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Aphanomyces astaci PsI-genotype isolates from different Finnish signal crayfish stocks show variation in their virulence but still kill fast

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ABSTRACT

Key-words: *crayfish plague, noble crayfish, potent killer,* Psorospermium haeckeli

We detected significant virulence differences among five tested (PsI-Puujärvi, PsI-Pyhäjärvi, PsI-Kukkia, PsI-Saimaa I and PsI-Saimaa II) PsI-genotype crayfish plague (*Aphanomyces astaci*) isolates against lake Mikitänjärvi noble crayfish population. The crayfish were inoculated with a dose of 300 m·L^{-1} *A. astaci* spores in ambient water under experimental conditions. Mortalities started from four to seven days after inoculation, depending on the PsI-genotype isolate. In all the experimentally infected groups it took no more than three days for all the crayfish to die after the first mortality. The PsI-Puujärvi isolate proved to be the most virulent strain, while PsI-Kukkia was the least virulent. The average day of death for these experimental groups was fifth and ninth day, respectively. We did not discover correlation between the average day of death and gender or level of additional *Psorospermium haeckeli* infestation. Our results show that there are, from the practical point of view, minor virulence differences among PsI-genotype *A. astaci* isolates, and that all the tested five isolates are highly virulent. The present results emphasize the necessity to prevent all further spread of highly virulent strains of *A. astaci* to aid and shelter successful conservations attempts of the native European crayfishes.

RÉSUMÉ

Les génotypes PsI d'*Aphanomyces astaci* isolés de différents stocks d'écrevisses signal finlandaises montrent une variabilité dans leur virulence mais tuent toujours rapidement

Mots-clés : *peste de l'écrevisse, écrevisse noble, tueur puissant,* Psorospermium haeckeli

Nous avons détecté des différences de virulence significatives entre les cinq génotypes PsI testés (PsI-Puujärvi, PsI-Pyhäjärvi, PsI-Kukkia, PsI-Saimaa I et PsI-Saimaa II) de la peste de l'écrevisse (*Aphanomyces astaci*) vis-à-vis de la population d'écrevisse noble du lac Mikitänjärvi. Les écrevisses ont été inoculées avec une dose de 300 m·L^{-1} de spores d'*A. astaci* dans l'eau ambiante dans des conditions expérimentales. Les mortalités ont commencé de quatre à sept jours après l'inoculation, selon le génotype PsI isolé. Dans tous les groupes infectés expérimentalement il n'a pas fallu plus de trois jours pour que toutes les écrevisses meurent après la première mortalité. L'isolat PsI-Puujärvi s'est avéré être la souche la plus virulente, tandis que PsI-Kukkia a été le moins virulent. Le nombre moyen de jours pour la mort de ces groupes expérimentaux est le cinquième et le neuvième jour, respectivement. Nous n'avons pas découvert de corrélation entre le jour moyen de décès et le sexe ou le niveau de l'infestation supplémentaire

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par *Psorospermium haeckeli*. Nos résultats montrent qu'il existe, du point de vue pratique, des différences de virulence mineures entre isolats de génotype PsI de *A. astaci* et que tous les cinq isolats testés sont très virulents. Les résultats actuels soulignent la nécessité de prévenir toute propagation de souches très virulentes de *A. astaci* pour contribuer au succès des tentatives de conservation des écrevisses européennes autochtones.

INTRODUCTION

The virulence variation of the crayfish plague (*Aphanomyces astaci*) strains from different epidemics had been long discussed (Huang *et al*., 1994; Fürst, 1995; Edgerton *et al*., 2004), until it was recently repeatedly documented (Makkonen *et al*., 2012b, 2013; Jussila *et al*., 2013a). In general, this disease has a bad reputation, but some *A. astaci* strains seem to show signs of avirulence (Jussila *et al*., 2011a; Makkonen, 2013). Certain strains of this parasite have likely adapted to their fairly recent European hosts, as the As-genotype is currently causing highly variable mortalities among native European crayfish both in laboratory experiments (Makkonen *et al*., 2013) and among wild populations (Jussila *et al*., 2011, Viljamaa-Dirks *et al*., 2011; Kokko *et al*., 2012; Svoboda *et al.*, 2012; Caprioli *et al*., 2013; Kušar *et al*., 2013). So far, however, the evidence of virulence differences and potential evolution among PsI-genotype, or other more recently introduced *A. astaci* genotypes, has been lacking. The assumption is, that those fairly recently introduced *A. astaci* strains, that were introduced together with their original North American host crayfish species, have had less pressure and time to adapt their virulence to enable lower mortality rates among the susceptible native European crayfish populations, as has been theorized for the pathogen-host relationship to evolve in similar situations (*e.g.* Gandon and Michalis, 2000; Bull and Ebert, 2008). On the other hand, the host-parasite relationship of a host with long life span and a parasite with long infective periods is supposed to create diversity among both hosts and parasites (Best *et al*., 2010), which could thus re-balance relationship of *A. astaci* and, at least, its North American hosts in Europe. Environmental conditions could, in their part, also be a driving force of the host-parasite co-evolution (Wolinska and King, 2009; Biron and Loxdale, 2013) and thus might also be affecting the aforementioned balance. The indications of a shifted balance between signal crayfish (*Pacifastacus leniusculus*) and *A. astaci* in Nordic countries seen as signal crayfish population collapses and severe symptoms of individual crayfish infected with *A. astaci* (Jussila *et al*., 2013a). The parasites could also manipulate their host's behavior or physiological processes to ensure that they could compelete their life cycle (Biron and Loxdale, 2013).

The PsI-genotype, which is one of the two known genotypes currently causing crayfish plague epidemics in Finland and other Nordic countries, has its original North American host species, the signal crayfish (*P. leniusculus*), present in Europe (Souty-Grosset *et al*., 2006). The signal crayfish has been intentionally introduced to inhabit most of the continental Europe (Westman, 2000; Souty-Grosset *et al*., 2006), thus assisting in the spread of the PsI-genotype crayfish plague (Unestam, 1969; Persson and Söderhäll, 1983; Diéguez-Uribeondo and Söderhäll, 1993; Diéguez-Uribeondo, 2006; Souty-Grosset *et al*., 2006). These *A. astaci* PsI-genotype strains could likely have had less pressure to adapt to novel European habitat than As-genotype strains, since they have easy access to host habitat that is often available as a result of signal crayfish introductions (Jussila *et al*., 2013a). So far, this assumption has been mostly based on speculations supported by theories on general evolution of the diseases entering novel territories and infecting susceptible new hosts (Read, 1994; Regoes *et al*., 2000; Ebert and Bull, 2008). New invasions, in general, often result in high initial virulence that is accompanied with notable killing rates among native host candidate species (Read, 1994). In case of PsI-genotype crayfish plague, the high virulence among the native European crayfishes can be evolutionary sustained because of the continuously existing more resistant host habitat reserve, *i.e.* an introduced alien crayfish stock that is always present (Jussila *et al*., 2013a).

The crayfish plague remains as the biggest threat to all the native European species like the noble crayfish (*Astacus astacus*) (Souty-Grosset *et al*., 2006; Jussila *et al*., 2013a), after the general decline of the aquatic pollution level and more ecologically friendly codes of practice in the management of the aquatic resources (EU, 2000; Jurvelius and Auvinen 2001; Tulonen *et al*., 2008, 2010). The signal crayfish has been more or less permanently introduced to numerous areas still inhabited by the noble crayfish (Jussila and Mannonen, 2004; Bohman *et al*., 2006; Johnsen *et al*., 2007), either according to national strategies or illegally. Crayfish plague is generally thought to be one of the main reasons for the difference in the crayfish introduction success rates between the noble crayfish and the signal crayfish (Erkamo *et al*., 2010). Initial reason to introduce the signal crayfish to Europe was the fact that many waterbodies were already eradicated from the noble crayfish by the first invasion of the crayfish plague. The situation has actually been further reinforced since the signal crayfish introductions have often been accompanied by the notably virulent PsI-genotype crayfish plague (Souty-Grosset *et al*., 2006; Jussila *et al*., 2013a). Furthermore, the more recently introduced *A. astaci* genotypes, namely PsI, PsII, Pc and Or (Kozubíková *et al*., 2011), are, if possible, an even greater threat to the native European crayfish than the originally introduced As-genotype, since unlike the As-genotype, they so far have not had to adapt to susceptible host species in Europe.

The status of the alien species, in this case both the crayfish plague and its introduced North American host crayfish species, has been debated intensively (Gherardi and Holdich, 1999). Therefore, the strategies concerning these aliens have been drafted on both national and EU level (EU, 2013). The crayfish plague and the signal crayfish have been listed as greatly harmful and harmful, respectively, in the Finnish alien species strategy (MMM, 2013). Thus, the attempts to understand the dynamics between different *A. astaci* strains, their native European crayfish hosts and an introduced alien host of North American origin are of utmost importance. The conservation actions success lies on the understanding of the dangers of chronically infected crayfish spreading high virulent strains of crayfish plague and acting as permanent sources of *A. astaci* spores.

The main aim of the study was to assess whether different isolates of PsI-genotype would differ in their virulence against the noble crayfish and whether they would all be high virulent killers. We were also interested to explore, if Lake Mikitänjärvi noble crayfish would show any resistance against this genotype, which would be different from what has been reported before in other noble crayfish populations. This interest was justified due to the fact that latent crayfish plague infection had been reported in this wild population (Jussila *et al*., 2011a). We also studied whether the individual crayfish being parasitized by *Psorospermium haeckeli* (Makkonen *et al*., 2013) would show different mortality rate, *i.e.* resistance or sensitivity, against *A. astaci* PsI-genotype. In order to fulfill our aims, we carried out a controlled infection trial under laboratory conditions.

MATERIAL AND METHODS

> THE EXPERIMENTAL CRAYFISH

The Lake Mikitänjärvi (ETRS-TM35FIN coordinates N 7158870, E 604356) noble crayfish, equal numbers of both sexes in each group, were used in the infection experiment. The crayfish were obtained from a commercial trapper and kept in the holding tank system in Muuruvesi campus aquaculture facilities from early September 2012 until the commencement of the experiment in early February 2013. The crayfish were maintained, sexes separated, in two interconnected tanks in prefiltered (5 μm) recirculating Lake Kallavesi water. During the holding, an ambient lake water temperature was maintained and partial water exchanges made every two weeks. Crayfish were given *ad libitum* sweet corn as food during the holding. The tank system contained a biofilter to lower the load from metabolic substances. Prior to the experiment, the crayfish were transported to the University of Eastern Finland RapuLatorio® (Jussila *et al*., 2011b) and placed in the experimental system for a two week acclimation. The crayfish sexes were determined and they were weighed (0.1 g) before placing them into the

Table I
The A. astaci PsI-genotype isolates used in the experiment. The references contain further details of the isolates in question. Epidemic and isolation expressed as the year when the wild stocks started showing gross symptoms for crayfish plague or collapsed for the first time, and when the isolation was carried out by the University of Eastern Finland.

Name	Code	Epidemic	Isolation	Location	Reference
PsI-Puujärvi	UEF8866-2	1996	2003	N 6683791, E 317391	Makkonen *et al*., 2012a
PsI-Saimaa I	Satr1	2007	2012	N 6800998, E 555776	Jussila *et al*., 2013b
PsI-Saimaa II	Satr2	2007	2012	N 6800998, E 555776	Jussila *et al*., 2013b
PsI-Pyhäjärvi	UEF8147-4	1995	2003	N 6770682, E 245801	Makkonen *et al*., 2012a
PsI-Kukkia	UEF7203	1992	2003	N 6801172, E 377743	Makkonen *et al*., 2012a

individual 2 L containers of the experimental system. Crayfish feeding behaviors were monitored during acclimation and those crayfish showing decreased condition were substituted.
The *P. haeckeli* infestation level for the experimental crayfish was investigated after the experiment using method described by Henttonen (1996). The crayfish were categorized by their infestation level ranking followingly: 0 = no *P. haeckeli*; 1 = 1–14 *P. haeckeli* and 2 ⩾ 14 *P. haeckeli* counted in the sample.

> THE CRAYFISH PLAGUE ISOLATES

We used five PsI-genotype isolates to infect the experimental crayfish. The isolates were obtained from signal crayfish (*P. leniusculus*) which were chronic carriers of *A. astaci*. The isolates will be referred to from hereon according to epidemic: PsI-Puujärvi, PsI-Saimaa I, PsI-Saimaa II, PsI-Pyhäjärvi and PsI-Kukkia (Table I). All these strains have been isolated and maintained by the University of Eastern Finland.

> PRODUCTION OF THE ZOOSPORES AND INOCULATION

The *A. astaci* isolates, maintained in PG1-agar (Unestam, 1965), were used in the infection experiments. Details of the methods for zoospore production, modified after Cerenius *et al*. (1988), are explained in Makkonen *et al.* (2012c). The inoculation dose for the infection groups was 300 zoospores $m \cdot L^{-1}$.

> THE EXPERIMENTAL SYSTEM

The experimental system consisted of individual 2 L tanks for the crayfish and a dual sump tank system equipped with pumps and filtering system to recirculate the water. The dual sump tank system consisted of two 100 L tanks, with the first receiving water from the experimental crayfish tanks. The water was first prefiltered through a porous foam mat and then pumped (Biltema DP 900 W, Sweden) through a filtering system consisting first of two filters back to back, the first being a 25 μm (Polypropylene Atlas Water Filter, Atlas, Italy) and the second a 5 μm filter (Spunflow QN, Dominic Hunter Technologies Ltd., England). These were followed by two parallel absolute filters (Pleatflow II, Prosep Filter Systems Ltd., England), with the filtered water ending into another 100 L tank and then being pumped (EHEIM Compact 1002, 6 pumps) into a pipeline distributing the water into the individual 2 L tanks. The filtering system used removes all the infectious agents, such as *A. astaci* spores, from the water column (Jussila *et al*., 2011b). The water pressure prior to the filters was monitored and the filters were replaced when the pressure exceeded 2 bar.
The water volume in the individual holding tanks was fully replaced hourly. The day and night rhythm were 12 h light on (fluorescent lights) and 12 off, over the night time. Water temperature was maintained stable by room air conditioning at 18 ± 1 °C. The water quality, DO-%,

Table II
Average day of death (mean± SD) of the PsI-genotype infected noble crayfish for each strain. Different superscript indicates statistically significant difference (Log Rank (Mantel-Cox), $p < 0.05$) among the groups. All control group crayfish survived the experiment.

Control	PsI-Puujärvi	PsI-Saimaa II	PsI-Saimaa I	PsI-Pyhäjärvi	PsI-Kukkia
n/a	5.0 ± 0.0^{d}	6.5 ± 0.2^{c}	$6.7 \pm 0.5^{b,c}$	8.0 ± 0.1^{b}	9.0 ± 0.2^{a}

temperature and pH, were monitored twice a week during the experiment. Gravel containing calcium was added to the sump tanks to maintain optimum water pH 7 in the recirculating water. Water quality remained within optimal range for crayfish throughout the study. The dissolved oxygen was 88.8 ± 10.3% (min−max, 66,1−98,9%), pH was 7.1 ± 0.2 (min–max, 6,7–7,4) and water temperature was 17.8 ± 0.6 °C (min–max, 17,0–18,9 °C).
The crayfish were randomly divided into the experimental groups ($N = 10$), having initially equal numbers of sexes. During acclimation, five crayfish declined to feed on a regular basis and they were substituted with reserve crayfish which were feeding. Thus, finally, the control group and PsI-Saimaa II group had male to female ratio of 6:4.
Crayfish were monitored at least twice a day for behavioral symptoms indicating *A. astaci* infection (*i.e.*, difficulties in maintaining orientation, paralysis, loosing limbs or claws) and the death. Notes were made on feeding behavior and observed symptoms.

RESULTS

> CRAYFISH MORTALITY AND A. ASTACI VIRULENCE

We observed significant differences in mortality among tested crayfish groups which were infected using different PsI-genotypes. The PsI-Puujärvi strain was the most virulent, then PsI-Saimaa I and PsI-Saimaa II, PsI-Pyhäjärvi and the least virulent strain was PsI-Kukkia (Table II, Figure 1). The differences in the average day of death between most and least virulent PsI-genotype isolates were roughly 2× (Table II). The predicted day of death in the earlier work by Makkonen *et al*. (2013) was, for the inoculation dose and *A. astaci* genotype used in this experiment, 5.8 days, which is thus lower than observed in this experiment, except for PsI-Puujärvi infected group. The control group crayfish survived the whole 12-day experiment and a two week follow-up period. The mortalities started in PsI-Puujärvi infected group four days after inoculation and in the PsI-Kukkia infected group seven days after inoculation (Figure 1). A 100% mortality rate was achieved rapidly, *i.e.* within three days after the first crayfish in the experimental group in question died, except for PsI-Puujärvi group in which crayfish died within two days.

> PSOROSPERMIUM HAECKELI

All the experimental crayfish were infested with *P. haeckeli*. The crayfish in experimental groups PsI-Puujärvi, PsI-Saimaa II, PsI-Pyhäjärvi and control group had both level 1 and 2 infestation, while PsI-Saimaa and PsI-Kukkia had only level 2 infestation. We did not observed differences in the average day of death among the two infestation levels within experimental groups (Mann-Whitney U-test, $p > 0.05$ in all valid cases).

DISCUSSION

We have shown significant, but from the practical point of view minor, differences in virulence among several highly lethal PsI-genotype crayfish plague (*A. astaci*) strains isolated from the signal crayfish (*P. leniusculus*) of Finnish origin. During the laboratory trials the mortality rates,

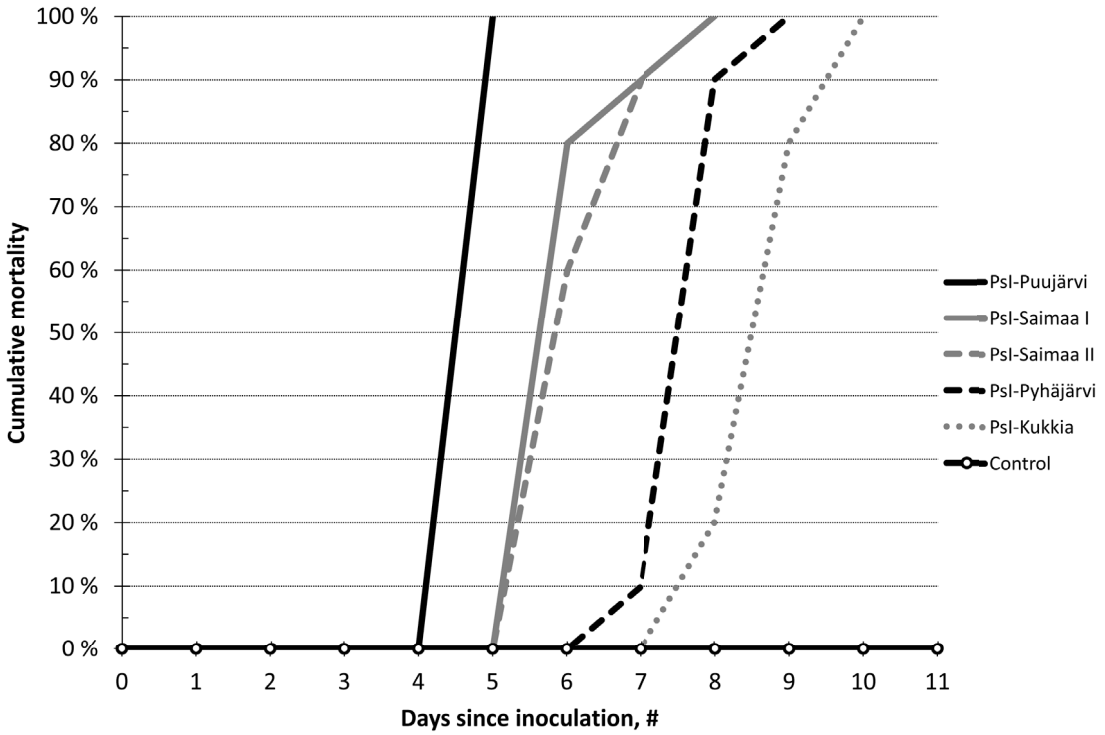

Figure 1
The mortality rates among the noble crayfish groups inoculated using different PsI-genotype isolates from Finland and the control crayfish group. The inoculation dose was 300 spores per m·L^{-1}. Statistically significant differences were observed among isolates from different geographical locations, from most virulent to least virulent: PsI-Puujärvi > PsI-Saimaa I = PsI-Saimaa II > PsI-Pyhäjärvi > PsI-Kukkia (Log Rank (Mantel-Cox), $p < 0.05$).

expressed as the average day of death of the experimental group, varied from five to nine days. This means roughly 100% variation when the most virulent isolate killing rate was compared to the least virulent isolate. We have to emphasize that all the crayfish died in every infected group and mortalities happened in little more than a week after the inoculation, in contrast to zero mortality in control group. This would have meant an instant and total eradication of a noble crayfish population in wild after an infection.

Our results regarding the PsI-genotype *A. astaci* killing rate are in line with our previous findings on the virulence of the PsI-genotype (Jussila *et al*., 2011b; Makkonen *et al*., 2012b; Makkonen *et al*., 2013), and, furthermore, there are also other reports indicating high virulence in the *A. astaci* genotypes that have been introduced with alien crayfish from North America (Diéguez-Uribeondo and Söderhäll 1993; Bohman *et al*., 2006; Souty-Grosset *et al*., 2006; Aquiloni *et al*., 2011). We also verified, while in a more narrow scale, the findings on the variable virulence of different crayfish plague isolates within genotype level, which have largely been previously obtained from studies on As-genotype (Makkonen *et al*., 2011; Makkonen *et al*., 2012b; Jussila *et al*., 2013a). On the other hand, we have previously observed a delayed mortality among PsI-Puujärvi infected Lake Mikitänjärvi noble crayfish (Makkonen *et al.* 2013; Makkonen, 2013), but reasons for the mortality rate differences among different experiments have not been analyzed. The possible reasons for this between-experiment variation could be differences in experimental crayfish condition among the different experiments, especially when crayfish from the same wild stock have been used, or crayfish plague spores behaving differently during different experiments due to minor differences during the spore production process.

On the other hand, we did not find the Lake Mikitänjärvi noble crayfish to be more vulnerable to crayfish plague infection when infested also with *P. haeckeli*, while such weakening of the

immune system has earlier been reported in the signal crayfish (Thörnqvist and Söderhäll, 1993). In the future, the effects of multiple infestations to the immune system status in freshwater crayfish should be thoroughly studied.

We observed clear behavioral symptoms indicating late stages of the infection, similarly to our previous laboratory studies (Makkonen, 2013). These symptoms include 1) crayfish starting to scratch their bodies with chelae and 2) lost limbs a day before death. Less than a day before death, the experimental crayfish usually lost their balance and could not maintain normal upright position. The behavioral symptoms were similar in all groups in this study and the initiation of the scratching was the point of no return, leading to death of the crayfish. Based on our observations, most of the As-genotype infected crayfish in our previous studies experienced a lengthy catatonic stage before dying (Makkonen, 2013), which is different from what was observed during this or our previous studies using the PsI-genotype for the noble crayfish challenging (Makkonen *et al*., 2012b, 2013).

The differences observed in the virulence among the tested PsI-genotype isolates in this study might be an indication of what we have reported before on the differences among PsI-genotype isolates' chitinase gene (Makkonen *et al*., 2012b). We have already observed differences on genome level within the PsI-genotype. These differencies could indicate the presently detected virulence differences. The previously observed differences in chitinase genes were more pronounced within As-genotype compared to the combined Ps-genotype, *i.e.* in both PsI and PsII-genotypes (Makkonen *et al*., 2012b), with the assumption that those differences could reflect also virulence differences. The differences in PsI-genotype chitinase genes, even though evident, were minor compared to As-genotype (Makkonen *et al*., 2012b), which has also shown wider virulence variation in previous studies compared to what we observed with PsI-genotype in this study (Makkonen, 2013). Chitinase production has been claimed to be adaptation to parasitic life style and one of the virulence factors (Unestam, 1966; Hochwimmer *et al*., 2009).

The reports of *A. astaci* adaptation to the European crayfish hosts (Jussila *et al*., 2011; Viljamaa-Dirks *et al*., 2011; Kokko *et al*., 2012; Svoboda *et al*., 2012; Kušar *et al*., 2013) are most probably all concerning As-genotype adaptation. The 150 year history in Europe, with high selection pressure, has caused a rather rapid co-evolution of both the host and the pathogen. The drive to less virulent parasite-like life style is not as strong in case of the PsI-genotype tested in this study, as it is expected to be in As-genotype (Makkonen *et al.*, 2012a). Taken together, the recent results give a strong signal about the case of an invading disease, that can use resistant host habitat species as a reserve, but simultaneuously being detrimental to those species susceptible to the disease, in this case crayfish native to Europe. Thus, the policies driving for the conservation of the native species and actions against harmful alien species are urgently needed. The attempt to implement a common alien species strategy within European Union is important, and tough measures are needed to prevent the spread of the aliens carrying high virulent strains of the crayfish plague.

We conclude, that the *A. astaci* PsI-genotypes, that were introduced to Europe with the signal crayfish, are high virulent, and that the tested isolates do not show signs of adaptation to co-existence with one of the threatened native European crayfish, the noble crayfish. Thus, the PsI-genotype crayfish plague been frequently carried with the signal crayfish is an utmost threat to the noble crayfish stocks and thus the spread of the alien crayfish and crayfish plague should be prevented. The conservation attempts of the European crayfish can succeed only, if the dilemma of the socioeconomic benefits of the alien species over native species and the negative impact on alien species to native species is thoroughly considered and understood.

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