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# The crayfish plague pathogen can infect freshwater-inhabiting crabs

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## SUMMARY

1. The oomycete *Aphanomyces astaci* is generally considered a parasite specific to freshwater crayfish, and it has become known as the crayfish plague pathogen. Old experimental work that reported transmission of crayfish plague to the Chinese mitten crab *Eriocheir sinensis*, and the ability of *A. astaci* to grow in non-decapod crustaceans, has never been tested properly.

2. We re-evaluated the host range of *A. astaci* by screening for the presence of *A. astaci* in two crab species cohabiting with infected crayfish in fresh waters, as well as in other higher crustaceans from such localities. The animals were tested with species-specific quantitative PCR, and the pathogen determination was confirmed by sequencing of an amplified fragment of the nuclear internal transcribed spacer. Furthermore, we examined microscopically cuticle samples from presumably infected crab individuals for the presence of *A. astaci*-like hyphae and checked for the presence of pathogen DNA in such samples.

3. Screenings of benthopelagic mysids, amphipods and benthic isopods did not suggest infection by *A. astaci* in non-decapod crustaceans. In contrast, both studied lake populations of crabs (a native semiterrestrial species *Potamon potamios* in Turkey, and an invasive catadromous *E. sinensis* in Sweden) were infected with this parasite according to both molecular and microscopic evidence.

4. Analyses of polymorphic microsatellite loci demonstrated that *A. astaci* strains in the crabs and in cohabiting crayfish belonged to the same genotype group, suggesting crayfish as the source for crab infection.

5. The potential for *A. astaci* transmission in the opposite direction, from crabs to crayfish, and potential impact of this pathogen on populations of freshwater crabs require further investigations, because of possible consequences for crayfish and freshwater crab conservation and aquaculture.

*Keywords*: *Aphanomyces astaci*, *Eriocheir sinensis*, host range, invasive species, *Potamon potamios*

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## Introduction

The oomycete *Aphanomyces astaci* (Oomycetes, Saprolegniales) has caused and still causes heavy losses of indigenous European freshwater crayfish populations (Alderman, 1996; Holdich *et al*., 2009). Due to its devastating impacts, it has been included among 100 of the worst invasive alien species in Europe and the whole world (Lowe *et al*., 2004; DAISIE, 2009). *A. astaci* has become one of the best-known pathogens of invertebrates (Alderman, 1996; Diéguez-Uribeondo *et al*., 2006), and it is usually considered as a parasite specific to freshwater crayfish (Decapoda, Astacidea) (e.g. Alderman, 1996; Söderhäll & Cerenius, 1999; Diéguez-Uribeondo *et al*., 2006).

A few studies have tried to evaluate the host range of this pathogen outside the group of freshwater crayfish. The growth of *A. astaci* on fish scales reported by Häll & Unestam (1980) was not confirmed by experiments *in vivo* (Oidtmann *et al*., 2002), and several planktonic crustaceans and one rotifer did not die after they had been exposed to *A. astaci* (Unestam, 1969b, 1972). One study, however, stands out among those evaluating the potential of crustaceans other than crayfish to be hosts of *A. astaci*. Benisch (1940) reported experimental transmission of the presumed crayfish plague pathogen from moribund individuals of the European noble crayfish *Astacus astacus* to the Chinese mitten crab *Eriocheir sinensis*. The experiments resulted in moderate death rates for the crabs. However, while some pathogen had indeed been transmitted to *E. sinensis*, it remains uncertain whether it was *A. astaci*, since the pathogen was not isolated in culture for direct tests of pathogenicity and species identification (see Cerenius *et al*., 1988; Oidtmann *et al*., 1999; Oidtmann, 2012). Considering Benisch's experiment with crabs, Unestam (1972) in his work on *A. astaci* specificity suggested that the parasite host range may include not only crayfish but freshwater decapods in general (i.e. higher crustaceans including crabs, crayfish and shrimps).

Surprisingly, no work evaluating the ability of *A. astaci* to parasitise decapods other than crayfish has been published since Benisch (1940), although the potential of *A. astaci* to infect other freshwater decapods would have important consequences for management of susceptible crayfish populations, especially in Europe and adjacent regions. Moreover, freshwater crabs and shrimps play important ecological roles in aquatic habitats (De Grave, Cai & Anker, 2008; Yeo *et al*., 2008), and they are important in the global aquaculture industry. The 2010 annual harvest of freshwater shrimps (Decapoda, Caridea) and Chinese mitten crabs was about 500 000 tons each, with a total value of over 6.4 billion USD (FAO, 2012). Reductions in yield or changes in population characteristics of freshwater decapods may thus impact ecosystem functioning as well as aquaculture and fisheries.

Apart from crayfish and possibly freshwater-inhabiting crabs, there has been no reliable evidence for other hosts of *A. astaci* (Unestam, 1969b, 1972; Oidtmann *et al*., 2002). Occasional reports of the occurrence of *A. astaci* in dead freshwater crustaceans (e.g. Czeczuga, Kozlowska & Godlewska, 2002; Czeczuga, Kiziewicz & Gruszka, 2004) were based on morphology only, and they seem unreliable since *A. astaci* morphological features are not specific enough (see Cerenius *et al*., 1988; Oidtmann *et al*., 1999; Oidtmann, 2012). For such screening, molecular detection, particularly species-specific quantitative PCR (qPCR), is more appropriate due to its high specificity and sensitivity (see Vrålstad *et al*., 2009; Tuffs & Oidtmann, 2011; Oidtmann, 2012).

We tested the hypothesis that freshwater crabs can serve as alternative hosts of the crayfish plague pathogen when cohabiting with infected crayfish. We used recently developed molecular methods allowing species-specific detection of *A. astaci* in host tissues (Oidtmann *et al*., 2006; Vrålstad *et al*., 2009) to analyse individuals representing two genera of crabs that may come into contact with *A. astaci*-infected crayfish in natural habitats. In the Western Palaearctic, such taxa include (i) the invasive catadromous Chinese mitten crab *E. sinensis* (Varunidae), one of the 100 worst invasive species in the world (Lowe *et al*., 2004), and (ii) several strictly freshwater to semiterrestrial species of a native crab genus *Potamon* (Potamidae), which are found in southern Europe and the Middle East (Brandis, Storch & Türkay, 2000). We obtained and screened samples of both crab genera from populations known to be in contact with *A. astaci*-infected crayfish: *E. sinensis* from a Swedish lake inhabited by North American signal crayfish *Pacifastacus leniusculus*, a natural vector of *A. astaci*, and *Potamon potamios* from a Turkish lake inhabited by infected narrow-clawed crayfish *Astacus leptodactylus*, a native Western Palaearctic species relatively susceptible to crayfish plague. In addition, we analysed samples of three benthic or benthopelagic crustacean species, representing other orders of higher crustaceans frequently found in fresh waters (Amphipoda, Isopoda, and Mysida), coexisting with infected North American crayfish.

## Methods

### *Crustacean samples*

Altogether seven crustacean species were tested in this study (Table 1). A total of 30 individuals of *P. potamios*

**Table 1** General overview of *A. astaci* detection in tested crustaceans. Results of *A. astaci*-specific qPCR in tested tissues of crabs (*Eriocheir sinensis*, *Potamon potamios*), coexisting crayfish (*Astacus leptodactylus*, *Pacifastacus leniusculus*), benthopelagic crustaceans *Mysis relicta* and *Pallasea quadrispinosa* coexisting with *A. astaci*-positive *P. leniusculus*, and benthic isopod *Asellus aquaticus* coexisting with *A. astaci*-positive *Orconectes limosus*. Countries are abbreviated as follows: CZ: Czech Republic, NO: Norway, SE: Sweden, TR: Turkey

| Locality (country code) | Vänern (SE) | | Eğirdir (TR) | | Øymarksjøen (NO) | | Smečno (CZ) |
|---|---|---|---|---|---|---|---|
| Species | *Eriocheir sinensis* | *Pacifastacus leniusculus* | *Potamon potamios* | *Astacus leptodactylus* | *Mysis relicta* | *Pallasea quadrispinosa* | *Asellus aquaticus* |
| No. individuals tested | 6 | 20 | 30 | 30 | 10 | 10 | 8 |
| No. individuals positive | 6 | 12 | 13 | 2 | 0 | 1‡ | 0 |
| Prevalence | 100% | 60% | 43% | 7% | 0% | 10% | 0% |
| 95% confidence interval | 42–100% | 36–81% | 25–63% | 1–22% | 0–41% | 0–45% | 0–48% |
| Agent levels* | | | | | | | |
| Negative (A0) | – | 8 | 17 | 28 | 10 | 9 | 8 |
| Very low (A2) | – | 7 | 1 | – | – | 1‡ | – |
| Low (A3) | 1 | 3 | 2 | 1 | – | – | – |
| Moderate (A4) | 2 | 1 | 7 | – | – | – | – |
| High (A5) | 1 | – | 2 | 1 | – | – | – |
| Very high (A6) | 2 | 1 | 1 | – | – | – | – |

*Results of *A. astaci* detection using *A. astaci*-specific qPCR according to Vrålstad *et al.* (2009) are given as semiquantitative categories. The scale is logarithmic; thus, each category usually represents one order of magnitude higher level of pathogen DNA than the previous one (for details, see Vrålstad *et al.*, 2009). For those individuals of which more than one sample of tissues was tested (*E. sinensis*, *P. potamios*), only the highest value found in any analysed tissue is listed (results of all tested samples are in the Tables S2 and S3).
‡This result is not considered as the evidence for the host infection, because it may have been caused by occasionally attached *A. astaci* spores (see Discussion).

crabs were caught in Lake Eğirdir (Turkey; 37.9°N, 30.9°E) where they coexist with the native population of the narrow-clawed crayfish (*Astacus leptodactylus*) recently shown to be infected by the crayfish plague pathogen (Svoboda *et al.*, 2012). The thirty *Potamon* individuals (14 males and 16 females; mean carapace length ± SD: 39 ± 5 mm) were captured in May 2010 and kept for 10 days in a common tank before being killed and dissected. Selected body parts from each individual were preserved in 96% ethanol. Thirty individuals of *A. leptodactylus* from Lake Eğirdir captured in November 2009 were already analysed for *A. astaci* presence in a previous study (Svoboda *et al.*, 2012).

Six individuals of the Chinese mitten crab (*Eriocheir sinensis*) were captured from the south-eastern part of Lake Vänern (Sweden; 58.8°N, 13.3°E) that is colonised by the invasive signal crayfish (*Pacifastacus leniusculus*), a natural host of *A. astaci* (Unestam, 1969b, 1972). The crabs (five males and one female; mean carapace length ± SD: 63 ± 4 mm) were captured in August 2009 for a behavioural experiment that lasted for 24 h, and then frozen at −20 °C. Samples of 20 *P. leniusculus* from this lake were captured in September 2011 and stored in 96% ethanol prior to testing for *A. astaci* infection.

Two benthopelagic crustacean species, *Mysis relicta* and *Pallasea quadrispinosa*, representing two orders (Mysida and Amphipoda) of higher crustaceans (Malacostraca), were collected in Lake Øymarksjøen (Norway; 59.33°N, 11.65°E) where they coexist with confirmed *A. astaci*-positive *P. leniusculus* (Vrålstad *et al.*, 2011). Ten individuals of each species were captured at 10 m depth in September 2012. Eight individuals of the benthic isopod *Asellus aquaticus* (Isopoda, Malacostraca) coexisting in a pond in Smečno (Czech Republic, 50.188°N, 14.047°E) with strongly infected *A. astaci*-positive *Orconectes limosus* (Kozubíková *et al.*, 2011b; Matasová *et al.*, 2011) were captured in May 2013. These crustacean samples were stored in 96% ethanol prior to further analyses.

### *Sample processing and DNA extraction*

Sample processing and DNA isolation differ slightly because samples from the involved localities were processed independently in two laboratories. *Eriocheir sinensis*, *P. leniusculus*, *M. relicta* and *P. quadrispinosa* were analysed at the Norwegian Veterinary Institute (NVI) in Oslo, and *P. potamios*, *A. leptodactylus* and *A. aquaticus* at the Charles University in Prague.

Tissues of *P. potamios* individuals were processed in two stages. At first, soft abdominal cuticle, soft cuticle from two joints, the second gonopods from every male, three endopods of pleopods from every female and any melanised pieces of cuticle (found in 24 of 30

individuals) were sampled. These tissues were pooled and ground in liquid nitrogen. A separate sterile mortar was used for tissues of each individual. DNA from up to 40 mg of ground tissues was extracted with the DNeasy tissue kit (Qiagen, Venlo, the Netherlands) by following the manufacturer's instructions to obtain one DNA isolate for each specimen. Additional tissue samples (telson, two joints of walking legs and either a gonopod in males or two endopods of pleopods in females) were processed separately for those *P. potamios* individuals that tested positive for *A. astaci* presence in the pooled DNA isolate. Individuals of *A. aquaticus* were analysed whole, using the same DNA extraction method as described above. For *A. leptodactylus*, pooled DNA isolates had previously been prepared (Svoboda *et al.*, 2012) from one uropod, soft abdominal cuticle, one eye stalk, one walking leg joint and prominent melanised cuticle regions of each crayfish. An environmental control and DNA extraction control to account for potential contamination were prepared during each isolation batch.

From each of the six *E. sinensis*, seven to ten pieces of tissue were dissected: the telson, the soft abdominal cuticle, soft cuticle from two leg joints, setae from the claw, two of the maxillipeds and up to three pieces of melanised tissues (which were observed in all six sampled specimens). Each tissue sample was subsequently processed separately. For *P. leniusculus*, the telson and two uropods were dissected as one tissue sample from each of the 20 individuals. Melanised spots were sampled if present, which was the case for three crayfish individuals. *Mysis relicta* and *Pallasea quadrispinosa* individuals were analysed whole. For all samples processed at the NVI in Oslo, DNA was extracted following the CTAB protocol provided by Vrålstad *et al.* (2009). An environmental control and DNA extraction control was included as above.

### *Quantitative real-time PCR*

All samples were analysed with *A. astaci*-specific qPCR (Vrålstad *et al.*, 2009), with minor modifications to increase the assay specificity (Strand, 2013). These included increased annealing temperature (from 58 to 62 °C) and decreased synthesis time (from 60 to 30 s). The TaqMan Environmental Master Mix (Life Technologies, Carlsbad, CA, U.S.A.) was used to reduce the potential PCR inhibition (see Strand *et al.*, 2011). The qPCR was performed on an iQ5 (Bio-Rad, Hercules, CA, U.S.A.) system for *P. potamios*, *A. leptodactylus* and *A. aquaticus* samples and a Mx3005 QPCR (Stratagene, La Jolla, CA, U.S.A.) system for *E. sinensis*, *P. leniusculus*, *P. quadrispinosa* and *M. relicta* samples. Undiluted and 10× diluted DNA isolates were used as templates for each sample, and an environmental control, DNA extraction control and a PCR blank control were included in each run. Four *A. astaci* calibrants were prepared and used to generate a standard curve to estimate the number of PCR-forming units (PFU), and then designate the semiquantitative agent level (A0-A7) for each analysed sample (for details, see Vrålstad *et al.*, 2009; Kozubíková *et al.*, 2011b). In the absence of inhibition, a mean PFU value per sample was estimated from both the undiluted and diluted DNA sample, while in the case of inhibition, only the diluted sample value was used (Kozubíková *et al.*, 2011b). We roughly estimated the number of *A. astaci* genomic units in the isolates from the PFU values, using conversion factors of PFU per spore previously obtained in each laboratory (for details, see Strand *et al.*, 2011; Svoboda *et al.*, 2013).

Considering the number of analysed specimens and the number of positive *A. astaci* detections, we estimated the prevalence of *A. astaci* in the studied populations. We then calculated its 95% confidence interval as in Filipová *et al.* (2013), using the function 'epi.conf' included in the library epiR (Stevenson *et al.*, 2013) for the statistical package R, v. 3.0 (R Core Team, 2013).

### *Sequencing*

The presence of *A. astaci* DNA in representative crab samples that yielded positive qPCR results was confirmed by sequencing of a 569-bp-long amplicons including parts of internal transcribed spacers (ITS) 1 and 2 and 5.8S rDNA according to Oidtmann *et al.* (2006) and as recommended by the World Organisation for Animal Health (Oidtmann, 2012). Purified PCR products of one *E. sinensis* and three *P. potamios* DNA isolates were sequenced in both directions on the ABi 3130 Genetic Analyser (Life Technologies). The resulting sequences representing the pathogen from both host species (GenBank accession numbers KF748131 and KF748132) were compared with publicly available sequences of *A. astaci*.

### *Microsatellite analyses*

We used a recently developed set of microsatellite markers (F. Grandjean, T. Vrålstad, J. Diéguez-Uribeondo, M. Jelić, J. Mangombi, C. Delaunay, L. Filipová, S. Rezinciuc, E. Kozubíková-Balcarová, D. Guyonnet, S. Viljamaa-Dirks, A. Petrusek, unpublished data) that distinguishes the five known genotype groups of *A. astaci* (A–E; Huang, Cerenius & Söderhäll,

1994; Diéguez-Uribeondo *et al.*, 1995; Kozubíková *et al.*, 2011a) and can be applied directly on mixed genome samples, that is, DNA isolates obtained from tissues of infected hosts. Nine microsatellite loci (Table 2, primer sequences are provided in Table S1) were selected out of a larger panel of candidate loci identified from 454 pyrosequencing of a library enriched with repetitive sequences. These loci showed variation among at least some of the reference strains representing *A. astaci* genotype groups (Table 2) and at the same time showed little cross-amplification with *Aphanomyces* species related to *A. astaci* and other oomycete taxa isolated from crayfish (F. Grandjean, T. Vrålstad, J. Diéguez-Uribeondo, M. Jelić, J. Mangombi, C. Delaunay, L. Filipová, S. Rezinciuc, E. Kozubíková-Balcarová, D. Guyonnet, S. Viljamaa-Dirks, A. Petrusek, unpublished data). Based on the protocol developed by the above team, we analysed the variation of these polymorphic loci to genotype the pathogen in *A. astaci*-positive isolates showing high agent level (A5–A6) from *E. sinensis* (three individuals), *P. potamios* (3), *A. leptodactylus* (1) and *P. leniusculus* (2) from the studied lakes. The resulting allele sizes were compared with those observed in axenic cultures of reference *A. astaci* strains (Table 2).

### *Microscopic examinations*

To support results obtained by the molecular detection methods described above, we searched for hyphae corresponding morphologically to *A. astaci* in tissues of *A. astaci*-positive *P. potamios* and *E. sinensis* specimens. For this purpose, we dissected small pieces of soft cuticle from abdomen and joints from each of the six *E. sinensis*, and one piece of soft abdominal cuticle from every *P. potamios* whose pooled sample of selected tissues tested positive in qPCR. The pieces of cuticle were cut with sterilised tools, cleaned of attached muscles and connective tissues with a scalpel, and immersed in distilled water. At 100× and 400× magnification, we searched for hyphae corresponding to features of *A. astaci* (for details, see Alderman & Polglase, 1986; Cerenius *et al.*, 1988; Oidtmann *et al.*, 1999). Such hyphae were documented by digital cameras attached to the microscopes. All examined pieces of *Eriocheir* cuticle and the pieces of *Potamon* cuticle in which *A. astaci*-like

**Table 2** Microsatellite analyses. The table compares allele sizes of nine microsatellite markers for reference strains of *A. astaci* genotype groups A–E and studied *A. astaci*-positive crabs and crayfish. The matching allele combinations between a reference strain and infected crabs and crayfish are highlighted by bold font

| *A. astaci* strain* | Host species | Origin and reference† | Fragment sizes at microsatellite loci | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Aast2 | Aast4 | Aast6 | Aast7 | Aast9 | Aast10 | Aast12 | Aast13 | Aast14 |
| VI03557 (group A) | *Astacus astacus* | Sweden (1962); H94 | 160 | 103 | 157 | 207 | 180 | 142 | – | 194 | 246 |
| VI03555 (group B) | *Pacifastacus leniusculus* | U.S.A. (1970); H94 | **142** | **87** | **148** | **215** | **164/182** | **132** | **226/240** | **202** | **248** |
| VI03558 (group C) | *Pacifastacus leniusculus* | Sweden (1978); H94 | 154 | 87 | 148 | 191 | 164/168 | 132 | 226 | 202 | 248 |
| VI03556 (group D) | *Procambarus clarkii* | Spain (1992); D95 | 138 | 131 | 148 | 203 | 180 | 142 | 234 | 194 | 250 |
| Evira4605 (group E) | *Orconectes limosus* | Czech Republic (2010); K11a | 150 | 87/89 | 148/157 | 207 | 168/182 | 132/142 | 234/240 | 194/202 | 248 |
| Crab and crayfish species (and no. of individuals) analysed | | | | | | | | | | | |
| | *Eriocheir sinensis* (3) | Sweden (2009) | **142** | **87** | **148** | **215** | **164/182** | **132** | **226/240** | **202** | **248** |
| | *Potamon potamios* (2) | Turkey (2010) | **142** | **87** | **148** | **215** | **164/182** | **132** | **226/240** | **202** | **248** |
| | *Pacifastacus leniusculus* (2) | Sweden (2011) | **142** | **87** | **148** | **215** | **164/182** | **132** | **226/240** | **202** | **248** |
| | *Astacus leptodactylus* (1) | Turkey (2009); S12 | **142** | **87** | **148** | **215** | **164/182** | **132** | **226/240** | **202** | **248** |

*VI numbers refer to assigned strain numbers in the culture collection of the Norwegian Veterinary Institute where the isolates are maintained. Evira numbers refer similarly to assigned strain numbers in the culture collection of the Finnish Food Safety Authority Evira (OIE reference laboratory for crayfish plague). Original codes for reference strains VI03557 (A), VI03555 (B), VI03558 (C) and VI03556 (D) are L1, P1, Kv and Pc, respectively (Huang *et al.*, 1994; Diéguez-Uribeondo *et al.*, 1995).

†References are abbreviated as follows: D95: Diéguez-Uribeondo *et al.* (1995), H94: Huang *et al.* (1994), K11a: Kozubíková *et al.* (2011a), S12: Svoboda *et al.* (2012).

hyphae were found were then tested for the presence of *A. astaci* DNA by qPCR as described above.

## Results

### *Molecular confirmation of A. astaci presence in crab tissues*

Tissues from all six examined individuals of *E. sinensis* and 13 of 30 individuals of *P. potamios* yielded qPCR results indicating *A. astaci* presence. Table 1 lists the highest agent level of *A. astaci* detected in any analysed tissue from each specimen together with results of *A. astaci* detection in cuticles of coexisting crayfish species. Positive DNA isolates from all species contained low to very high agent levels (A2–A6 according to Vrålstad *et al*., 2009). Levels A2 and A6 corresponded to approximately 1–10 and 20 000–200 000 genome units in the original sample, since *c*. 100 PFU corresponds to one genomic unit (Strand *et al*., 2011; Svoboda *et al*., 2013 and unpublished data). The ITS sequences acquired to confirm the qPCR results (one from *E. sinensis*, three from *P. potamios*) were identical to publicly available reference sequences of *A. astaci*. The negative controls included in qPCR analyses remained negative for all runs.

*Aphanomyces astaci* DNA was found in all body parts tested in both crab species, but its distribution was heterogeneous and did not match between the two crab hosts. Of the tissues tested separately, 75 % of *P. potamios* and 83 % of *E. sinensis* samples yielded positive *A. astaci* detection (see Tables S2 and S3 in Supporting Information). For *E. sinensis*, the highest concentrations of the pathogen DNA were quantified in the soft abdominal cuticle, walking leg joints and melanised tissues. In contrast, the lowest agent levels of *A. astaci* was found in joints of *P. potamios*, while the highest concentrations were quantified in the mixture of different tissues from this species (soft abdominal cuticle, joints, melanised spots and gonopods or pleopod endopods).

No trace of *A. astaci* DNA was detected in mysids *Mysis relicta* or isopods *Asellus aquaticus* (Table 1). Only one sample of an amphipod *Pallasea quadrispinosa* was weakly positive, just above the limit of detection (level A2). Due to the low levels of *A. astaci* DNA in this apparently positive sample, it was not possible to conduct sequencing or microsatellite analyses, so the result cannot be regarded as a reliable confirmation of an *A. astaci*-carrier status for this amphipod. However, due to the modest number of individuals analysed, the 95% confidence intervals of prevalence remain wide (up to 48 %; Table 1), and thus the negative results also cannot be considered conclusive at the whole-population level.

### *Microsatellite analysis*

The *A. astaci* genotype group B, corresponding to the genotype isolated from the signal crayfish *P. leniusculus* (Huang *et al*., 1994), was identified in all the tested tissue samples from the crabs *E. sinensis* and *P. potamios* and crayfish *A. leptodactylus* and *P. leniusculus*. The genotype found in all four species was strictly identical with the reference strain of *A. astaci* genotype B (Pl isolated from *P. leniusculus*; Table 2), without any allele variation at all nine microsatellite loci analysed (Table 2).

### *Microscopic examinations*

Microscopic screening of soft cuticles from presumably infected crab hosts resulted in observation of characteristic oomycete hyphae (Fig. 1) in two of 13 (*Potamon*) and in one of six (*Eriocheir*) examined crab individuals. The observed hyphae were aseptate, with rounded tips and a diameter of *c.* 4–13 μm (Fig. 1a–d). The tissue immediately adjacent to the hyphae was melanised in some areas of the cuticle from one *Potamon* individual (Fig. 1a, the outer edge of the melanised area is indicated by an arrow), while elsewhere in the same sample and in the cuticle of *Eriocheir*, melanisation was not observed (Fig. 1b–d). In some areas of the *Potamon* cuticle, the hyphae were frequently branching, forming a three-dimensional net (Fig. 1b). Despite their relatively small area (*c.* 3 × 3 and 5 × 5 mm), the two pieces of *Potamon* cuticle with observed hyphal growth contained high and moderate levels of *A. astaci* DNA (agent levels A5, A4) corresponding to *c.* 15 000 and 1000 genomic units, respectively. The DNA isolate obtained from the cuticle of *Eriocheir* with detected hyphae (Fig. 1c,d) also tested *A. astaci*-positive, with high level of the pathogen DNA (A5, i.e. *c.* 15 000 genomic units in the original sample). Most other pieces of *Eriocheir* cuticle (10 of 13) examined also tested positive for *A. astaci* DNA (agent levels from A2–A4, corresponding to 1–2000 genomic units), although we had not succeeded in observing any *A. astaci*-like hyphae in them.

## Discussion

Our study demonstrates that *A. astaci*, the crayfish plague pathogen, was present in cuticles of the freshwater-inhabiting crabs *P. potamios* and *E. sinensis*, both coexisting with *A. astaci*-positive crayfish. Substantial proportions of

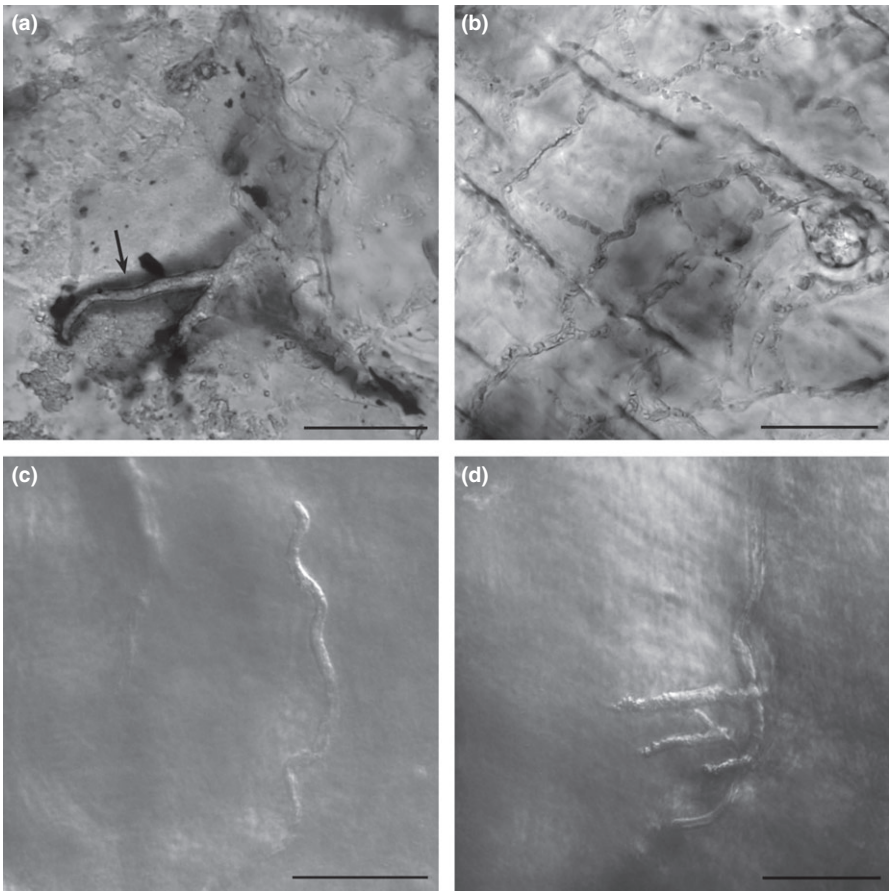


**Fig. 1** Photomicrographs of *Aphanomyces astaci*-like hyphae in the cuticle of freshwater-inhabiting crabs. Hyphae corresponding to morphological features of *A. astaci* were found in the soft abdominal cuticle of both tested species, *Potamon potamios* (a, b) and *Eriocheir sinensis* (c, d). The darker area adjacent to hyphae in (a) (indicated by an arrow) is likely due to melanin deposition. In contrast, no such melanisation was observed along hyphae shown in (b), *c.* 1 mm from the location of (a), as well as along hyphae from *E. sinensis* tissues (c, d). Hyphae in some parts of the cuticle of *P. potamios* formed dense three-dimensional net (b). Scale bars in all photos indicate 50 μm.

crab individuals within the affected populations (100 % and 43 % of the analysed *E. sinensis* and *P. potamios* specimens, respectively) were apparently infected. The analyses were carried out by comparable methods in two independent laboratories, no analysis of control samples indicated laboratory contamination, and the results were consistent for different crab species coexisting at two distant localities with different crayfish species. In both crab species, the pathogen load found in certain tissues exceeded in many cases any level that could be regarded as a chance attachment of pathogen zoospores on the body surface. Instead, the highest observed levels, corresponding to several thousands of genomic units, suggested an extensive infection.

Furthermore, microscopic evaluations of the soft abdominal cuticle of one *E. sinensis* and two *P. potamios* specimens revealed aseptate hyphae matching the morphological features of *A. astaci* (for details, see Alderman & Polglase, 1986; Cerenius *et al.*, 1988; Oidtmann *et al.*, 1999). In some areas of a *Potamon* cuticle, these hyphae were apparently melanised as observed in North American carrier crayfish (Cerenius *et al.*, 1988; Söderhäll & Cerenius, 1999; Aquiloni *et al.*, 2011) or in native European crayfish with a persistent infection (Viljamaa-Dirks *et al.*, 2011). Although the cuticle pieces with visible hyphae were small and their surface was thoroughly cleaned, they contained high and moderate *A. astaci* DNA levels. This strongly supports the conclusion that we indeed observed hyphae of *A. astaci*.

With respect to the infection of *E. sinensis* reported by Benisch (1940), Unestam (1972) suggested that *A. astaci* might be limited to freshwater decapods in general. However, Benisch's study only describes infection of the crabs under laboratory conditions and the identification of the pathogen as *A. astaci* would not be considered convincing based on current state of the art (see Cerenius *et al.*, 1988; Oidtmann *et al.*, 1999; Oidtmann, 2012). Thus, no alternative crustacean hosts have recently been considered when the pathogen transmission pathways and natural reservoirs were reviewed (see Oidtmann *et al.*, 2002; Small & Pagenkopp, 2011; Oidtmann, 2012). However, our results confirm that *A. astaci* can infect crabs in freshwater habitats. Moreover, the match of the pathogen genotype groups between coexisting crayfish and crabs strongly suggests that the pathogen was transmitted between these taxa. In experiments by Benisch (1940), the crayfish plague was apparently transmitted to *E. sinensis* from moribund

crayfish. However, that study did not reveal whether *A. astaci* is able to complete its life cycle in crabs, that is, to sporulate and infect additional hosts. As far as crayfish are concerned, conditions resulting in high *A. astaci* sporulation apparently occur after moulting (presumably in exuviae) or soon after death of infected North American crayfish host species (Strand *et al.*, 2012; Svoboda *et al.*, 2013) as well as after death of infected susceptible European crayfish *A. astacus* (Makkonen *et al.*, 2013). Nevertheless, sporulation from *A. astaci* hyphae does not depend on interactions with crayfish tissues and can be induced (by washing with water) even from mycelia cultivated on artificial media (Cerenius *et al.*, 1988). Since the amount of infection in some crabs was as high as in susceptible crayfish dying from the crayfish plague (see Vrålstad *et al.*, 2009), *A. astaci* spore release from such hosts seems likely, at least when their immune system is impaired. The possibility of zoospore release from infected crabs, their exuviae, or cadavers, thus warrants further attention.

If infected crabs are indeed able to release zoospores, crabs should be considered true hosts of *A. astaci*. More important, however, are potential consequences for susceptible crayfish species that may get in contact with those crabs, especially in Europe where *E. sinensis* has invaded numerous regions (for details, see Herborg *et al.*, 2003, 2007; Dittel & Epifanio, 2009). Despite spending most of its lifetime in fresh water, adult *Eriocheir* reproduce and die in the sea, and their larval stages are found in marine zooplankton (Kobayashi & Matsuura, 1995). Since *A. astaci* does not survive in marine or brackish water (Unestam, 1969a), the crab's planktonic larvae should not be infected. However, juvenile crabs can become *A. astaci* carriers if they enter watersheds with *A. astaci* reservoirs, such as infected crayfish (or possibly crabs). Since they can migrate hundreds of kilometres upstream and then back (Herborg *et al.*, 2003; Dittel & Epifanio, 2009), infected specimens might spread the pathogen faster and further (up to two orders of magnitude) in comparison with invasive American crayfish species (see Holdich, Haffner & Noël, 2006), which are the most important carriers of *A. astaci* in Europe (Diéguez-Uribeondo *et al.*, 2006; Oidtmann, 2012).

*Potamon potamios* is independent of the sea for completion of its life cycle (Cumberlidge *et al.*, 2009), though as a semiterrestrial species, it does not spend entire life in fresh waters either (Warburg & Goldenberg, 1984). We have presented evidence that infected population of the crab coexists with *A. leptodactylus* crayfish in Lake Eğirdir (Turkey). Since these freshwater crabs and crayfish are widespread in Turkey (Brandis *et al.*, 2000; Harlıoğlu, 2008; Bolat *et al.*, 2010) and at least some Turkish populations of *A. leptodactylus* are persistently infected with *A. astaci* (Kokko *et al.*, 2012; Svoboda *et al.*, 2012), other populations of *P. potamios* are probably infected as well. This particular species is restricted to the Middle East and some Greek islands but its congeners are distributed in other parts of the Western Palearctic such as Italy, Turkey, Iran and the Pontocaspian region (Brandis *et al.*, 2000), where they may possibly get into contact with crayfish (see Holdich *et al.*, 2006). It is not presently clear whether *Potamon* populations in other countries also coexist with infected crayfish, for example with *Procambarus clarkii*, which is widespread in southern Europe (see Holdich *et al.*, 2006). Nevertheless, while these crabs might serve as local reservoirs of *A. astaci*, their potential for long-range transmission of the pathogen seems much more limited than for catadromous *E. sinensis*, since *Potamon* do not perform long-distance migrations.

Some of the tested tissues of both crab species contained *A. astaci* DNA at levels corresponding to infected tissues of susceptible crayfish that died from crayfish plague (see Vrålstad *et al.*, 2009). Despite that, crabs tested in our study were captured alive. Similarly, mortalities of *E. sinensis* were spread over months from the first exposure of the crabs to crayfish infected with *A. astaci* (Benisch, 1940). The present data thus correspond with Unestam's (1969b) suggestion that *E. sinensis* is a species of moderate resistance to the pathogen. As the two crab species included in our study belong to different higher taxa (*Potamon*: family Potamidae, subsection Heterotremata; *Eriocheir*: Varunidae, Thoracotremata; De Grave *et al.*, 2009), and have different geographic origins and life cycles, the moderate level of resistance to *A. astaci* might be shared by freshwater-inhabiting crabs in general.

The resistance of crabs to *A. astaci* might also depend on the particular strain of *A. astaci*. As was shown for crayfish, the virulence of *A. astaci* strains can differ, especially when strains from different genotype groups are compared (Makkonen *et al.*, 2012; Jussila *et al.*, 2013). According to our analyses, both crab species were infected with a strain from the genotype group B. Although strains from this group are highly virulent to European crayfish (Makkonen *et al.*, 2012; Jussila *et al.*, 2013), we did not notice any signs of a serious disease of the tested crabs before they were killed. In the first half of the 20th century, when Benisch (1940) performed his experiments, the genotype group B had probably not yet been introduced to Europe, and the strain most likely belonged to the group A (see Huang *et al.*, 1994). This

means that crabs that died in Benisch's experiment were probably exposed to the genotype group A, which has been recently reported to show lower virulence to crayfish (Makkonen *et al*., 2012). Nonetheless, the virulence of different *A. astaci* strains to crayfish and crabs can hardly be compared across studies separated by decades, especially as the virulence of the pathogen is likely to evolve through time (Makkonen *et al*., 2012) and depends on many factors, such as the spore dose and temperature (e.g. Alderman, Polglase & Frayling, 1987). Thus, any potential negative impact of *A. astaci* on crab population dynamics remains to be assessed by further studies, which should also consider variability in virulence of different *A. astaci* strains. Considering the extent and value of *E. sinensis* aquacultures (see FAO, 2012), such a study is highly desirable particularly for that species, even though it shows at least some resistance to *A. astaci*.

*Astacus leptodactylus* has also been classified as a species of moderate resistance to *A. astaci* by Unestam (1969b), and its populations do coexist with the crayfish plague pathogen in several Turkish lakes (Kokko *et al*., 2012; Svoboda *et al*., 2012) and apparently also in the Danube (Pârvulescu *et al*., 2012; Schrimpf *et al*., 2012). It has been supposed that *A. astaci* had been present in *A. leptodactylus* populations in some Turkish lakes since the first outbreaks in the 1980s (Harlıoğlu, 2008; Kokko *et al*., 2012; Svoboda *et al*., 2012). However, an *A. astaci* strain of the genotype group A was isolated from a crayfish in Turkey in the 1980s (Huang *et al*., 1994), whereas we detected an *A. astaci* strain of the group B in crayfish and crabs from Lake Eğirdir. This suggests that the history of the crayfish plague pathogen in Turkish lakes may be more complex, involving more than one introduction, and that the massive crayfish plague outbreaks in Turkey in the 1980s (see Harlıoğlu, 2008) might have been caused by a different strain from the recent one.

Samples of benthopelagic and benthic crustaceans representing other malacostracan orders (mysids, amphipods and isopods) remained negative in *A. astaci*-specific qPCR tests, except for one sample with a very low agent level (A2) corresponding to <10 genomic units. Samples analysed from zooplankton hauls from a lake with confirmed *A. astaci*-infected signal crayfish (Strand, 2013) were also mostly negative, and the few positive samples had only very low agent level. In our opinion, the few cases of detection of low levels of *A. astaci* DNA in non-decapod crustaceans (i.e. *Pallasea quadrispinosa* and crustacean zooplankton) are not an evidence of infections. They may rather represent traces of spores released from coexisting infected crayfish that were either randomly attached to animal bodies or ingested by filter feeders. This view is further supported by analyses of water samples collected at the same time, which contained *A. astaci* spore concentrations coinciding with the highest levels detected in the plankton samples (Strand, 2013). Our data therefore do not suggest that the tested species were parasitised by *A. astaci* at the time of their capture. The results also correspond with the experiments of Unestam (1969b, 1972), who observed that the mortality rates of *Mysis relicta*, several planktonic crustaceans (cladocerans and copepods) and a rotifer did not increase after exposure to *A. astaci*. However, lack of increased mortality does not exclude the presence of non-lethal *A. astaci* infections, and we tested only moderate number of individuals of benthopelagic and benthic crustaceans from a few localities. As the wide 95% confidence intervals (Table 1) clearly show, much more thorough screening or experimental work is needed to conclude whether these crustaceans can or cannot be parasitised by *A. astaci*.

It also remains to be explored if *A. astaci* has a potential to infect other freshwater decapods, as Unestam (1972) suggested. Apart from crabs and crayfish, the order Decapoda includes two other infraorders (Caridea and Anomura) with some freshwater species (De Grave *et al*., 2008, 2009; Cumberlidge *et al*., 2009). Unlike the relatively unimportant freshwater anomurans, freshwater shrimps are highly diverse, are present in all biogeographical regions except Antarctica (De Grave *et al*., 2008), and some have substantial economic value (FAO, 2012). As the early detection and control of diseases and pathogens is vital for freshwater shrimp aquaculture (Kutty, 2005), experimental work evaluating the susceptibility of these species to *A. astaci* is highly desirable.

Our results clearly demonstrate that the freshwater-inhabiting crab species *E. sinensis* and *P. potamios* can be infected by *A. astaci*. This is not only a rehabilitation of the conclusions from Benisch (1940), who considered *E. sinensis* as a species susceptible to *A. astaci*, but may also suggest that such crabs can serve as long-term, symptom-free carriers of the pathogen. Hence, both conservation and fishery management of susceptible crayfish species in Europe should consider that not only crayfish, but also crabs may serve as *A. astaci* hosts. The screening of other crustacean orders does not support such a conclusion for non-decapod crustaceans. Our work has also re-opened numerous questions that are important from conservational, parasitological and even economic points of views. These include the real ranges of decapod hosts and symptom-free carriers of *A. astaci*, the carrier status of invasive *E. sinensis* populations

across Europe, and the potential impact of different *A. astaci* genotype groups to a broader range of freshwater Decapoda in nature and aquaculture.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Additional characteristics of the analysed microsatellite loci for *Aphanomyces astaci*: primer sequences and repeat motifs.

**Table S2.** Results of the *A. astaci*-specific qPCR analyses of different tissues of *Eriocheir sinensis*.

**Table S3.** Results of the *A. astaci*-specific qPCR analyses of different tissues of *Potamon potamios*.

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