of the As genotype in Europe resulted from one accidental release in Italy in 1860s (Alderman 1996) and the remarkable variation of the growth rate as well as other variable features in this genogroup could result from development of subtypes in separated water catchments during its long history in European waters.

It is also very difficult to judge the connections between the isolates in this study. It seems plausible that two isolates from the same population represent the same strain even when there is a time interval between the isolation, when there has been no fishing and no restocking known to have occurred (Lake Taulajärvi 35.311), or the samples come from the same captive population (River Perhonjoki cage experiment 49.023). Also isolates from different locations could be of the same origin, representing one infection spreading through crayfish transfers or some other possible mechanism. Farmed crayfish and cage experiments could involve crayfish from several sources. Further studies involving genetic characterization could reveal relationships between the isolates, while the RAPD-PCR profiles are unfortunately too similar inside an *A. astaci* genotype group for this purpose (own unpublished results).

The variation of the virulence factors between different *A. astaci* strains is not well understood. The virulence can be influenced by the ability of the zoospores, the infective stage of the organism, to successfully attach to a suitable host, germinate and invade the crayfish cuticle (Cerenius *et al.* 1988). It has been shown that *A. astaci* possesses the ability for repeated zoospore emergence, which can enhance its possibilities to locate a suitable host animal (Cerenius & Söderhäll 1984a, 1985), as well as chemotaxis towards nutrients (Cerenius & Söderhäll 1984b). Once the spore has started the hyphal growth phase in the host animal cuticle, it produces a continuous release of chitinase (Söderhäll, Svensson & Unestam 1978; Anderson & Cerenius 2002) and also other enzymes (Söderhäll & Unestam 1975; Söderhäll *et al.* 1978; Persson, Häll & Söderhäll 1984) that may be involved in the pathogenic process (Dièguez-Uribeondo & Cerenius 1997, Bangyeekhun, Cerenius & Söderhäll 2001). Except the reported differences in the chitinase genes between the genotypes As and Ps1 (Makkonen *et al.*2012a), variations in virulence factors between the genogroups have not been studied extensively so far. Although it was not possible for practical reasons to deliver any definite evidence for the connection between the hyphal growth rate and the virulence in an experimental set-up, we selected one slow-growing and one fast-growing representative from the genogroups As and Ps1 to study the eventual differences in virulence. A clear and statistically significant difference between the genotypes As and Ps1 could be seen in the ability to cause mortality in noble crayfish. The lower virulence of As isolates compared with Ps1

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genotype has been demonstrated also in later experiments (Makkonen *et al.* 2012b, 2014). Several studies (Alderman *et al.* 1987; Makkonen *et al.* 2012b, 2014; Jussila *et al.* 2013), including this study, have revealed that the Ps1 genotype is responsible for causing an acute crayfish plague that leads to total mortality in a relatively short time period, although in this study the development of mortality in Ps1Slow groups was slower than with Ps1Fast. AsSlow, with its very limited growth capacity, seemed to induce a slow progressing infection, only evoking total mortality after 5 months. The medium high zoospore density (Cerenius *et al.* 1988), combined with the temperature that should favour the growth of the plague organism (Alderman *et al.* 1987), and suboptimal environment for the crayfish as judged by the low but constant mortality in the control groups, clearly did not create the circumstances where this strain could cause rapid mortality. The AsFast strain had an even less pathogenic effect as the mortality in AsFast groups did not differ from that encountered in the control groups. During the time of this trial, no molecular methods were available to ascertain the successful establishment of the infection, but the microscopic pathology as well as the re-isolation of the crayfish plague agent from the test groups verified the presence of viable *A. astaci* in the test tanks. Although AsFast did not cause any elevated mortality in this trial, it was originally associated with a mortality episode in a small lake. Later it was found that there was not 100% crayfish mortality in this lake, and a crayfish plague isolate genotype As was again detected in the lake in 2011, 8 years after the initial isolation (own unpublished results). We conclude that this strain has lost another factor of the many-faceted process of growth in the host animal and this should be clarified in further studies. Variable virulence within the As genotype group was also demonstrated by Makkonen *et al.* (2012b, 2014). It becomes clear that these kinds of low-virulent strains can easily survive for extended time periods in natural surroundings, where for most of the time the water temperature will be much lower, and where there will be less infection pressure towards the host animal, even in an acute phase of the epizootic attack (Strand *et al.* 2014) than in this trial.

Due to the unexpected lack of virulence showed by the AsFast strain, we could not link the virulence with the growth rate. With the other three tested strains, Ps1Fast, Ps1Slow and AsSlow, the mortality developed according to the diminishing growth capacity. While the overall comparison between the isolates of the two genotypes revealed a clearly slower growth in As group, the question remains if this has an effect on the reported lower virulence of As genotype isolates. In its struggle for survival in its new surroundings, the crayfish plague agent may have developed several strategies, but low virulence must have been the key for a successful parasitic life in a situation where there is a lack of natural hosts. A reduced growth rate could be one of the strategies, but certainly not the only one. This is clearly demonstrated by the infection trial, where the fast-growing As strain did not seem to cause any elevated mortality despite the parasite's undeniably successful attachment and germination in the host. The greater variations of the growth rate and virulence inside the As genotype group could reflect its long history in Finland, when the pathogen has had to cope with the low resistance of the novel host and was thus subjected to harsh selection pressure. High virulent forms causing total mortality of noble crayfish might be able to exist as long as new host populations are readily available, but not in the kinds of situations that have been prevalent since the initial epidemic waves of crayfish mortalities in Finland. It must be kept in mind that during the first 70 years, the pathogen managed to survive in Finland supported only by the highly susceptible European host, and even the introduction of the signal crayfish may not have offered more choices of host, as the As genotype has never been recovered from signal crayfish in natural conditions (own unpublished results). The original host species of the As genotype is unknown and most probably until today not present in the open waters of Europe. It would add considerably to our knowledge of the adaptive ability of this pathogenic oomycete, if the original host could be recognized as the study of the more naturally supported strains would then become possible.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Pairwise log rank comparisons of the test groups of the infection trial.

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