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Aphanomyces astaci in wild crayfish populations in Slovenia: first report of persistent infection in a stone crayfish *Austropotamobius torrentium* population

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ABSTRACT: All 5 crayfish species inhabiting Slovenian freshwaters, of which 3 are indigenous crayfish species (ICS: *Astacus astacus*, *Austropotamobius pallipes*, and *A. torrentium*) and 2 are non-indigenous (NICS: *Pacifastacus leniusculus* and *Cherax quadricarinatus*), were inspected for the presence of *Aphanomyces astaci*, the causative agent of crayfish plague. Wild crayfish populations showing no clinical signs of infection were inspected using *A. astaci*-specific real-time PCR. In addition, a conventional PCR assay was employed and confirmative sequencing was performed. Out of 88 analyzed crayfish, 15/27 (55.6%) specimens of *A. torrentium* from Borovniščica Brook and 4/35 (11.4%) of *P. leniusculus* from the Mura River tested positive, showing low to moderate levels of infection (agent levels A1–A4 and A1–A3, respectively). Results revealed the presence of *A. astaci* not only in the resistant NICS but also in ICS, since the infected population of *A. torrentium* presumably had no contact with the NICS carrier and appeared to sustain *A. astaci* infection in the 2 sampling years. Although the *A. astaci* genotype has not yet been identified, a connection between the latent infection in ICS and a Group A strain of *A. astaci*, co-evolving with *A. torrentium* since its first introduction to Slovenia, is suggested as the most plausible conclusion. This is the first reported population of the genus *Austropotamobius* with persistent infection, in addition to the already known populations of the genus *Astacus*. Findings of the presumed co-evolution of *A. astaci* and ICS hosts open new perspectives, necessitating additional studies on the presence of *A. astaci* genotypes in the persistently infected ICS populations.

KEY WORDS: Crayfish plague · Group A (Genotype As) · Latent infection · Indigenous species · Invasive species · *Austropotamobius torrentium* · *Pacifastacus leniusculus* · Slovenia

INTRODUCTION

Freshwater crayfish suffer from many diseases, among which the crayfish plague caused by the parasitic oomycete *Aphanomyces astaci* is the most detrimental for European, Australian, and Asian native species (Edgerton et al. 2002). At the end of the 1850s, the plague was introduced to Europe from North America (Alderman 1996). The introduction was followed by mass mortalities and local extinctions of susceptible populations of indigenous crayfish species (ICS), e.g. the noble crayfish *Astacus astacus*, the white-clawed crayfish *Austropotamobius pallipes*, and the stone crayfish *A. torrentium*. Local extinctions in European freshwaters have continued from the end of the 19th century until recent times (Alderman 1996, Souty-Grosset et al. 2006, Kozubíková et al. 2008). In Europe, resistant non-

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indigenous crayfish species (NICS), i.e. the signal crayfish *Pacifastacus leniusculus*, the red swamp crayfish *Procambarus clarkii*, and the spiny-cheek crayfish *Orconectes limosus*, represent permanent crayfish plague reservoirs, as they harbor the parasite in the cuticle but do not contract the actual disease unless they are stressed (Persson & Söderhäll 1983, Vey et al. 1983, Diéguez-Uribeondo & Söderhäll 1993). NICS are spreading rapidly across the European continent, exerting an adverse ecological and economical impact (Holdich et al. 2009).

Previously, a conclusive diagnosis of crayfish plague has been difficult to obtain since it strongly depended on experience and involved laborious procedures for isolation in pure culture with subsequent infection experiments on susceptible species (Cerenius et al. 1988). As an alternative to the formerly established protocols, molecular methods based on the polymerase chain reaction (PCR) can be employed as a reliable diagnostic tool for the identification of *Aphanomyces astaci* in crayfish material (Oidtmann et al. 2004, 2006, Diéguez-Uribeondo 2009, Hochwimmer et al. 2009, Vrålstad et al. 2009). In the last 2 decades, plague outbreaks in wild crayfish populations have been reported, and *A. astaci* was diagnosed in Great Britain (Lilley et al. 1997), Finland (Vennerström et al. 1998), Germany (Oidtmann et al. 1999), Sweden (Edsman 2004, Bohman et al. 2006, 2011), the Czech Republic (Kozubíková et al. 2006, 2008), Spain (Diéguez-Uribeondo 2006), Switzerland (Hefti & Stucki 2006), Austria (Hochwimmer et al. 2009), Estonia (Paaver & Hurt 2009), Hungary (Kozubíková et al. 2010), Italy (Cammà et al. 2010), Slovakia (Kozubíková et al. 2011), Norway (Vrålstad et al. 2011), France (Filipová 2012), Romania (Pârvulescu et al. 2012), and Turkey (Kokko et al. 2012). Identification of *A. astaci* was successful not only in live crayfish (Hefti & Stucki 2006, Kozubíková et al. 2006, Aquiloni et al. 2011), but also in dead specimens of ICS found after mass mortalities (Vennerström et al. 1998, Kozubíková et al. 2008, Cammà et al. 2010).

Slovenia, a part of Central Europe, still holds viable and well-distributed populations of 3 ICS: *Astacus astacus*, *Austropotamobius pallipes*, and *A. torrentium* (Govedič 2006, Govedič et al. 2007). Crayfish plague outbreaks resulting in mass mortalities were recorded for the period 1880 to 1909 (Fig. 1), and later, unsuccessful reintroductions of *A. astacus* which continued until 1935 (Franke 1889, Hubad 1894, Šulgaj 1937) may have resulted from the presence of *Aphanomyces astaci* as also suggested by Viljamaa-Dirks et al. (2011). The disease had spread from the Danube, with recorded outbreaks in the river basins of the Drava, Kolpa, Krka, Mura, and Sava Rivers (Fig. 1), and largely affected populations of *A. astacus* but also of *A. torrentium*. In later periods, no reoccurrences of the crayfish plague were recorded in Slovenia, and national programs for repopulation of *A. astacus* were more or less successful (Budihna 1996, Bertok et al. 2003, Govedič 2006).

Since *Aphanomyces astaci* cannot survive without crayfish hosts for longer periods, and local epizootic extinctions of crayfish populations cure the formerly plague-infected streams (Souty-Grosset et al. 2006), populations of ICS in Slovenia have probably recovered in the past century (Govedič et al. 2007). However, first occurrences of NICS were recently recorded as *Pacifastacus leniusculus* invaded the Mura and Drava Rivers in 2003 and 2007, respectively (Bertok et al. 2003, Hudina et al. 2009), and in 2009, a population of the Australian redclaw *Cherax quadricarinatus* was discovered in the Sava River oxbow (Jaklič & Vrezec 2011). In the near future, new invasions of NICS are expected, both due to their rapid expansion in neighboring countries, e.g. Croatia (Hudina et al. 2009) and Italy (Holdich et al. 2009), and because of the free-market availability of live specimens. This strongly increases the potential for new crayfish plague outbreaks.

The aim of the present work was to obtain a first insight into recent *Aphanomyces astaci* infection of selected populations of all ICS and NICS known to inhabit Slovenian freshwaters. Some recent studies have revealed the persistence of *A. astaci* in crayfish populations without mass mortalities recorded even in some ICS (Jussila et al. 2011, Viljamaa-Dirks et al. 2011, Kokko et al. 2012, Schrimpf et al. 2012, Svoboda et al. 2012), although only moderate resistance was previously known in the narrow-clawed crayfish *Astacus leptodactylus* (Unestam 1969). Consequently, our study was focused on the identification of *A. astaci* based only on specimens taken from vital wild populations of ICS and NICS with no clinical signs of infection or plague outbreak. This is an essential approach when setting up a preventive scheme to monitor the health status of crayfish populations, which is needed for identification of the areas with higher plague-outbreak risk and initiating effective management plans to limit disease transmission and spread (Oidtmann et al. 2002, Souty-Grosset et al. 2006). In addition, crayfish populations co-existing with possibly adapted low-virulent *A. astaci* strains, or themselves showing an evolved resistance, could be discovered in this manner, even among ICS populations (Viljamaa-Dirks et al. 2011).

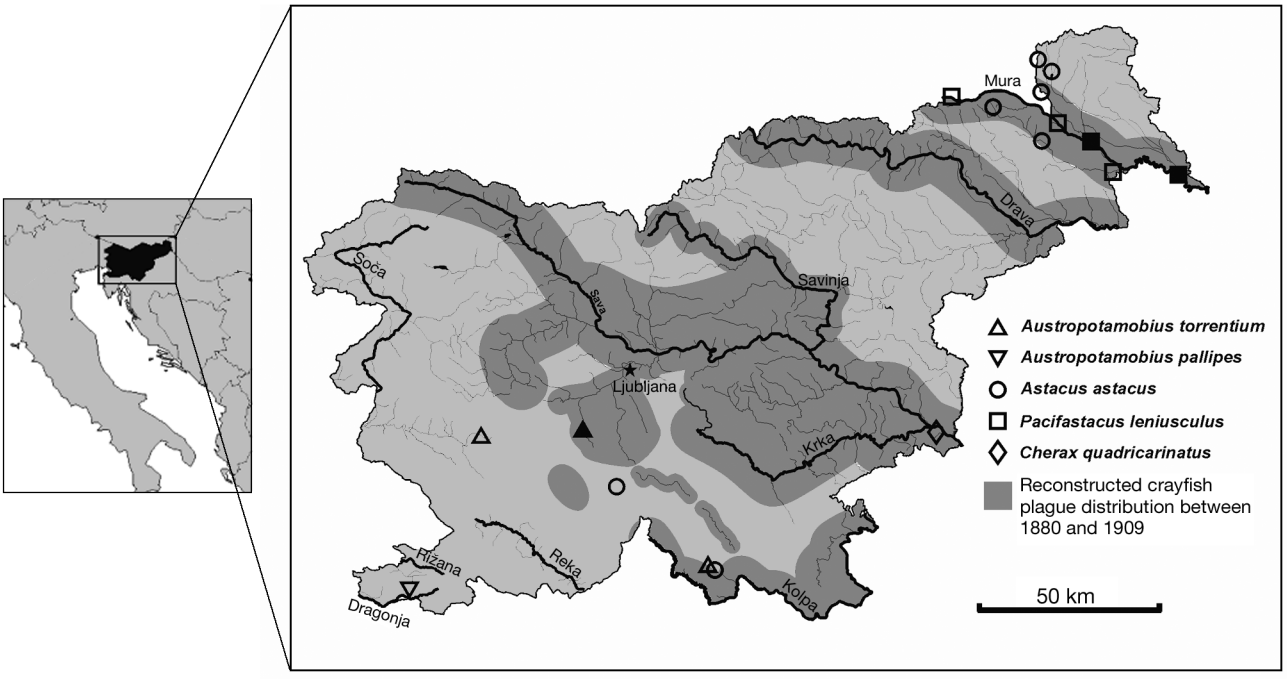

Fig. 1. *Aphanomyces astaci*. Reconstructed distribution in Slovenia between 1880 and 1909 (dark grey area) and sampling sites of different crayfish populations selected in the present study (symbols with affiliated crayfish species; filled symbols indicate *A. astaci* infection) (map by A. Kapla)

MATERIALS AND METHODS

Crayfish samples

All 5 crayfish species that inhabit Slovenian freshwaters were inspected for the presence of *Aphanomyces astaci*. The first series of samples was collected in September to December 2009 and the second series in July 2011 from established wild populations in Slovenia (Fig. 1): *Austropotamobius torrentium* (3 sites in the Kolpa, Sava, and Soča River basins), *A. pallipes* (1 site in the Dragonja River basin), *Astacus astacus* (7 sites in the Kolpa, Mura, and Sava River basins), *Pacifastacus leniusculus* (5 sites in the Mura River basin, representing a population distributed along the main stream), and *Cherax quadricarinatus* (1 site in the Sava River basin). All specimens were collected alive using classical cylindrical crayfish traps (76 × 23 cm) with a funnel at either end (Holdich 2002) or by manual searching under stones according to a standardized protocol to enable estimations of the relative abundances that can be compared between sites (Peay 2003). Relative abundances of crayfish populations were expressed as the number of crayfish per trap night, i.e. the 1-night catch of a crayfish trap, or as the number of crayfish per 10 inspected stones (Govedič et al. 2007, Jaklič & Vrezec 2011). Differences in relative abundances of infected and non-infected crayfish populations were assessed by a χ^2 test with significance set to $p < 0.05$. At the sampling sites, crayfish specimens were collected randomly to obtain a representative sample of the inspected population. After collection, animals were transported alive in cooling bags to the laboratory and stored at −20°C until further manipulation.

Detection of *Aphanomyces astaci*

Crayfish samples were subjected to molecular detection of the crayfish plague causative agent. On the day of analysis, frozen crayfish were left at room temperature for 1 to 2 h. Presence or absence of melanized spots, visible by eye, in the crayfish cuticle was inspected. Samples for the analysis were collected by sterile dissection tools which were changed for each individual. Prior to sample collection, any superficial contamination was removed from the target crayfish surfaces by wiping with clean disposable cotton towels wetted with sterile water (OIE 2009).

In each crayfish, 3 to 5 segments of soft abdominal cuticle were collected according to the OIE (2009)

recommendations. For *Pacifastacus leniusculus*, 3 to 5 segments of tail-fan, including uropod and telson tissue, were collected in addition to the abdominal cuticle, and each sample type was processed separately (Oidtmann et al. 2006, Vrålstad et al. 2011). Samples were subjected to molecular analysis: total genomic DNA extraction followed by *Aphanomyces astaci*-specific real-time PCR detection. All samples collected in 2011 and positive samples from 2009 were also subjected to conventional PCR analyses and, when applicable, confirmative sequencing as recommended for the crayfish plague diagnostics (OIE 2009).

The prevalence of *Aphanomyces astaci* infection was estimated for each crayfish population in which infected animals were detected. Prevalence was calculated as the ratio between the number of animals that tested positive and the total number of investigated crayfish from the same population (Hefti & Stucki 2006).

DNA extraction

According to the OIE (2009) recommendations and additional instructions published by Oidtmann et al. (2004, 2006), total DNA was extracted using the commercial DNeasy Blood & Tissue Kit (Qiagen) preceded by mechanical tissue disintegration using 1.4 mm ceramic beads in 3 disruption periods of 90 s at 6500 rpm followed by 2 min cooling periods (MagNA Lyser Instrument; Roche Diagnostics) following the manufacturer's instructions for insect tissue manipulation. In order to control the potential carry-over contamination of crayfish samples, environmental controls (as described by Vrålstad et al. 2009) and extraction blank controls (sterile water in place of crayfish tissue) were included in each DNA extraction series and subsequent real-time PCR analysis. The extracted DNA was stored at −20°C until further analysis.

Real-time PCR

The extracted DNA was subjected to real-time PCR amplification using primer pair AphAstITS-39F/AphAstITS-97R combined with TaqMan MGB probe AphAstITS-60P for quantitative and highly species-specific detection of *Aphanomyces astaci* in crayfish (Vrålstad et al. 2009). Reactions contained 12.5 µl of TaqMan Universal PCR Master Mix (Applied Biosystems) with the passive reference dye ROX, 500 nM of each primer, 200 nM of probe, 5 µl of template DNA, and sterile water to a final volume of 25 µl. Amplification and detection were performed in 96-well MicroAmp Fast Optical Reaction Plates (Applied Biosystems) sealed with MicroAmp Optical Adhesive Film (Applied Biosystems) on the 7500 Fast Real-Time PCR System (Applied Biosystems). The PCR program included an initial decontamination step of 2 min at 50°C, a following step of 10 min at 95°C and 50 successive cycles of amplification consisting of 15 s at 95°C and 1 min at 58°C. The real-time PCR data were analyzed using the 7500 Software v2.0.5 (Applied Biosystems). Results are reported in threshold cycle (Ct) values. In addition to the environmental and extraction blank control, positive (*A. astaci* DNA from the strains EviraK47/99 [PsI] and EviraK86/99 [As], obtained from the Finnish Food Safety Authority Evira) and negative (sterile water) amplification controls were included to rule out possible amplification failures and reagent contamination, respectively.

A dilution series was prepared from *Aphanomyces astaci* DNA (EviraK47/99, Lot. K47-2 positive control obtained from Evira, Finland), and each standard dilution was amplified in 3 replicates to calibrate the real-time PCR assay adopted from Vrålstad et al. (2009) prior to the in-house use (Table 1, Table S1 & Fig. S1 in the Supplement; www.int-res.com/articles/suppl/d103p157_supp.pdf). In brief, assay variability, expressed by the coefficient of variation (CV) for each standard dilution, was determined. Rough estimations of PCR-forming units (PFU, as defined by Holst-Jensen & Berdal 2004) per PCR were estimated on the basis of a stochastic effect at very low amplifiable target copy numbers, i.e. below 10 PFU per reaction (Ellison et al. 2006). According to the obtained assay variability, the limit of quantification (LOQ) could roughly be determined since CVs are markedly larger below LOQ (Vaerman et al. 2004). The limit of detection (LOD) was determined accordingly, i.e. 5- to 10-fold lower than LOQ in complex samples (Berdal & Holst-Jensen 2001), but not below the theoretical limit of 3 PFU per PCR (Bustin et al. 2009). Both of the estimated values were compared to the results of Vrålstad et al. (2009). In addition, the obtained Ct values of replicates were also employed for determination of the Ct cut-off value according to Mehle et al. (2012).

As suggested by Vrålstad et al. (2009), real-time PCR results of the analyzed crayfish were translated into the semi-quantitative agent levels A0–A7 (see 'Results' below and Fig. S1 in the Supplement).

Table 1. *Aphanomyces astaci* in crayfish specimens from the present study. Semi-quantitative agent levels A0–A7 adopted from Vrålstad et al. (2009). LOD: limit of detection; LOQ: limit of quantification; PFU: PCR-forming units; Ct: threshold cycle value; ud: undetermined (negative); obs: observed. For determination of LOD, LOQ, and Ct cut-off values, see Table S1 in the Supplement, and for translation of real-time PCR results into agent levels A0–A7, see Fig. S1 in the Supplement; www.int-res.com/articles/suppl/d103p157_supp.pdf

Agent level	Explanation (level of agent DNA)	PFU per PCR	Expected Ct (this study)
A0	No agent (negative)	0 or PFU_{obs} < cut-off	ud or Ct_{obs} > 40.5 (Ct cut-off)
A1	Possible presence (sub-LOD)	Cut-off ≤ PFU_{obs} < LOD	40.5 ≥ Ct_{obs} > 38.3
A2	Very low (sub-LOQ)	LOD ≤ PFU_{obs} < LOQ	38.3 ≥ Ct_{obs} > 34.8
A3	Low	LOQ ≤ PFU_{obs} < 10^3 PFU	34.8 ≥ Ct_{obs} > 30.0
A4	Moderate	10^3 ≤ PFU_{obs} < 10^4 PFU	30.0 ≥ Ct_{obs} > 26.4
A5	High	10^4 ≤ PFU_{obs} < 10^5 PFU	26.4 ≥ Ct_{obs} > 22.8
A6	Very high	10^5 ≤ PFU_{obs} < 10^6 PFU	22.8 ≥ Ct_{obs} > 19.2
A7	Exceptionally high	10^6 ≤ PFU_{obs}	19.2 ≥ Ct_{obs}

Conventional PCR

In addition to the real-time PCR amplification, samples collected in 2011 and positive samples from 2009 were also subjected to 3 conventional PCR assays. PCR products were analyzed and visualized using the QIAxcel instrument in combination with the QIAxcel Screening Kit (Qiagen) according to the manufacturer's instructions.

Decapod-specific PCR amplification was performed using the primers 143F (5′ TGC CTT ATC AGC TNT CGA TTG TAG 3′) and 145R (5′ TTC AGN TTT GCA ACC ATA CTT CCC 3′) adopted by Lo et al. (1996) to verify the quality of the extracted DNA. A 50 µl reaction mixture contained 5 µl of DNA, 2.5 U of Platinum *Taq* DNA Polymerase (Invitrogen), 1.5 mM $MgCl_2$ and 1× PCR buffer supplied by the manufacturer, 1 µM of each primer, and 0.2 mM of each dNTP (Applied Biosystems). Amplification was performed in GeneAmp PCR System 2700 (Applied Biosystems) according to the following protocol: initial denaturation at 94°C for 2 min, 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 3 min, and final extension at 72°C for 7 min.

The widely applied PCR method described by Oidtmann et al. (2006) was also employed in our study for confirmative purposes. For the standard PCR assay, primers 42 (5′ GCT TGT GCT GAG GAT GTT CT 3′) and 640 (5′ CTA TCC GAC TCC GCA TTC TG 3′) were employed, generating a 569 bp fragment of the *Aphanomyces astaci* internal transcribed spacer (ITS) region (Oidtmann et al. 2006). A second PCR assay was conducted with identical amplification parameters, but employing a modified forward primer, primer 42m (5′ GCT TGT GCT GAG GAT GTT CTT 3′; this study) in combination with primer 640; 42m had an additional T added at the 3′ end according to the sequence alignment presented in Fig. 2 of Oidtmann et al. (2006).

For both assays, a 50 µl reaction mixture contained 2 µl of DNA, 1 U of Phusion Hot Start II High-Fidelity DNA Polymerase (Finnzymes), 1× Phusion HF buffer (with $MgCl_2$) supplied by the manufacturer, 0.5 µM of each primer, and 0.2 mM of each dNTP (Applied Biosystems). Amplification was performed in a GeneAmp PCR System 2700 (Applied Biosystems) according to the following protocol adopted for the Phusion polymerase: initial denaturation at 98°C for 1 min, 45 cycles of denaturation at 98°C for 10 s, annealing at 62.5°C for 30 s, and extension at 72°C for 25 s, and final extension at 72°C for 10 min.

Sequencing

The selected 42/640 or 42m/640 amplicons were electrophoretically separated and purified from agarose gels using the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions and were sequenced in both directions (Macrogen Europe) using the amplification primers 42 and 640. The retrieved forward and reverse sequence fragments were edited and assembled into nearly complete 42/640 sequences employing the SeqMan II v.5.05 program (DNASTAR) and were subjected to BLAST (megablast; Zhang et al. 2000) similarity analysis accessed through the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov/). The obtained *Aphanomyces astaci* sequences, comprising partial ITS-1, complete 5.8S rRNA, and partial ITS-2 sequences, were submitted to GenBank under accession numbers JX272183 to JX272201.

RESULTS

Of the 88 analyzed crayfish, 19 tested positive for the crayfish plague employing *Aphanomyces astaci*-specific real-time PCR detection: 15 specimens of *Austropotamobius torrentium* from the Borovniščica Brook (45° 53′ N, 14° 22′ E) in the Sava River basin and 4 *Pacifastacus leniusculus* from the Mura River mainstream (45° 41′ N, 16° 9′ E; Table 2, Fig. 1; information on body length/weight of the collected specimens is presented in Table S2 in the Supplement; www.int-res.com/articles/supp/d103p157_supp.pdf). The estimated level of infection, expressed by the semi-quantitative agent levels (Table 1), ranged from A0 (no infection) to A4 (moderate; only 1 specimen) for the infected population of *A. torrentium* and from A0 to A3 (low) for *P. leniusculus* (Table 2). Two of the 4 positive *P. leniusculus* crayfish tested positive for the soft abdominal cuticle only and 1 for the tail-fan only. Melanized spots could be observed on the abdominal cuticle of 2 of the positive *P. leniusculus* and 3 of the positive *A. torrentium* specimens (Table 2).

The prevalence of *Aphanomyces astaci* in infected crayfish populations was 55.6% (N = 27 ind.) and 11.4% (N = 35 ind.) for *Austropotamobius torrentium* and *Pacifastacus leniusculus*, respectively. The infected population of *A. torrentium* was sampled twice, once in 2009 and later in 2011, with 33.3% out of 6 and 61.9% out of 21 samples positive for *A. astaci*, respectively. In the tested populations of *A. torrentium*, relative density in the infected population (7.0 ind. per 10 inspected stones, N = 30 stones) was significantly higher than in the non-infected populations (1.4 to 1.9 ind. per 10 searched stones, N = 143 stones; $\chi^2 = 16.3$, $p < 0.0001$; data given for 2009). Relative abundances of *P. leniusculus* in the Mura River (0.2 to 10.0 ind. per trap night, N = 23 trap nights), including the main stream only, was comparable to relative abundances of *Astacus astacus* found in other streams of the Mura River basin (0.2 to 6.5 ind. per trap night, N = 22 trap nights; $\chi^2 = 0.1$, $p = 0.80$). No mass mortalities were recorded in any of the infected crayfish populations.

The decapod-specific PCR assay generated positive results in all but one case of a *Pacifastacus leniusculus* specimen, sample type T, where negative results were also obtained after the *Aphanomyces astaci*-specific real-time and conventional PCR amplifications (Table 2). Differences could be observed between the results of the 42/640 and the 42m/640 PCR assays. For many samples where positive real-time PCR results were obtained, both assays generated positive results as expected (10 samples); however, in the remaining samples, only 42m/640 (n = 5), only 42/640 (n = 2), or neither (n = 3) generated amplification products (Table 2). Real-time PCR results were given the greatest significance, although we aimed for confirmation by conventional PCR followed by sequencing; the latter was not possible in 7 cases (Table 2). Namely, sequencing was performed for all samples where enough of the amplification product was obtained. For some samples, the conventional PCR generated a positive result, but later amplification intended for the generation of material for sequencing was unsuccessful (3 cases of *Austropotamobius torrentium*). In one case of *A. torrentium*, enough material was obtained and sequencing generated good results, but no similarity hits could be found after a BLAST search, and the sequence was not deposited in GenBank.

DISCUSSION

Since the crayfish plague outbreaks in Slovenia occurred well in the past, a recent presence of *Aphanomyces astaci* would especially, though not exclusively, be expected in connection with new invasions of plague-resistant NICS-like *Pacifastacus leniusculus* (Bertok et al. 2003, Hudina et al. 2009). Our results not only confirmed this presumption, but also revealed the presence of *A. astaci* in ICS, namely in a population of *Austropotamobius torrentium* from central Slovenia. According to these results, Slovenia has joined the European countries where *A. astaci* was recently confirmed in crayfish populations. As a result of sharing crayfish populations that have confirmed infections, it is highly probable that *A. astaci* is also present in neighboring countries, e.g. Croatia (Hudina et al. 2009).

Most detections of *Aphanomyces astaci* in ICS were made only in moribund or dead individuals found after crayfish mass mortalities (Vennerström et al. 1998, Kozubíková et al. 2008, Cammà et al. 2010). Recently, populations of European ICS with persistent *A. astaci* infection were found in 2 species of the genus *Astacus*, namely *A. astacus* (Jussila et al. 2011, Viljamaa-Dirks et al. 2011) and *A. leptodactylus* (Kokko et al. 2012, Pârvulescu et al. 2012, Schrimpf et al. 2012, Svoboda et al. 2012). Our study revealed that persistently infected populations can also occur in the genus *Austropotamobius*, namely in *A. torrentium*. In addition to the lack of mass mortality or evident clinical signs, the sub-clinical status of infection in the *A. torrentium* population was reflected by the

Table 2. *Aphanomyces astaci* in crayfish specimens from the present study. Tis.: tissue; A: soft abdominal cuticle; T: tail-fan; Ct: threshold cycle value; SD: standard deviation; ud: undetermined (negative); na: not applicable; –: negative; +: positive; nd: not determined; *Aa*: *Aphanomyces astaci*-identical sequence. Real-time PCR was adopted from Vrålstad et al. (2009); results averaged from 2 to 3 Ct values obtained from assays performed on different days and translated into agent levels (see Table 1). For PCR, we used primers 143F/145R adopted from Lo et al. (1996) and 42/640 from Oidtmann et al. (2006), with modification introduced into primer 42 (42m in this study). For information on body lengths and weights of the animals, see Table S2 in the Supplement at www.int-res.com/articles/supp/d103p157_supp.pdf

Collection of crayfish specimens			n	Melani-	Real-time PCR						PCR		Sequencing (JX272…)		
River basin	Water body *Location*	Date (mo/yr)		zation	Tis.	Ct Mean	Ct SD	Agent level	Diagnosis Sample	Diagnosis Animal	143F/ 145R	42/ 640	42m/ 640	42/ 640	42m/ 640	Result
Astacus astacus																
Mura	Mura River tributary *Various*	09/2009	5		A	ud	na	A0	–	–	nd	nd	nd	nd	nd	nd
Kolpa	Mokri Potok Stream *Kočevska Reka*	10/2009	5		A	ud	na	A0	–	–	nd	nd	nd	nd	nd	nd
Sava	Bloščica Stream (wetland) *Bloke*	10/2009	1		A	ud	na	A0	–	–	nd	nd	nd	nd	nd	nd
Austropotamobius pallipes																
Dragonja	Pinjevec Stream *Župančiči*	10/2009	6		A	ud	na	A0	–	–	nd	nd	nd	nd	nd	nd
Austropotamobius torrentium																
Kolpa	Mokri Potok Stream *Kočevska Reka*	10/2009	5		A	ud	na	A0	–	–	nd	nd	nd	nd	nd	nd
Soča	Bela Stream *Vipava*	10/2009	1		A	ud	na	A0	–	–	nd	nd	nd	nd	nd	nd
Sava	Borovniščica Brook *Borovnica*	10/2009	4		A	ud	na	A0	–	–	nd	nd	nd	nd	nd	nd
		07/2011	8		A	ud	na	A0	–	–	+	–	–	nd	nd	nd
		10/2009	1		A	36.03	0.57	A2	+	+	+	+	+	...184	nd	*Aa*
			1		A	38.56[a]	1.20	A1	+	+	+	–	+	nd	...194	*Aa*
		07/2011	1		A	38.58	0.28	A1	+	+	+	–	+	nd	nd	nd
			1		A	39.68[b]	na	A1	+	+	+	–	+	nd	nd	nd
			1	+	A	36.53	1.59	A2	+	+	+	–	+	nd	...195	*Aa*
			1	+	A	32.77	0.19	A3	+	+	+	+	+	...189	...198	*Aa*
			1		A	33.67	0.04	A3	+	+	+	+	+	...185	...196	*Aa*
			1		A	35.25	0.67	A2	+	+	+	+	–	...186	nd	*Aa*
			1		A	35.61	0.74	A2	+	+	+	+	+	...187	nd	*Aa*
			1		A	29.40	0.37	A4	+	+	+	+	+	...190	...199	*Aa*
			1		A	32.29	0.15	A3	+	+	+	+	+	...188	...197	*Aa*
			1	+	A	31.53	0.08	A3	+	+	+	+	+	...191	...200	*Aa*
			1		A	36.75[b]	na	A2	+	+	+	–	–	nd	nd	nd
			1		A	37.68	0.01	A2	+	+	+	+	+	nd	nd	nd
			1		A	38.79	0.36	A1	+	+	+	–	+	nd	+	–[e]
Cherax quadricarinatus																
Sava	Topla Oxbow *Prilipe*	12/2009	3		A	ud	na	A0	–	–	nd	nd	nd	nd	nd	nd
Pacifastacus leniusculus																
Mura	Mura River main stream *Various locations*	09/2009	31		A	ud	na	A0	–	–	nd	nd	nd	nd	nd	nd
					T	ud	na	A0	–		nd	nd	nd	nd	nd	nd
		09/2009	1		A	36.47	0.63	A2	+	+	+	+	+	nd	…192	*Aa*
					T	34.08	0.20	A3	+		+	+	+	…183	…193	*Aa*
			1		A	39.42[c]	na	A1	+	+	+	–	–	nd	nd	nd
					T	ud	na	A0	–		–	–	–	nd	nd	nd
			1	+	A	35.37	1.00	A2	+	+	+	+	–	…201	nd	*Aa*
					T	45.90[d]	3.06	A0	–		+	–	–	nd	nd	nd
			1	+	A	ud	na	A0	–	+	+	–	–	nd	nd	nd
					T	37.74	0.18	A2	+		+	–	–	nd	nd	nd

[a]2/3 replicates positive; [b]1/2 replicates positive; [c]1/3 replicates positive; [d]regarded as negative (above the Ct cut-off value). [e]For the last *A. torrentium* specimen in the list, a sequence of adequate quality and length was obtained, but no similarity hits of considerable coverage could be detected (therefore, it was not deposited in GenBank)

low agent levels detected (ranging from A0 to A3, with 1 sample showing A4), distinctive of symptom-free carriers (Vrålstad et al. 2009). Melanizations observed in some of the specimens could reveal the immune response activity previously described in carrier crayfish (e.g. Cerenius et al. 2003, Oidtmann et al. 2006). Until now, this species was regarded as highly susceptible to crayfish plague infection and thus subjected to mass mortality outbreaks (e.g. Souty-Grosset et al. 2006, Kozubíková et al. 2008). The *A. astaci*-positive population of *A. torrentium* from the Borovniščica Brook showed no signs of increased mortality during the 2 sampling years. Compared to the plague prevalence recorded so far in the persistently infected ICS of the genus *Astacus* (Jussila et al. 2011, Viljamaa-Dirks et al. 2011, Kokko et al. 2012, Pârvulescu et al. 2012, Schrimpf et al. 2012, Svoboda et al. 2012), ranging from 3 to 100% (median 30%, quartile range 14 to 60%, N = 9 infected populations with 5 or more specimens tested), albeit being only a rough estimation due to a limited number of animals inspected, the prevalence in the infected *A. torrentium* population from Slovenia appears to be intermediate (56%).

Carrier NICS can transmit the disease to all susceptible crayfish in a water body which then usually succumb in 1 to 2 wk after the initial infection (Unestam 1976, Persson & Söderhäll 1983, Vey et al. 1983, Diéguez-Uribeondo & Söderhäll 1993); successful re-introduction of crayfish of the susceptible species is thought to be possible only after all of the crayfish in the affected water body have been eradicated (Fürst 1995). However, no NICS population has been discovered in central Slovenia to date, and therefore, the source of infection is highly questionable there. Since *Aphanomyces astaci* cannot remain without the presence of a host crayfish population (Souty-Grosset et al. 2006), the most plausible explanation for the observed phenomenon would be that it persisted in the population of *Austropotamobius torrentium*. Viljamaa-Dirks et al. (2011) proposed that *A. astaci* persisted in *Astacus astacus* due to the low density of the crayfish population; however, our data obtained for *A. torrentium* do not support this presumption due to the high relative density recorded. The area where the infected *A. torrentium* population was found is known to be in the range of crayfish plague distribution at the beginning of the 20th century (Šulgaj 1937, Fig. 1). We could speculate that the discovered infected population is a result of an adaptive co-evolutionary process of the host–parasite system, leading to an increased resistance of local crayfish, which appeared to be a powerful mechanism in many cases of co-existence (Tokeshi 1999, Svoboda et al. 2012). This might be the case in the resistant NICS (Evans & Edgerton 2002) and is also suggested in the case of persistently infected or resistant ICS (Kokko et al. 2012, Svoboda et al. 2012). However, a co-evolutionary process leading to a reduced virulence of some *A. astaci* strains (Diéguez-Uribeondo et al. 1995, Viljamaa-Dirks et al. 2011) would give a more plausible explanation, since the evolutionary rate depends, among other factors, on the generation time of organisms (Martin & Palumbi 1993). Therefore, oomycetes are expected to adapt to the long-term co-existence faster than crayfish hosts. Indeed, it has been shown that differences exist in the virulence of different *A. astaci* strains, i.e. Groups A–D described according to RAPD-PCR typing (Huang et al. 1994, Diéguez-Uribeondo et al. 1995, OIE 2012). Namely, strains that have been present in Europe for more than 150 yr (Group A strains, i.e. *Astacus* or As-genotype strains) appear to show a decreased pathogenicity in comparison to strains introduced more recently (Viljamaa-Dirks et al. 2011, Makkonen et al. 2012, OIE 2012, S. Viljamaa-Dirks pers. comm.). In their study, Makkonen et al. (2012) confirmed that a Group B (i.e. *Pacifastacus* I or PsI-genotype) strain was highly virulent and killed *A. astacus* crayfish within few days; however, Group A strains showed a variance of virulence with a dose-dependent mortality rate.

In our study, *Aphanomyces astaci* strains were not genotyped, since a pure culture would be obligatory for the RAPD-PCR typing; also, differences in the polymorphisms observed in ITS regions were not shown to support the grouping of strains (Makkonen et al. 2011). However, a PCR-based genotyping, not dependent on cultivation of *A. astaci* in pure cultures, was developed for differentiation of genotypes As and PsI (Heinikainen & Viljamaa-Dirks 2010). In addition, microsatellite markers for assessing variation of *A. astaci* directly in host tissues were discovered (Grandjean et al. 2012), but both protocols are awaiting publication. In the scope of our study, more crayfish specimens from Slovenian freshwaters will be inspected for the plague agent. Genotyping of *A. astaci* is planned, as collaboration was kindly offered by the Finnish Food Safety Authority Evira (S. Viljamaa-Dirks pers. comm.) and the University of Poitiers (F. Grandjean pers. comm.). Nevertheless, the collected data on *A. astaci* virulence can lead to the presumption that persistent infections in ICS, including the *Austropotamobius torrentium* population from our study, are in connection with Group A strains and not due to the increased resistance of

crayfish. If this holds true, a crash of the *A. torrentium* population from the Borovniščica Brook cannot be completely excluded, as low-virulent *A. astaci* strains can show a long period of latency (Viljamaa-Dirks et al. 2011), with mortality rates depending on the number of *A. astaci* zoospores (Makkonen et al. 2012). However, a high relative density of the infected *A. torrentium* population was recorded in our study, challenging the weak-population proposal for the phenomenon of chronic plague (Viljamaa-Dirks et al. 2011). In this population, the prevalence of *A. astaci* infection seemed to double when comparing sampling in 2009 (33%) and 2011 (62%). On one hand, this might be due to the low number of animals collected in 2009, but on the other hand, this finding necessitates regular follow-up of the infected *A. torrentium* population.

In Europe, studies detecting *Aphanomyces astaci* in live crayfish specimens from wild populations and estimating the prevalence of infection are scarce and fairly recent, both for ICS (e.g. Jussila et al. 2011, Viljamaa-Dirks et al. 2011, Kokko et al. 2012, Pârvulescu et al. 2012, Svoboda et al. 2012) and NICS (e.g. Kozubíková et al. 2011, Matasová et al. 2011, Skov et al. 2011, Filipová 2012, Pârvulescu et al. 2012). The prevalence levels in the resistant NICS were reported to vary greatly from 0 to 100% (Oidtmann et al. 2006). For *Pacifastacus leniusculus*, according to the published data based on 62 populations where 5 or more individuals were tested (Oidtmann et al. 2006, Kozubíková et al. 2009, 2010, 2011, Skov et al. 2011, Vrålstad et al. 2011, Filipová 2012, this study), it appears that about 63% of populations are infected with *A. astaci*. The prevalence of *A. astaci* in the infected populations varies from 5 to 86% (median 29%, quartile range 14 to 47%; in our study, 11.4%), which places the population of *P. leniusculus* from our study at the lower range of prevalence. Since low prevalence is associated with low levels of *A. astaci* DNA detected in crayfish (Kozubíková et al. 2011), our results are in accordance with this correlation; none of the analyzed *P. leniusculus* samples contained higher agent levels than A3. This is also in congruence with the results of Vrålstad et al. (2009), showing the agent levels of symptom-free carrier crayfish ranging from A1 to A3. Invasion of *P. leniusculus* in the Slovenian part of the Mura River is fairly recent, with the first specimens detected in 2003 (Bertok et al. 2003), supposedly as a consequence of its spread from Austria in the north (Füreder 2009). Spread of the population in the river mainstream was rapid, with downstream dispersal rate estimated at 18 to 24 km yr^{-1} (Hudina et al. 2009). Prevalence of *A. astaci* infection in the crayfish population may be low in the early stages of infection, but will gradually increase over time (OIE 2009). This could explain the low prevalence of *A. astaci* in the recently established *P. leniusculus* population in the Mura River as also suggested by Kozubíková et al. (2011). In addition, large populations of susceptible ICS, namely *Astacus astacus*, persisted in subsidiary streams of the Mura River with no known recent mass mortality outbreaks, and moreover, no infected animals were found in these populations. Regular monitoring of these highly vulnerable ICS populations is thus extremely important, as well as monitoring the spread and increase of *A. astaci* prevalence in *P. leniusculus* populations.

Crayfish plague diagnostic requirements have changed with the publication of molecular methods (Oidtmann et al. 2004, 2006, Hochwimmer et al. 2009, Vrålstad et al. 2009), representing a much more reliable and fast alternative to classical methods for the detection of *Aphanomyces astaci* from a broad range of materials (Finnish Food Safety Authority Evira 2008, Tuffs & Oidtmann 2011). According to OIE instructions, *A. astaci*-specific real-time PCR adopted from Vrålstad et al. (2009) or a conventional 42/640 PCR assay adopted from Oidtmann et al. (2006) followed by confirmative sequencing are suggested (OIE 2012). The former was also given the greatest importance in our study, as it proved to be highly specific (Vrålstad et al. 2009) and the most sensitive (Tuffs & Oidtmann 2011) in comparison to the other published real-time PCR (Hochwimmer et al. 2009) and the recommended 42/640 assay (Oidtmann et al. 2006). In addition, it was selected because it was also suitable for the detection of *A. astaci* in symptom-free carrier crayfish (Finnish Food Safety Authority Evira 2008, Vrålstad et al. 2009, Tuffs & Oidtmann 2011), or even directly from water samples (Strand et al. 2011), as the majority of positive specimens in our study were lacking clinical signs of infection. Prior to the in-house implementation, the real-time PCR assay was re-validated and calibrated. Both of the estimated values for LOD and LOQ were in congruence with the results of Vrålstad et al. (2009). The obtained Ct cut-off value of 40.5 was employed when interpreting the results of crayfish samples, as justified before (Kozubíková et al. 2011), and the semi-quantitative agent levels were determined as recommended by Vrålstad et al. (2009). For some of the negative samples, DNA dilution was employed to test for possible inhibition and avoid false negative results (Vrålstad et al. 2011); inhibition was not discovered as a major problem as also repor-

ted previously (Kozubíková et al. 2011, Pârvulescu et al. 2012). For confirmative purposes, conventional PCR assays supplemented with sequencing were employed. Decapod-specific PCR (Lo et al. 1996) was adopted to confirm the presence of DNA of satisfactory quality after extraction and the results were in accordance with the expectations; for 1 sample, no amplification product was obtained; therefore, the results of real-time and conventional PCR assays were excluded. The recommended 42/640 assay was also performed and was, if possible, followed by sequencing; however, not all of the real-time PCR results could be confirmed by the latter. Unsuccessful amplification could be assigned to e.g. DNA instability in solution after storage and repeated manipulation for PCR assay purposes as reported before (Oidtmann et al. 2006) or the phenomenon of uneven distribution and gradual depletion of ITS target copies in DNA samples (Kozubíková et al. 2011). In our study, the observed discrepancies between real-time and conventional PCR assay results were never observed in samples showing agent levels A3 or A4; a marked drop of amplification efficiency of the conventional PCR was described previously when comparing samples belonging to categories A3 and A2 (Kozubíková et al. 2011). The observed discrepancies are also in congruence with a demonstrated 10-fold lower sensitivity of 42/640 conventional PCR in comparison to the real-time PCR assay of Vrålstad et al. (2009) (Tuffs & Oidtmann 2011). In addition, no clear relationship between the *A. astaci* infection ratio and body length of the tested crayfish could be observed in our study.

For one *Austropotamobius torrentium* sample, an interesting observation was made, namely that amplification and sequencing were both successful but no similarity hits could be found for the obtained sequence. Although new discoveries are continuously calling the reported specificities of *Aphanomyces astaci* diagnostic tests into question (Kozubíková et al. 2011) and several new *Aphanomyces* species have been described recently (Diéguez-Uribeondo et al. 2009), possibly leading to cross-reaction with the established PCR assays, this is probably not the case here as the obtained sequence showed no similarity even to any of the known sequences belonging to the *Aphanomyces* genus. It was reported that sequencing of 42/640 PCR products has resulted in only one discovery of a false positive result to date (Diéguez-Uribeondo et al. 2009, Kozubíková et al. 2009), and thus our study describes the second case. Nevertheless, the sample was considered an *A. astaci*-positive result, since the latter was obtained not only with conventional *A. astaci*-specific PCR but also with real-time PCR, showing high specificity in all studies undertaken to date.

When inspecting the 42 and 640 primer sequences from Oidtmann et al. (2006), the alignment of *Aphanomyces* spp. sequences in their Fig. 2 encouraged us to test the effect of an additional T at the 3′ end of primer 42, namely primer 42m constructed for the present work, since it is generally known that the 3′ end of the primer molecule markedly affects the efficiency of primer annealing. A modification of primer 42 was tested before, but the variant sequence 42v2 was not further recommended as it showed reduced specificity (Tuffs & Oidtmann 2011). In our study, both primer variants enabled amplification. However, when amplicons were not generated by both of them, 42m/640 proved somewhat more effective, but no further conclusions should be made based on these scarce results.

In Europe, the stone crayfish *Austropotamobius torrentium* is a species of high conservation importance, since it has the most restricted range among European ICS, lives in small populations, is considered the most sensitive ICS to pollution, and crayfish plague is listed as one of its biggest threats (Souty-Grosset et al. 2006, Peay & Füreder 2011). *A. torrentium* is among the most genetically diverse crayfish species in Europe, with many isolated populations, some of which even represent genetically distinct haplotype clades (Trontelj et al. 2005). Findings of the persistently infected ICS opened new perspectives, since not only NICS could be regarded as plague carriers but also some ICS populations (Kokko et al. 2012, Pârvulescu et al. 2012, Schrimpf et al. 2012, Svoboda et al. 2012). This is a new challenge in the conservation of ICS, also in terms of the refuge sites that are less accessible to NICS (Weinländer & Füreder 2012), since ICS as new plague carriers might lead to a more efficient plague spread due to the higher adaptive rates to more extreme freshwater environments (e.g. colder montane brooks) than NICS. We suggest that future studies should test physiological and immunological responses of ICS with latent infection in order to evaluate their resistance level and survival rate. Persistently plague-infected populations of ICS place the problem of crayfish plague in Europe into a new evolutionary perspective in connection with biotic interactions, which might influence evolution of non-native as well as native species (e.g. Jones & Gomulkiewicz 2012). In other words, this would mean stable establishment of *Aphanomyces astaci* in European freshwater eco-

systems, which requires a low-virulent pathogen as well as sufficiently plague-tolerant ICS populations. However, a permanent presence of the already resistant NICS, which act as a reservoir of the naive (not co-evolved) *A. astaci* pathogen, would slow down or even stop the co-evolutionary process between the pathogen and its native hosts. Thus, discovery of the persistently infected ICS populations does not decrease the problem of NICS in Europe. Nevertheless, these issues are possibly indicating the peak of an iceberg awaiting further examination.

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LITERATURE CITED

Alderman DJ (1996) Geographical spread of bacterial and fungal diseases of crustaceans. Rev Sci Tech Off Int Epizoot 15:603–632

Aquiloni L, Martin MP, Gherardi F, Diéguez-Uribeondo J (2011) The North American crayfish *Procambarus clarkii* is the carrier of the oomycete *Aphanomyces astaci* in Italy. Biol Invasions 13:359–367

Berdal KG, Holst-Jensen A (2001) Roundup Ready® soybean event-specific real-time quantitative PCR assay and estimation of the practical detection and quantification limits in GMO analyses. Eur Food Res Technol 213: 432–438

Bertok M, Budihna N, Povž M (2003) Strokovne osnove za vzpostavljanje omrežja Natura 2000: Ribe (*Pisces*), Piškurji (*Cyclostomata*), Raki deseteronožci (*Decapoda*). Zavod za ribištvo Slovenije, Ljubljana (in Slovene)

Bohman P, Nordwall F, Edsman L (2006) The effect of the large-scale introduction of signal crayfish on the spread of crayfish plague in Sweden. Bull Fr Peche Piscicult 380–381:1291–1302

Bohman P, Degerman E, Edsman L, Sers B (2011) Exponential increase of signal crayfish in running waters in Sweden — due to illegal introductions? Knowl Manag Aquat Ecosyst 401:23

Budihna N (1996) Potočni raki (*Astacidae*). In: Gregori J, Martinčič A, Tarman K, Urbanc-Berčič O, Tome D, Zupančič M (eds) Narava Slovenije, stanje in perspektive. Društvo ekologov Slovenije, Ljubljana, p 228–233 (in Slovene)

Bustin SA, Benes V, Garson JA, Hellemans J and others (2009) The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55:611–622

Cammà C, Ferri N, Zezza D, Marcacci M, Paolini A, Ricchiuti L, Lelli R (2010) Confirmation of crayfish plague in Italy: detection of *Aphanomyces astaci* in white clawed crayfish. Dis Aquat Org 89:265–268

Cerenius L, Söderhäll K, Persson M, Ajaxon R (1988) The crayfish plague fungus *Aphanomyces astaci* — diagnosis, isolation and pathobiology. Freshw Crayfish 7:131–144

Cerenius L, Bangyeekhun E, Keyser P, Söderhäll I, Söderhäll K (2003) Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci.* Cell Microbiol 5:353–357

Diéguez-Uribeondo J (2006) The dispersion of the *Aphanomyces astaci*-carrier *Pacifastacus leniusculus* by humans represents the main cause of disappearance of the indigenous crayfish *Austropotamobius pallipes* in Navarra. Bull Fr Peche Piscicult 380–381:1303–1312

Diéguez-Uribeondo J (2009) Current techniques, approaches and knowledge in diagnosis of crayfish plague and other crayfish diseases. Knowl Manag Aquat Ecocsyst 394–395:02

Diéguez-Uribeondo J, Söderhäll K (1993) *Procambarus clarkii* Girard as a vector for the crayfish plague fungus, *Aphanomyces astaci* Schikora. Aquacult Res 24:761–765

Diéguez-Uribeondo J, Huang T, Cerenius L, Söderhäll K (1995) Physiological adaptation of an *Aphanomyces astaci* strain isolated from the freshwater crayfish *Procambarus clarkii.* Mycol Res 99:574–578

Diéguez-Uribeondo J, García MA, Cerenius L, Kozubíková E and others (2009) Phylogenetic relationships among plant and animal parasites, and saprotrophs in *Aphanomyces* (Oomycetes). Fungal Genet Biol 46:365–376

Edgerton BF, Evans LH, Stephens FJ, Overstreet RM (2002) Synopsis of freshwater crayfish diseases and commensal organisms. Aquaculture 206:57–135

Edsman L (2004) The Swedish story about import of live crayfish. Bull Fr Peche Piscicult 372-373:281–288

Ellison SLR, English CA, Burns MJ, Keer JT (2006) Routes to improving the reliability of low level DNA analysis using real-time PCR. BMC Biotechnol 6:33

Evans LH, Edgerton BF (2002) Pathogens, parasites and commensals. In: Holdich DM (ed) Biology of freshwater crayfish. Blackwell Science, Oxford, p 337–438

Filipová L (2012) Genetic variation in North American crayfish species introduced to Europe and the prevalence of the crayfish plague pathogen in their populations. PhD thesis, Charles University, Prague & University of Poitiers

Finnish Food Safety Authority Evira (2008) Crayfish disease diagnostics – towards a Nordic standard. Evira publications, 2/2008. Finnish Food Safety Authority Evira, Helsinki

Franke J (1889) Zur Krebsfrage in Krain. Mitteilungen des österreichischen Fischvereins, Wien, p 2–7 (in German)

Füreder L (2009) Flusskrebse: Biologie-Ökologie-Gefährdung. Folio Verlag, Bozen (in German)

Fürst M (1995) On the recovery of *Astacus astacus* L. populations after an epizootic of the crayfish plague (*Aphanomyces astaci* Schikora). Freshw Crayfish 8:565–576

Govedič M (2006) Potočni raki Slovenije: razširjenost, ekologija, varstvo. Center za kartografijo favne in flore, Miklavž na Dravskem polju (in Slovene)

Govedič M, Bedjanič M, Grobelnik V, Kapla A and others (2007) Dodatne raziskave kvalifikacijskih vrst Natura 2000 s predlogom spremljanja stanja — raki. Center za

kartografijo favne in flore, Miklavž na Dravskem polju (in Slovene)

Grandjean F, Jelic M, Mangombi J, Delaunay C and others (2012) Isolation of microsatellite markers in *Aphanomyces astaci*: new perspectives for the identification of pathogen strains in infected crayfish. IAA 19 Conf 2012, Innsbruck, 26–31 August, p 24 (Abstract)

Hefti D, Stucki P (2006) Crayfish management for Swiss waters. Bull Fr Peche Piscicult 380-381:937–950

Heinikainen S, Viljamaa-Dirks S (2010) PCR method for differentiation of two *Aphanomyces astaci* genogroups directly from crayfish tissue. Autumn School in Biodiversity of *Saprolegnia* (Oomycetes), Madrid, 1–4 November, p 7–8 (Abstract)

Hochwimmer G, Tober R, Bibars-Reiter R, Licek E, Steinborn R (2009) Identification of two GH18 chitinase family genes and their use as targets for detection of the crayfish-plague oomycete *Aphanomyces astaci*. BMC Microbiol 9:184

Holdich DM (2002) Background and functional morphology. In: Holdich DM (ed) Biology of freshwater crayfish. Blackwell Science, Oxford, p 3–29

Holdich DM, Reynolds JD, Souty-Grosset C, Sibley PJ (2009) A review of the ever increasing threat to European crayfish from non-indigenous crayfish species. Knowl Manag Aquat Ecosyst 394-395:11

Holst-Jensen A, Berdal KG (2004) The modular analytical procedure and validation approach and the units of measurement for genetically modified materials in foods and feeds. J AOAC Int 87:927–936

Huang TS, Cerenius L, Söderhäll K (1994) Analysis of genetic diversity in the crayfish plague fungus, *Aphanomyces astaci*, by random amplification of polymorphic DNA. Aquaculture 126:1–9

Hubad J (1894) O račji kugi. Izvestje c. kr. državne nižje gimnazije v Ljubljani, Ljubljana, p 15–23 (in Slovene)

Hudina S, Faller M, Luci A, Klobučar G, Maguire I (2009) Distribution and dispersal of two invasive crayfish species in the Drava River basin, Croatia. Knowl Manag Aquat Ecosyst 394-395:09

Jaklič M, Vrezec A (2011) The first tropical alien crayfish species in European waters — the redclaw *Cherax quadricarinatus*. Crustaceana 84:651–665

Jones EI, Gomulkiewicz R (2012) Biotic interactions, rapid evolution, and the establishment of introduced species. Am Nat 179:E28–E36

Jussila J, Makkonen J, Vainikka A, Kortet R, Kokko H (2011) Latent crayfish plague (*Aphanomyces astaci*) infection in a robust wild noble crayfish (*Astacus astacus*) population. Aquaculture 321:17–20

Kokko H, Koistinen L, Harlio lu MM, Makkonen J, Aydin H, Jussila J (2012) Recovering Turkish narrow clawed crayfish (*Astacus leptodactylus*) populations carry *Aphanomyces astaci*. Knowl Manag Aquat Ecosyst 404:12

Kozubíková E, Petrusek A, uriš Z, Kozák P, Geiger S, Hoffmann R, Oidtmann B (2006) The crayfish plague in the Czech Republic — review of recent suspect cases and pilot detection study. Bull Fr Peche Piscicult 380–381: 1313–1323

Kozubíková E, Petrusek A, uriš Z, Martín MP, Diéguez-Uribeondo J, Oidtmann B (2008) The old menace is back: recent crayfish plague outbreaks in the Czech Republic. Aquaculture 274:208–217

Kozubíková E, Filipová L, Kozák P, Ďuriš Z and others (2009) Prevalence of the crayfish plague pathogen *Aphanomyces astaci* in invasive American crayfishes in the Czech Republic. Conserv Biol 23:1204–1213

Kozubíková E, Puky M, Kiszely P, Petrusek A (2010) Crayfish plague pathogen in invasive North American crayfish species in Hungary. J Fish Dis 33:925–929

Kozubíková E, Vrålstad T, Filipová L, Petrusek A (2011) Re-examination of the prevalence of *Aphanomyces astaci* in North American crayfish populations in Central Europe by TaqMan MGB real-time PCR. Dis Aquat Org 97: 113–125

Lilley JH, Cerenius L, Söderhäll K (1997) RAPD evidence for the origin of crayfish plague outbreaks in Britain. Aquaculture 157:181–185

Lo CF, Leu JH, Ho CH, Chen CH and others (1996) Detection of baculovirus associated with white spot syndrome (WSBV) in penaeid shrimps using polymerase chain reaction. Dis Aquat Org 25:133–141

Makkonen J, Jussila J, Henttonen P, Kokko H (2011) Genetic variation in the ribosomal internal transcribed spacers of *Aphanomyces astaci* Schikora from Finland. Aquaculture 311:48–53

Makkonen J, Kokko H, Vainikka A, Kortet R, Jussila J (2012) Differences in the virulence of crayfish plague (*Aphanomyces astaci*) strains confirmed with infection trials. IAA 19 Conf 2012, Innsbruck, 26–31 August, p 36 (Abstract)

Martin AP, Palumbi SR (1993) Body size, metabolic rate, generation time, and molecular clock. Proc Natl Acad Sci USA 90:4087–4091

Matasová K, Kozubíková E, Svoboda J, Jarošík V, Petrusek A (2011) Temporal variation in the prevalence of the crayfish plague pathogen, *Aphanomyces astaci*, in three Czech spiny-cheek crayfish populations. Knowl Manag Aquat Ecosyst 401:14

Mehle N, Nikolić P, Gruden K, Ravnikar M, Dermastia M (2012) Real-time PCR for specific detection of three phytoplasmas from the apple proliferation group. In: Dickinson M, Hodgetts J (eds) Phytoplasma: methods and protocols. Methods in molecular biology, Vol 938. Humana Press, New York, NY, p 269–281

Oidtmann B, Cerenius L, Schmid I, Hoffmann R, Söderhäll K (1999) Crayfish plague epizootics in Germany — classification of two German isolates of the crayfish plague fungus *Aphanomyces astaci* by random amplification of polymorphic DNA. Dis Aquat Org 35:235–238

Oidtmann B, Heitz E, Rogers D, Hoffmann RW (2002) Transmission of crayfish plague. Dis Aquat Org 52:159–167

Oidtmann B, Schaefers N, Cerenius L, Söderhäll K, Hoffmann RW (2004) Detection of genomic DNA of the crayfish plague fungus *Aphanomyces astaci* (Oomycete) in clinical samples by PCR. Vet Microbiol 100:269–282

Oidtmann B, Geiger S, Steinbauer P, Culas A, Hoffmann RW (2006) Detection of *Aphanomyces astaci* in North American crayfish by polymerase chain reaction. Dis Aquat Org 72:53–64

OIE (World Animal Health Organisation) (2009) Manual of diagnostic tests for aquatic animals. Chapter 2.2.1. Crayfish plague (*Aphanomyces astaci*). Office International des Epizooties, Paris, p 63–77

OIE (2012) Manual of diagnostic tests for aquatic animals, 7th edn. Chapter 2.2.1. Crayfish plague (*Aphanomyces astaci*). Office International des Epizooties, Paris, p 101–118

Paaver T, Hurt M (2009) Status and management of noble crayfish *Astacus astacus* in Estonia. Knowl Manag Aquat Ecosyst 394-395:18

Pârvulescu L, Schrimpf A, Kozubíková E, Cabanillas Resino S, Vrålstad T, Petrusek A, Schulz R (2012) Invasive crayfish and crayfish plague on the move: first detection of the plague agent *Aphanomyces astaci* in the Romanian Danube. Dis Aquat Org 98:85–94

Peay S (2003) Monitoring the white-clawed crayfish *Austropotamobius p. pallipes*. In: Parr L (tech ed) Conserving Natura 2000 Rivers, Monitoring Series No. I. English Nature, Peterborough

Peay S, Füreder L (2011) Two indigenous European crayfish under threat — How can we retain them in aquatic ecosystems for the future? Knowl Manag Aquat Ecosyst 401:33

Persson M, Söderhäll K (1983) *Pacifastacus leniusculus* (Dana) and its resistance to the parasitic fungus *Aphanomyces astaci* Schikora. Freshw Crayfish 5:292–298

Schrimpf A, Pârvulescu L, Copila -Ciocianu D, Petrusek A, Schulz R (2012) Crayfish plague pathogen detected in the Danube Delta — a potential threat to freshwater biodiversity in southeastern Europe. Aquat Invasions 7:503–510

Skov C, Aarestrup K, Sivebæk F, Pedersen S, Vrålstad T, Berg S (2011) Non-indigenous signal crayfish *Pacifastacus leniusculus* are now common in Danish streams: preliminary status for national distribution and protective actions. Biol Invasions 13:1269–1274

Souty-Grosset C, Holdich DM, Nöel PYN, Reynolds JD, Hauffner P (2006) Atlas of crayfish in Europe. Muséum National d'Histoire Naturelle, Paris

Strand DA, Holst-Jensen A, Viljugrein H, Edvardsen B, Klaveness D, Jussila J, Vrålstad T (2011) Detection and quantification of the crayfish plague agent in natural waters: direct monitoring approach for aquatic environments. Dis Aquat Org 95:9–17

Šulgaj A (1937) Naš potočni rak. Zveza ribarskih društev Dravske banovine, Ljubljana (in Slovene)

Svoboda J, Kozubíková E, Kozák P, Kouba A and others (2012) PCR detection of the crayfish plague pathogen in narrow-clawed crayfish inhabiting Lake Eğirdir in Turkey. Dis Aquat Org 98:255–259

Tokeshi M (1999) Species coexistence. Ecological and evolutionary perspectives. Blackwell Science, London

Trontelj P, Machino Y, Sket B (2005) Phylogenetic and phylogeographic relationships in the crayfish genus *Austropotamobius* inferred from mitochondrial COI gene sequences. Mol Phylogenet Evol 34:212–226

Tuffs S, Oidtmann B (2011) A comparative study of molecular diagnostic methods designed to detect the crayfish plague pathogen, *Aphanomyces astaci.* Vet Microbiol 153:343–353

Unestam T (1969) Resistance to the crayfish plague in some American, Japanese and European crayfishes. Rep Inst Freshw Res Drottningholm 49:202–209

Unestam T (1976) Defence reactions in and susceptibility of Australian and New Guinean freshwater crayfish to European-crayfish-plague fungus. Aust J Exp Biol Med Sci 53:349–359

Vaerman JL, Saussoy P, Ingargiola I (2004) Evaluation of real-time PCR data. J Biol Regul Homeost Agents 18: 212–214

Vennerström P, Söderhäll K, Cerenius L (1998) The origin of two crayfish plague (*Aphanomyces astaci*) epizootics in Finland on noble crayfish, *Astacus astacus.* Ann Zool Fenn 35:43–46

Vey A, Söderhäll K, Ajaxon R (1983) Susceptibility of *Orconectes limosus* Raff. to the crayfish plague, *Aphanomyces astaci* Schikora. Freshw Crayfish 5:284–291

Viljamaa-Dirks S, Heinikainen S, Nieminen M, Vennerström P, Pelkonen S (2011) Persistent infection by crayfish plague *Aphanomyces astaci* in a noble crayfish population — a case report. Bull Eur Assoc Fish Pathol 31: 182–188

Vrålstad T, Knutsen AK, Tengs T, Holst-Jensen A (2009) A quantitative TaqMan® MGB real-time polymerase chain reaction based assay for detection of the causative agent of crayfish plague *Aphanomyces astaci.* Vet Microbiol 137:146–155

Vrålstad T, Johnsen SI, Ferreira Fristad R, Edsman L, Strand D (2011) Potent infection reservoir of crayfish plague now permanently established in Norway. Dis Aquat Org 97:75–83

Weinländer M, Füreder L (2012) Associations between stream habitat characteristics and native and alien crayfish occurrence. Hydrobiologia 693:237–249

Zhang Z, Schwartz S, Wagner L, Miller W (2000) A greedy algorithm for aligning DNA sequences. J Comput Biol 7: 203–214

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