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

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

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

INTRODUCTION

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

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

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

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

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

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

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

MATERIALS AND METHODS

Sample material

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

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

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

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

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

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

Isolation and identification of *Aphanomyces astaci*

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

Randomly amplified polymorphic DNA-PCR

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

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

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

Table 1 (continued)

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

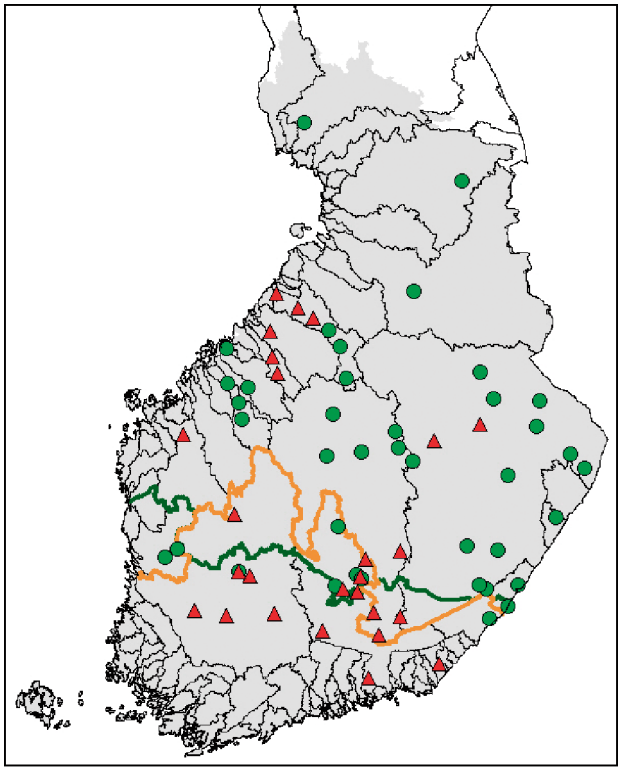

Fig. 1. Locations of crayfish plague *Aphanomyces astaci* identified in noble crayfish in 1996–2006, overlaid on the map of the main water catchment areas of Finland. Green circles: *A. astaci* genotype As; red triangles: *A. astaci* genotype Ps1; orange line: northern border of the signal crayfish stocking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

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

RESULTS

Genotypes of Finnish *Aphanomyces astaci* isolates

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

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

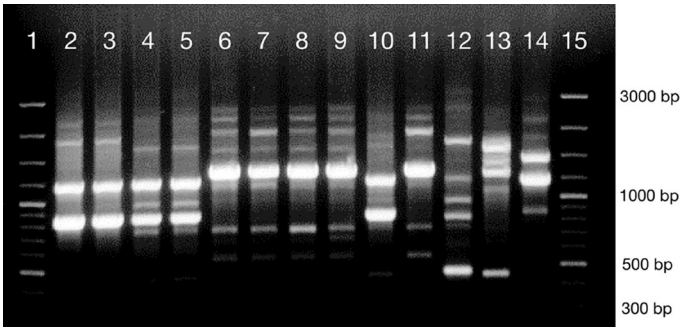

Fig. 2. RAPD-PCR analysis of some Finnish *Aphanomyces astaci* isolates. Lanes 1 and 15: molecular weight marker 100+ bp. Lanes 2–5: isolates representing Pasifastacus strain I type (Ps1). Lanes 6–9: isolates representing Astacus type (As). Lanes 10–14: reference strains Si (Ps1), Da (As), Kv (Ps2),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

Geographical distribution of the genotypes

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

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

Epidemiological features of the genotypes

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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