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


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

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AFLP-PCR</td>
<td>amplified fragment length polymorphism PCR</td>
</tr>
<tr>
<td>As</td>
<td><em>Aphanomyces astaci</em> genotype group Astacus (genotype A)</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ICS</td>
<td>indigenous crayfish species</td>
</tr>
<tr>
<td>ITS</td>
<td>the internal transcribed spacer</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NACS</td>
<td>North American crayfish species</td>
</tr>
<tr>
<td>NICS</td>
<td>non-indigenous crayfish species</td>
</tr>
<tr>
<td>OIE</td>
<td>World Organization for Animal Health (Office International des Epizooties)</td>
</tr>
<tr>
<td>Or</td>
<td><em>Aphanomyces astaci</em> genotype group Orconectes (genotype E)</td>
</tr>
<tr>
<td>Pc</td>
<td><em>Aphanomyces astaci</em> genotype group Procamburus (genotype D)</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>proPO</td>
<td>prophenoloxidase</td>
</tr>
<tr>
<td>Ps1</td>
<td><em>Aphanomyces astaci</em> genotype group Pacifastacus I, or PsI (genotype B)</td>
</tr>
<tr>
<td>Ps2</td>
<td><em>Aphanomyces astaci</em> genotype group Pacifastacus II, or PsII (genotype C)</td>
</tr>
<tr>
<td>RAPD</td>
<td>random amplification of polymorphic DNA</td>
</tr>
<tr>
<td>RAPD-PCR</td>
<td>random amplification of polymorphic DNA- polymerase chain reaction</td>
</tr>
<tr>
<td>rDNA</td>
<td>ribosomal deoxyribonucleic acid</td>
</tr>
<tr>
<td>WCA</td>
<td>water catchment area (according to the Finnish Environment Institute)</td>
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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 *Astroptamobius* 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 Fuereeder 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-Uribendoza 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 °C 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.

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<th>Isolate identification</th>
<th>Sample identification</th>
<th>Sample location</th>
<th>Water catchment area (3rd level)</th>
<th>Coordinate N/lat</th>
<th>Coordinate E/lon</th>
<th>Host</th>
<th>Background information</th>
<th>Genotype</th>
<th>Publication</th>
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<td>Ps1</td>
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<td>Pond Smečno, Czech Republic</td>
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<td>Or</td>
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<td>Pond Smečno, Czech Republic</td>
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<td>O.I.</td>
<td>Symptomatic spiny-cheek crayfish</td>
<td>Or</td>
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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 MgCl₂, 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 °C.

The selected *A. astaci* isolates were incubated in PG-1 broth at 20 °C 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](image-url)

**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.

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](image)

**Figure 6.** The radial growth rate of *Aphanomyces astaci* isolates belonging to the genotypes As and Ps1 in PG-1 medium at 20 °C. (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 Evira6672/03. 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, Psl, 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), Psl = 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 (Harlioglu 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 preventative 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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9 References


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