Latent crayfish plague (Aphanomyces astaci) infection in a robust wild noble crayfish (Astacus astacus) population

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A B S T R A C T
Apparently healthy wild noble crayfish (Astacus astacus) sampled from Lake Mikitänjärvi, Kainuu, Finland in 2009 and 2010 were discovered to be PCR-positive for Aphanomyces astaci. Detection prevalence was lower in 2009 than in 2010. Despite the detected sublethal infection, the Lake Mikitänjärvi population has been productive and is being under commercial exploitation. Even though it has been suggested frequently that noble crayfish might harbor subclinical crayfish plague infections, this is the first confirmed report, based on molecular evidence, of healthy A. astacus carrying A. astaci.

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1. Introduction

Crayfish plague caused by Aphanomyces astaci is among the most severe diseases affecting aquatic organisms exploited commercially (Edgerton et al., 2004; Edgerton and Jussila, 2004; Holdich, 2002). During the last century, the invasive oomycete extensively eliminated native European crayfish populations (Souty-Grosset et al., 2006). Currently, the last commercially exploitable native European populations exist in Nordic and Baltic countries (Jussila and Mannonen, 2004; Souty-Grosset et al., 2006). In Finland, the commercial value of the annual noble crayfish catch varies from 4 to 6 million € (Jussila, 1995; Kirjavainen and Sipponen, 2004) which exceeds the value of most Finnish finfish fisheries (Jussila and Mannonen, 2004).

Based on current knowledge, two distinct major A. astaci types exist in Finland (Makkonen et al., 2011; Vennerström et al., 1998). Annually, both types cause lethal infections in numerous wild noble crayfish populations in Finland (Mannonen et al., 2006). The Po–type, that is believed to have originated from recent signal crayfish introductions (Westman, 2000) normally causes 100% mortality, while the As-type that is believed to have existed longer in Europe might cause lower mortality and might be less virulent (manuscript in preparation).

It has been suggested that certain A. astaci strains might establish subclinical infections in noble crayfish and that these infections might be perpetuated over several years due to low host densities, slow infection dynamics, spatial refuges for hosts or decreased virulence in some strains (Alderman et al., 1987; Fürst, 1995; Kirjavainen and Sipponen, 2004; Skov et al., 2011; Svärdson, 1965; Westman, 2000; Westman and Nylund, 1979). In addition, due to it causing an invasive disease, it might be expected that A. astaci virulence is selected against during adaptation to a new host (Bull and Ebert, 2008) to avoid evolutionary suicide (Gandon et al., 2001; Gandon and Michalakis, 2000; May and Nowak, 1995; Nowak and May, 1994).

In Finland, before noble crayfish stockings, sample of healthy crayfish are routinely kept in cages to monitor for survival and reproduction success as well as for the presence of A. astaci in target water systems. These cage trials are usually undertaken over short time periods or over winter and utilize healthy noble crayfish of both genders, preferably originating from the planned stocking population, and they direct decisions on the management strategies. Recently it was suggested that these cage trials could be expanded to accommodate PCR screening for A. astaci (Souty-Grosset and Reynolds, 2009).

As part of the crayfisheries management project in Kainuu, Finland, we found, using PCR-methods, that apparently healthy wild noble crayfish can carry subclinical A. astaci infections. This novel finding is important as such infected crayfish pose disease risks if used for stocking or for cage-based monitoring in disease-free waters.

2. Materials and methods

2.1. Crayfish populations sampled

The health status of four wild noble crayfish populations within the Lake Hyyrönjärvi watershed area of Kainuu in Finland was examined over 2 consecutive years. Noble crayfish were obtained from local trappers in Lake Mikitänjärvi (2009 and 2010), Lake Luvanjärvi (2009 and 2010), Lake Ristijärvi (2009) and Lake Hietajärvi (2010) (Fig. 1).
2.2. Crayfish cage trials

Both short and long term cage trials of overtly healthy noble crayfish were undertaken in Lake Hyrynjärvi to detect possible *A. astaci* infection and to monitor crayfish reproductive success. The cages (120 × 40 × 40 cm: L × W × H) were wooden, equipped with 20 stumps of drain pipes as hides and stocked with raw potatoes and carrots as food. The cages were placed along the path of the water flow to maximize possible exposure to *A. astaci*. Tissue melanisation and other possible gross signs of crayfish plague were monitored for during the trials.

In the short-term trial, 3 sterilized cages stocked with 15 females and 5 males originating from Lake Mikitänjärvi were set in early August 2009 (Fig. 1). After a month, crayfish were transferred to laboratory tanks, to be held at +4 °C in Kuopio for monitoring and PCR testing for *A. astaci*. The key study population resided in Lake Mikitänjärvi (N 7158870, E 604356, 915 ha), which is the uppermost large lake in the Lake Luvänjarvi (841 ha) water course. This lake was first populated with noble crayfish in 1968 and 1969 (Martti Niskanen, personal communication) and since then it has maintained a robust crayfish plague (Mannonen et al., 2006) and in 2007 (Pasi Korhonen, personal communication), with *A. astaci* infection being suspected as the cause. However, prior to present work, all the study populations were considered healthy.

In the long-term trial, another set of 2 similar sterilized cages with 15 females and 5 males were set in September (Fig. 1) to test the overwintering and reproduction success and to detect the possible presence of *A. astaci*. Cages were examined 4 times over winter and dead crayfish were removed on each occasion. Surviving males were transferred to tanks at Kuopio in March and surviving females were transferred in late May. Samples of eggs were also taken from females and samples from all crayfish were tested by PCR for *A. astaci*.

2.3. Sample preparation and PCR detection of *A. astaci* DNA

Two uropods and telson (pooled as one sample), abdominal cuticle and leg joint tissues were dissected aseptically from individual crayfish and frozen in separate disposable tubes for subsequent PCR analysis, out of which pooled uropod and telson samples were analysed routinely and other samples kept if confirmation of the analyses was required. Tissue was disrupted aseptically in a FastPrep® FP120 (BIO101 Thermo Savant) together with ceramic beads and sterile sea sand (Merck) for 2 × 30 sec with 6.0 m/sec speed and DNA was extracted using the EZ.N.A. Insect DNA Isolation kit (Omega Bio-Tek) according to the manufacturers protocol. The DNA quantity and quality was measured with a NanoDrop-spectrophotometer (Thermo Fisher Scientific). The ITS-PCR (25 μL) contained 2 mM MgCl₂, 1 mM dNTP mixture, 1 U Maxima Hot Start Taq PCR -enzyme, 1× Maxima Hot Start Taq PCR -buffer (Fermentas) and 10 pM each primer AAF: 5′- ATG TTC TTC GGG ACG ACC -3′ and AAR: 5′-GAC GGC TAA GTT TAT CAG TAT GTT -3′ and 1 ng DNA sample. The PCR amplifies a 100 bp ITS1-region of *A. astaci* DNA. Thermal cycling at 95 °C for 4 min, 35 × (95 °C for 30 s, 54 °C for 60 s, 72 °C for 90 s) and 72 °C for 7 min was performed using a PTC-200 instrument (MJ Research). Blank sample and pure DNA of noble crayfish were included into analyses as negative controls, and *A. astaci* DNA (isolate UEF8866-2) as positive control, and also *A. invadans* (SAP307) and *A. frigidophilus* (SAP472) DNA (Diéguez-Uribeondo et al., 2009) were used as controls (Fig. 2). Amplified DNA was detected by electrophoresis of a PCR aliquot in a 1.5% gel agarose gel containing 0.5 μg mL⁻¹ ethidium bromide. Due to unique nature of our findings, all sampled populations with ITS-PCR positive detections were confirmed using a validated TaqMan® MGB PCR performed as described previously using standard dilutions and threshold values (Vrålstad et al., 2009). Agent level scoring for the detection levels obtained in this study were done according to Vrålstad et al. (2009): A0 are negative subsamples, A1 = 5 PFU (PCR forming units), 5 PFU ≥ A2 ≥ 50 PFU, 50 PFU ≥ A3 ≥ 10⁴ and 10⁴ ≥ A4 ≥ 10⁵ PFU. An A2 agent level was considered as a reliable but low-level *A. astaci* DNA detection. The 95% confidence interval for *A. astaci* DNA detection prevalence was estimated using binomial probabilities (AV Bio-Statistics 4.9).
3. Results

3.1. Crayfish cage trials

During the short-term cage trial, one crayfish with no apparent indications of disease died. However, all surviving crayfish died subsequently over a 2–6 month period after being transferred to laboratory tanks. During the long-term cage trial, male mortality until late March was 40% in Cage #1 and 80% in Cage #2 and female crayfish mortality was 47% in both cages over the 8 month period. When examined periodically, surviving crayfish generally appeared healthy and virile, except for the discovery of melanised spots in the tail membrane of two females.

3.2. PCR analyses

In the ITS-PCR, tissues from all crayfish sampled from the short-term cage trial were _A. astaci_ DNA-positive (Table 1); an example of positive PCR results in the gel shown in Fig. 2. In addition, 40% of crayfish sampled from Lake Mikitänjärvi and 3% of crayfish sampled from Lake Hietajärvi in 2010 were _A. astaci_ DNA-positive. In short-term cage samples, 10 out of 22 telson samples were ITS-PCR positive and 17 out 22 abdominal cuticle samples were ITS-PCR positive, resulting in 100% detection level. Lake Mikitänjärvi year 2010 and lake Hietajärvi ITS-PCR positive results were obtained from telson samples (Table 1). With Lake Mikitänjärvi crayfish, TaqMan realtime PCR analyses detected _A. astaci_ DNA in 10% of the samples collected in 2009 and all samples collected in 2010. Disease agent levels amongst crayfish caged short-term were scored as A3–A4 except for two scored as A2 and disease agent levels amongst Lake Mikitänjärvi crayfish were scored as A2 for 2009 samples and A2–A3 for 2010 samples (Table 2).

4. Discussion

Our observation, PCR detection of _A. astaci_ DNA in tissues, uropods and leg joints, of overtly healthy wild noble crayfish, indicates that these crayfish can be chronically infected by subclinical _A. astaci_. Even when _A. astaci_ DNA was detected at relatively high prevalence in our sample, no increased mortality was evident in the wild population, nor amongst our experimental crayfish housed under supposedly stressful cage conditions.

Interestingly, so far _A. astaci_ DNA has not been detected in crayfish populations downstream of the Lake Mikitänjärvi population in the watershed system in which it was detected by PCR. The low level infection detected could be the reason for Lake Mikitänjärvi noble crayfish showing no gross symptoms of infection. These crayfish could act as latent carriers, since _A. astaci_ was clearly detected by TaqMan® MGB PCR, similarly to what has been suggested for signal crayfish ( _Pacifastacus leniusculus_ ) (Tuffs and Oidtmann, 2011).

### Table 1

<table>
<thead>
<tr>
<th>Population</th>
<th>Year</th>
<th>Number tested</th>
<th>Number of ITS-PCR positive (%)</th>
<th>95% confidence level range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Mikitänjärvi</td>
<td>2009</td>
<td>10</td>
<td>0 (0)</td>
<td>0 – 30.9%</td>
</tr>
<tr>
<td>Lake Luvanjärvi</td>
<td>2010</td>
<td>10</td>
<td>4 (40)</td>
<td>12.3 – 73.8%</td>
</tr>
<tr>
<td>Lake Hyrynjärvi</td>
<td>2009</td>
<td>10</td>
<td>0 (0)</td>
<td>0 – 30.5%</td>
</tr>
<tr>
<td>Lake Ristijärvi</td>
<td>2010</td>
<td>10</td>
<td>0 (0)</td>
<td>0 – 30.9%</td>
</tr>
<tr>
<td>Lake Hietajärvi</td>
<td>2010</td>
<td>10</td>
<td>0 (0)</td>
<td>0 – 30.9%</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Population</th>
<th>Year</th>
<th>Number tested</th>
<th>Agent level</th>
<th>Number of qPCR positive (%)</th>
<th>95% confidence level range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lake Mikitänjärvi</td>
<td>2009</td>
<td>10</td>
<td>A0 A1 A2 A3 A4</td>
<td>1 (10)</td>
<td>0.3 – 44.5%</td>
</tr>
<tr>
<td>Lake Luvanjärvi</td>
<td>2010</td>
<td>10</td>
<td>5 0 5 0 0</td>
<td>10 (100)</td>
<td>69.2 – 100%</td>
</tr>
<tr>
<td>short term caging</td>
<td>2009</td>
<td>39</td>
<td>5 0 5 0 0</td>
<td>10 (100)</td>
<td>69.2 – 100%</td>
</tr>
</tbody>
</table>

1 crayfish originating from Lake Mikitänjärvi; 2 tissue samples tested from both uropod and leg joint; 3 ten uropod samples and five leg joint samples. Tissue samples were taken from uropod, except when indicated otherwise. Agent level rating is according to Vrålstad et al. (2009) and indicated as number of crayfish in each category. Number of the sampled crayfish is in brackets after the detection prevalence result. The 95% confidence interval at 95% confidence level.
Conflicting PCR results on crayfish housed in cages short-term and long-term encouraged analysis of wild crayfish populations. We identified A. astaci DNA in the noble crayfish population in Lake Mikkilänjärvi rather than the alternative explanation that the caged crayfish originating from Lake Mikkilänjärvi became infected subsequently to being placed in Lake Hyrylnjärvi. The difference in A. astaci detection prevalence in short-term and long-term caged crayfish might have resulted from crayfish representing different subsamples trapped at differing locations and times (early August and September) in Lake Mikkilänjärvi. The detection of apparently subclinical A. astaci infections might explain some speculated chronic crayfish plague cases (Kirjavainen and Sipponen, 2004). In these cases, either unknown intermediate hosts or sparse crayfish populations have been proposed to maintain the oomycete, which can emerge to cause acute infections when crayfish densities increase (Fürst, 1995). In this scenario, a few crayfish could carry a subclinical infection that progresses to an acute state under more stressful conditions, from which disease can eventuate and be transmitted. In our laboratory trials we have discovered that noble crayfish could survive even intensive crayfish plague infection of both PsI strain (Jussila et al., 2011) and As strain infections (manuscript in preparation). Alternatively, A. astaci could exist as more and less virulent forms, with the latter enhancing its survival in crayfish.

The commercial crayfish trapping continues in Lake Mikkilänjärvi, with several fishermen involved. The catch during the year 2011 season has been high according to the local fishermen, with estimated catch per unit effort for the first half of the season indicating dense population. Thus, the Lake Mikkilänjärvi noble crayfish population still appears vital, even though a small proportion of caught crayfish showing minor melanised spots or symptoms indicating burn spot disease (Makkonen et al., 2010) during our recent field study.

As the analyses reported here were planned to accommodate the objectives of a crayfish management project, with some exceptions, sample numbers were generally limited to ten crayfish. It is possible, therefore, that populations in which A. astaci DNA was not detected by PCR could contain a low prevalence of infection. At 95% confidence, a sample size of ten could have missed infection in up to a third of the population, and sampling of 500 crayfish would have been required to detect the oomycete at a 99% probability level (Harte, 2002). With the PCR detection methods used, processing of such large sample sizes to declare populations free of A. astaci may be prohibitive. However, this is not relevant among the population in Lake Mikkilänjärvi where A. astaci was detected. In general our discovery emphasizes the value of the pre-testing overly healthy noble crayfish used for restocking crayfish fisheries.

With A. astaci being a blacklisted species in the current draft EU Alien Species Strategy (MMM.fi, 2011), it is important to eliminate opportunities for the accidental spread of crayfish plague. Further studies are warranted to examine the virulence qualities and spatial heterogeneity of A. astaci.

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