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# *Fusarium avenaceum* causes burn spot disease syndrome in noble crayfish (*Astacus astacus*)

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ABSTRACT

Burn spot disease has been causing epidemics both in the Estonian mainland and in Saaremaa Island in the threatened noble crayfish (*Astacus astacus*) stocks. To study the cause of the disease, we isolated several *Fusarium* spp. from Estonian noble crayfish (*A. astacus*) populations suffering from burn spot disease syndrome. We first identified fungi directly from melanised cuticle by their ITS sequences. Then we isolated *Fusarium* spp. from melanised spots of crayfish showing burn spot disease symptoms, such as melanisation and shell erosion, from two different crayfish populations and watercourses in Estonia. The isolates were then identified based on ITS and EF1α-gene sequences. Isolates of *Fusarium* spp. taken from two separate Estonian noble crayfish populations were used in infection studies. Koch postulates confirmed that the studied agent was causing burn spot disease symptoms including shell erosion in the noble crayfish, which were significantly more severe after molts. After the infection period, an identical *Fusarium* spp. was re-isolated from carapace lesions and was thus shown to be the disease agent causing burn spot disease syndrome and shell erosion in noble crayfish. Based on GenBank database searches, the isolates causing burn spot disease symptoms were identified as *Fusarium avenaceum* in mainland Estonia and *F. solani* in Saaremaa crayfish.



## 1. Introduction

Burn spot disease, which is often accompanied by shell erosion, has been reported from many freshwater and marine crustaceans (Bian and Egusa, 1981; Evans and Edgerton, 2002). Diverse symptoms of the syndrome include melanisation, lesions with coloration and erosion of the exoskeleton. The syndrome is less common in normal crayfish, but in a dense population or aquaculture facilities it has been reported to infect up to 50% of the crayfish (Evans and Edgerton, 2002). The Saaremaa Island in Estonia has been one of the last safe havens of the noble crayfish (*Astacus astacus*) due to absence of alien species and rather pristine environment (Paaver and Hurt, 2009). On the Estonian mainland diseases like crayfish plague (*Aphanomyces astaci*) and burn spot disease are quite common, burn spot disease was officially reported in Saaremaa in the 1990s and crayfish plague as recently as 2006 (Makkonen et al., 2010; Paaver and Hurt, 2009). The estimated burn spot disease infection rate was up to 70–80% in historic samplings in the Estonian mainland (Järvekülg, 1958). These epidemics have partially eliminated viable noble crayfish populations and hindered crayfish trade due to substandard quality of market size crayfish.

*Fusarium* spp. has been reported to cause melanisation on the shell and gills in crustaceans such as freshwater crayfish (Alderman and Polglase, 1985, 1988; Chinain and Vey, 1988; Vey, 1988; Edgerton, 2002; Quaglio et al., 2006; Dörr et al., 2012), lobster (Lightner and Fontaine, 1975) and prawn and shrimp (Solangi and Lightner, 1976; Bian and Egusa, 1981; Hose et al., 1984; Colorni, 1989; Souheil et al., 1999; Khoa et al., 2004). The effects of *Fusarium* spp. infections reported for marine species are most dramatic under aquaculture conditions, where whole crops can be lost during the epidemics. Epidemics in wild stocks have been rarely studied but observations from aquaculture indicate that the infections caused *Fusarium* spp. could have significant effects among wild populations. Furthermore, crayfish plague has caused similar gross symptoms in wild and laboratory conditions in the noble crayfish.

*Fusarium* spp. has also been described as a saprophyte or a superficial contaminant on white-clawed crayfish (*Austropotamobius pallipes*) (Quaglio et al., 2006) with mostly no apparent harmful effect on this host. Epidemics could be more severe if several fungi or other disease agents act concurrently, as has been suggested by Dörr et al. (2012). Due to the current lack of knowledge, *Fusarium* spp. among other potential pathogenic fungi has been suggested to be a risk for the European freshwater crayfish (Edgerton, 2002).

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The morphospecies of *F. solani* comprises at least 60 phylogenetically distinct species (O'Donnell et al., 2008, 2012) members of the *F. solani* species complex (FSSC), typically reported as *F. solani*. They have been reported to cause disease in shrimp (Lightner and Fontaine, 1975), spiny lobster (McAleer and Baxter, 1983) and clawed lobster (Alderman, 1981) and also infection in sea turtle (*Caretta caretta*) with a resulting failure in hatching nests and suboptimal juveniles (Sarmiento-Ramirez et al., 2010). Furthermore, over hundred plant species from almost 90 genera are host of the FSSC (Kolattukudy and Gamble, 1995) and it has impacts on animals (D'Mello et al., 1999). Members of the FSSC have been reported to cause infection also in humans with immunosuppression (Venditti et al., 1988; Ammari et al., 1993; O'Donnell et al., 2008) with a high mortality rate.

We investigated potential pathogens that could cause the burn spot disease syndrome in Estonian noble crayfish. The aim was to isolate and identify the disease agent and use Koch postulate to test whether similar symptoms would be produced in Finnish noble crayfish stocks using the organism we isolated in controlled infection trials.

## 2. Materials and methods

### 2.1. Sampling of diseased noble crayfish population from Saaremaa

Noble crayfish were collected during two periods: first in 2008 from the Saaremaa Estonia Kuke River (coords. 58.472426, 23.008604), Kurdla River (coords. 58.447553, 23.000536), Laugi River (coords. 58.342749, 22.663565) and Angla crayfish farm of OÜ Vähilakk (coords. 58.505802, 22.748601), and secondly in a set of diseased noble crayfish that were collected in the autumn 2010 from the Mustoja River (coords. 59.570549, 26.178088) and Kamariku old gravel quarry (coords. 59.02386, 26.262385), both in mainland Northern Estonia. Crayfish were stored live in specific holding tanks in the Department of Agriculture, Tartu University of Life Sciences (Estonia) before they were transferred to the University of Eastern Finland.

### 2.2. Identification of Fusarium spp. from melanised carapace lesions by ITS sequencing

Melanised carapace samples were isolated from noble crayfish originating from the Kuke, Kurdla and Laugi Rivers and the Angla crayfish farm of OÜ Vähilakk. Samples for DNA isolation were dissected from distinct melanised lesions or spots (Fig. 1) where the disease agent appeared to be present.

Tissue samples were stored at −20 °C in tubes containing absolute ethanol. For DNA extraction, samples were first homogenized by vigorous shaking for 90 s at a speed of 4 m $s^{-1}$ in FastPrep FP120 (MP Biomedicals, Solon, OH, USA) with 350 µl lysis buffer from the DNA extraction kit, ceramic beads and sea sand (Merck). Then 25 µl of Proteinase K (Omega Bio-tek, Norcross, CA, USA) was added and samples were lysed for 3 h at +60 °C. DNA was extracted using the E.Z.N.A Insect DNA isolation kit (Omega Bio-tek, Norcross, CA, USA) according to the manufacturer's instructions. The concentration and purity of the DNA was measured with NanoDrop (Thermo Scientific, Wilmington, DE, USA) and diluted for PCR to 10 µg $ml^{-1}$ concentration.

PCR was run to amplify ITS rDNA present in the melanised lesion. PCR reactions were performed with universal primers ITS1 and ITS4 (White et al., 1991). The reaction volume of 25 µl contained 1 U of Hot Start *Taq* DNA polymerase (Fermentas GmbH, Germany), 1× volume of Hot Start *Taq* PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 10 pM of primers (ITS1 and ITS4) and 10 ng of DNA template. Samples were then amplified in a PTC-200 PCR cycler (MJ Research, USA) using a program containing 4 min denaturation step at 95 °C, 35 cycles with 30 s at 95 °C, 45 s at 54 °C, 90 s at 72 °C and the final elongation step for 7 min at 72 °C.

PCR products were checked in an agarose gel (1.5% agarose, 5 µg $ml^{-1}$ EtBr). When several bands were obtained, PCR products were isolated from the gel with the QiaQuick Gel Extraction kit (Qiagen, Valencia, CA, USA). Samples with a single visible band were purified with the QiaQuick Spin PCR Purification Kit (Qiagen, Valencia, CA, USA) or ExoSAP-IT® (USB, Cleveland, OH, USA) using the manufacturer's protocol. Purified samples were sequenced using the MegaBACE 750 (GE Healthcare, England) in A.I.V. Institute (Kuopio, Finland) sequencing service. Resulting sequence chromatograms were analyzed with Geneious 5.4 (Drummond et al., 2011) and combined as consensus sequences. ITS sequences obtained were identified with Blast-searches in three different databases: NCBI GenBank Megablast program against nr-database (http://blast.ncbi.nlm.nih.gov/Blast), FUSARIUM-ID (http://isolate.fusariumdb.org/index.php) and *Fusarium MLST* (http://www.cbs.knaw.nl/fusarium/). Sequences were submitted to NCBI GenBank with accession numbers JX402195–JX402202.

### 2.3. Isolation of Fusarium spp. from live symptomatic noble crayfish

Noble crayfish were trapped in the autumn of 2010 from the Mustoja River and the Kamariku old gravel quarry both located in mainland Northern Estonia. Putatively diseased crayfish were stored live in specific holding tanks in the Department of Agriculture, Tartu University of Life Sciences (Estonia), before they were transferred to the University of Eastern Finland for preparation of crayfish and isolation of microbes from diseased tissues.

Colored and melanised cuticle samples (i.e. reddish soft tissue around melanised spots) were dissected, surface sterilized with 70% ethanol for 1 min and washed in sterile Milli-Q water. Tissue was divided into three parts, approximately 4 $mm^2$ each, and placed on three different agars; (1) Mycological agar (Difco, NJ, USA) and (2) PG1-agar (Unestam, 1965) with 10 mg $l^{-1}$ of both ampicillin and oxolinic acid (Alderman and Polglase, 1986), (3) PDA-agar (Difco, NJ, USA) with 1 g $l^{-1}$ of streptomycin and ampicillin. Plates were incubated in a 20 °C incubator for two weeks during which fungal growth was transferred into new plates daily to obtain pure cultures (Tables 1 and 2).

### 2.4. Identification of isolates with ITS rDNA and EF1α-sequences

For DNA extractions, 50 mg of aerial hyphae was cut from PDA-agar. DNA extractions from pure cultures were made with the E.Z.N.A Insect DNA isolation kit (Omega Bio-tek, Norcross, CA, USA), as described earlier. Two different genes were amplified via PCR, ITS rDNA (White et al., 1991) and EF1α according to Geiser et al. (2004). PCR-products were purified with QiaQuick Spin PCR-purification Kit (Qiagen, Valencia, CA, USA) and sequenced at the A.I.V. Institute Sequencing Service in University of Eastern Finland. Resulting sequence chromatograms were analyzed with Geneious 5.4 (Drummond et al., 2011) and species were identified with Blast-searches as described earlier. Sequences were submitted to NCBI GenBank as accessing numbers JX402176–JX402182.

### 2.5. Infection experiments

#### 2.5.1. Production of Fusarium spp. microconidia

Aerial hyphae and conidias were transferred onto two growth media, Czapeck-Dox with 10% yeast extract (Chinain and Vey, 1988) and Oatmeal Broth were tested of production conidia. Fungi were grown for 14 days at 20 ± 2 °C temperature with mild continuous shaking. Conidia densities were estimated with a Bürker