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Detection of *Aphanomyces astaci* in North American crayfish by polymerase chain reaction

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ABSTRACT: We present a PCR based method to detect *Aphanomyces astaci* in North American crayfish. Primers were designed to specifically amplify parts of the internal transcribed spacer (ITS) regions and the 5.8 rRNA gene of *A. astaci*. A single round and a semi-nested assay were tested for their sensitivity and specificity. Specificity of the PCR assays was tested against several closely related *Aphanomyces* species, other Oomycetes and some non-*A. astaci* DNA that might be found in or on crayfish. The single round assay was fully specific against all DNA tested. In the semi-nested assay, cross-reaction was seen when the equivalent of 40 000 or more genomic units of *A. invadans* or *A. frigidophilus* were entered into the PCR reaction. The lower detection limit of both assays lies around 1 genomic unit of *A. astaci*. Investigation of various parts of the exoskeleton of 3 North American crayfish species revealed that for *O. limosus* and *P. leniusculus* the telson and soft abdominal cuticle yielded a positive PCR reaction most frequently. For the third species, *Procambarus clarkii*, only 1 individual tested positive, so no conclusion as to preferred infestation site(s) could be drawn.

KEY WORDS: *Aphanomyces astaci* · Oomycetes · Detection · Polymerase chain reaction · North American crayfish · *Orconectes limosus* · *Pacifastacus leniusculus* · Internal transcribed spacer region

INTRODUCTION

Aphanomyces astaci (Oomycetes) is the causative agent of crayfish plague in susceptible species. Highly susceptible crayfish species are those native to Europe, Asia and Australia (Unestam 1969a, 1972). *A. astaci* is highly adapted to parasitise crayfish cuticle, as is reflected in its ability to produce chitinase (but no cellolytic or pectinolytic enzymes), allowing it to penetrate crayfish cuticle (Unestam 1966, 1969b). Other indicators for its specialisation on crayfish as hosts are its restriction to glucose and amino acids as carbon nutrition sources (Unestam 1965, 1966) and its limited survival time outside a crayfish host (Söderhäll & Cerenius 1999). These features distinguish *A. astaci* from closely related saprophytic Oomycetes.

North American crayfish have been shown to carry *Aphanomyces astaci* in melanised spots in their cuticle, while remaining otherwise largely unaffected (Unestam 1972, Persson & Söderhäll 1983, Vey et al. 1983, Diéguez-Uribeondo & Söderhäll 1993). Several factors seem to contribute to the low susceptibility of North American crayfish, e.g. enhanced melanisation of *A. astaci* hyphae by the crayfish host (Unestam & Weiss 1970), which is likely to be an effect of continuously high mRNA levels of the melanin producing enzyme prophenoloxidase (Cerenius et al. 2003). North American crayfish species are regarded as the natural hosts of *A. astaci* (Unestam 1972, Cerenius et al. 1988, Alderman et al. 1990).

The first outbreaks of crayfish plague in Europe date back to the latter half of the 19th century (Alderman 1996). Infections in populations of crayfish species native to Europe lead to rapid epidemics and have usually resulted in the total extinction of affected populations. Since its first arrival in Europe (in either Italy or France), the disease has reached most European countries (Alderman 1996). It's spread is closely linked to

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that of North American crayfish (Henttonen & Huner 1999, Holdich 2003). Although North American crayfish had been imported into Europe earlier, their biggest impact and spread was due to widespread programmes introducing signal and red swamp crayfish for farming purposes that started in the 1960s (Rogers & Holdich 1995, Alderman 1996, Rogers 1996).

North American crayfish populations can now be found in almost every European country (Holdich 2003). Their impact on native crayfish populations is severe. In Sweden, where the situation is probably best documented, 90% of all native crayfish populations have disappeared (Edsman 2000).

In the past it has been assumed that every North American crayfish population is infected with *Aphanomyces astaci*. However, there is increasing evidence that this may not be the case. An increasing number of reports describe the coexistence of North American and European crayfish (Holdich & Domaniewski 1995, Söderbäck 1995, Peay & Rogers 1999), suggesting that some North American crayfish populations might not be carrying the pathogen.

PCR methods are especially useful where cultivation of the pathogen is difficult and other methods of diagnosis such as clinical symptoms or pathology are not sufficient for reliable diagnosis. This is the case with *Aphanomyces astaci*. Cultivation already poses a problem in heavily infected susceptible crayfish, due to frequently present water moulds overgrowing the slow growing pathogen or due to bacteria inhibiting *A. astaci* growth (Oidtmann et al. 1999). Since isolation of *A. astaci* from North American crayfish is difficult (Cerenius et al. 1988, Oidtmann unpubl.), a PCR based approach appears to be the most suitable method of choice.

The purpose of the study was to develop a method which detects *Aphanomyces astaci* in clinically inapparent carriers, i.e. crayfish of North American origin. In order to decide where material should be collected from for DNA extraction, it was important to gather data on the distribution of infection across the exoskeleton in the North American crayfish species. From a previous study on the histopathology of crayfish plague in Noble crayfish *Astacus astacus* (Oidtmann et al. unpubl.), we knew that soft cuticle is the preferred site of entry in a susceptible species. In this study we therefore compared parts of the exoskeleton, which consist to a greater or lesser extent of soft cuticle (soft abdominal cuticle, proximal walking leg, dorsal abdomen, telson). Since it was not known whether *A. astaci* would infect different parts of the exoskeleton in a species of low susceptibility, we also sampled hard cuticle (cephalothorax). Although the telson had not been recognised as being infected in Noble crayfish, we included it in this study with the view that (if infected) this part of the exoskeleton might serve as a non-lethal sampling site. We also had no prior knowledge on prevalence of infection within North American crayfish populations or on infestation levels in individual crayfish. As infestation levels would be expected to be much lower in North American crayfish than in clinically diseased susceptible crayfish, the test would have to be highly sensitive.

Recently, some new *Aphanomyces* strains (provisionally termed *A. repetans*) have been described, which have been isolated from North American crayfish species. When tested for their virulence to crayfish species susceptible to *A. astaci*, these strains failed to cause disease in the European Noble crayfish *Astacus astacus* and the Australian *Cherax destructor* (Royo et al. 2004). Additionally, we recently isolated an *Aphanomyces* strain from the signal crayfish *Pacifastacus leniusculus*. This strain shared 100% sequence identity in the ITS region with *A. frigidophilus* (Accession No. AY647192), which has been isolated from Japanese char eggs in Japan. At present, geographical areas affected by *Aphanomyces astaci* overlap very little with areas affected by *A. invadans*. Since this is likely to change over time, we wanted to ensure that our test would not cross-react with any closely related *Aphanomyces* species.

MATERIALS AND METHODS

Oomycetes and other organisms tested. The *Aphanomyces astaci* strains as well as the other organisms from which DNA was extracted to test the specificity of the primers used in the method presented are listed in Table 1.

DNA extraction. The methods used to extract DNA from pure oomycete and fungal cultures as well as from crayfish muscle tissue and crayfish pathogens such as *Psorospermium haeckeli* or *Citrobacter freundlii* have been described in a previous study (Oidtmann et al. 2004).

DNA extraction from crayfish cuticle: Crayfish were euthanised by exposure to chloroform vapours. In the areas of the body to be sampled, the cuticle was gently wiped clean with single use tissues and cotton swabs moistened with sterile distilled water to remove external contamination. We excised 30 to 50 mg of cuticle per sampling site using scissors and forceps. This was then cleaned of attached muscle or connective tissue and ground in liquid nitrogen. DNA was extracted using the DNeasy tissue kit (Qiagen) following the manufacturer's instructions. An additional centrifugation step was introduced after the digestion step and before loading the DNA onto the column. Only the fluid supernatant was transferred onto the extraction

Table 1. Species used in specificity test of PCR assays. ID No.: identification no.; *Hph*I digest: product of Primers 525/640 digested by *Hph*I; +: yes; –: no; (+): weak band

Species	ID No.	PCR product with primers 42/640	PCR product with primers 525/640	*Hph*I digest
Aphanomyces astaci				
Genetic Group A	L1	+	+	+
A	Sv	+	+	+
A	Ra	+	+	+
A	Da	+	+	+
A	Vl	+	+	+
Genetic Group B	Ti	+	+	+
B	Yx	+	+	+
B	Hö	+	+	+
B	FDL457	+	+	+
B	M96/1	+	+	+
B	M96/2	+	+	+
B	Si	+	+	+
B	Pl	+	+	+
B	SA	+	+	+
Genetic Group C	Kv	+	+	+
Genetic Group D	Pc	+	+	+
	F	+	+	+
	01	+	+	+
***Saprolegnia* spp.**				
S. parasitica	CBS 540.67	–	–	
S. declina		–	–	
S. litoralis		–	–	
S. furkata		–	–	
S. ferax	ATCC 26116	–	–	
S. terrestris	UK2102	–	–	
Other Oomycetes				
Leptolegnia sp.	CBS 177.86			
Pythium flevoense (female)	CBS 232.72	–	–	
Isoachlya turoloides, syn. *Saprolegnia monilifera*	CBS 558.67	–	–	
Isoachlya eccentrica		–	–	
Other *Aphanomyces* species				
A. frigidophilus		–	(+)	+
A. invadans	B99C	–	(+)	–
	UM3	–	(+)	–
	NJM 9701	–	(+)	–
	PA –8	–	(+)	–
	WIC1	–	(+)	–
	PA 7	–	(+)	–
	T99G2	–	(+)	–
A. laevis	CBS 107.52	–	–	
A.helicoides	CBS 210.82	–	–	
A. irregulare	CBS 278.81	–	–	
A. stellatus, syn. *Achlya racemosa*	CBS 578.67	–	–	
A. brassicae	CBS 121.80	–	–	
A. cochloides	CBS 477.71	–	–	
A. iridis	CBS 524.87	–	–	
A. euteiches	CBS 154.73	–	–	
A. cladogamus	CBS 108.29	–	–	
Crayfish parasites				
Psorospermium haeckeli		–		
Thelohania contejeani		–		
Bacterial species				
Citrobacter freundii		–		
Aeromonas hydrophila		–		
Hafnia alvei		–		
Crayfish muscle tissue				
Orconectes limosus		–		
Pacifastacus leniusculus		–		
Procambarus clarkii		–		
Fungal species				
Fusarium solani	CBS 181.29			
Mucor sp.		–	–	
Aspergillus sp.		–	–	
Candida albicans		–	–	
Trichosporon beigelii = *T. cutaneum*	DSM 70675	–	–	

columns, since partially undigested cuticle particles would otherwise have blocked these.

DNA extraction from Aphanomyces astaci spores: Spores were obtained from *A. astaci* Isolate M96/1, using the methods described previously by Cerenius et al. (1988). A spore solution was adjusted to 10 000 spores ml^{-1}. The spore concentration was confirmed by counting in a Neubauer counting chamber. From this stock solution, a 10-fold dilution series was prepared down to a theoretical spore concentration of 0.1 spore ml^{-1}. The concentration of 1000 spores ml^{-1} was confirmed using the Neubauer counting chamber. (At lower concentrations, the result of counting becomes unreliable because the number of spores counted is too low.)

To reduce volume, 1 ml of the spore solution was centrifuged at 10 000 × *g* for 10 min, and 950 µl of the supernatant were removed after centrifugation. The pellet was resuspended in the remaining water and the DNA extraction was performed following the instructions provided by the manufacturer.

DNA extraction of cuticle spiked with Aphanomyces astaci spores: The *A. astaci* spore dilution series (see last subsection above) was used to spike crayfish cuticle. We reduced 1 ml of each concentration of spore solution to a smaller volume as described above. Fifty mg of soft abdominal cuticle, obtained from a healthy Noble crayfish *Astacus astacus* after euthanasia, was ground in liquid nitrogen and the powder transferred to the reaction tube containing the concentrated spore solution. The DNA extraction procedure was performed as described above for crayfish cuticle.

Selection of *Aphanomyces astaci* specific primers. Sequences of the ITS 1 and 2 region and the ribosomal RNA gene from a number of *Aphanomyces* spp. were aligned with those of *A. astaci* using Megalign (DNAstar Lasergene). Sequences were either our own sequence data or sequences that were publicly accessible in GenBank (www.ncbi.nlm.nih.gov/). Primer 42 (5′ GCT TGT GCT GAG GAT GTT CT 3′) was chosen based on 100% homology with sequences from various *A. astaci* strains and greatest divergence from

other *Aphanomyces* species. Primers 525 (5′ AAG AAG GCT AAA TTG CGG TA 3′) and 640 (5′ CTA TCC GAC TCC GCA TTC TG 3′) have been described in an earlier study (Oidtmann et al. 2004).

PCR protocols to confirm presence of sufficient amounts of DNA when testing non-*Aphanomyces astaci* DNA. To ensure that the absence of an amplicon using Primers 42, 525 and 640 was not due to insufficient amounts of DNA in the PCR reaction, control PCRs were run using primers that would amplify a product with the DNA of the organisms in question. For all Oomycetes, Primers ITS1 and ITS4 (ITS 1: 5′-TCC GTA GGT GAA CCT GCG G-3′; ITS 4: 5′-TCC TCC GCT TAT TGA TAT GC-3′; White et al. 1990) were used; the primers applied for the other organisms have been described previously (Oidtmann et al. 2004).

PCR protocol. ***Single round PCR with Primers 42 and 640:*** PCR amplifications were carried out in a 50 µl reaction volume containing Reddy Mix PCR Mastermix (ABgene AB-0575, with 1.5 mM $MgCl_2$) and Primers 42 and 640 at a final concentration of 0.5 µM each. The mixture was denatured at 96°C for 5 min, followed by 50 cycles of 1 min at 96°C, 1 min at 59°C, 1 min at 72°C, followed by a final extension step of 7 min at 72°C.

Semi-nested PCR: PCR conditions for the PCR first round were as described in the preceding subsection, except that only 20 amplification cycles were run.

For the second PCR round, 1 µl from the first round were added to a mix containing Reddy Mix PCR Mastermix (ABgene AB-0575, with 1.5 mM $MgCl_2$) and Primers 525 and 640 at a final concentration of 0.5 µM each. The following cycling conditions were applied: 5 min at 95°C, 35 cycles of 95, 59, and 72°C for 1 min respectively, followed by a final extension step of 7 min at 72°C.

Test for sensitivity of PCR assays. Sensitivity of the PCR protocols was investigated using DNA extracted from mycelium and spores of *Aphanomyces astaci* strain M96/1.

After extraction of DNA from the mycelium, the DNA concentration of the extract was measured in a spectrophotometer, and 10-fold serial dilutions were prepared and subjected to analysis.

For testing the sensitivity of the PCR assay using *Aphanomyces astaci* spores, 10 µl of the DNA solution obtained from the DNA extraction were submitted to the PCR reaction. Sensitivity was tested additionally using a 10-fold serial dilution of the DNA solution obtained from the extraction of 10 000 spores.

When testing the sensitivity of the assay using crayfish cuticle spiked with *Aphanomyces astaci* spores, 10 µl of the DNA solution obtained from the DNA extraction were submitted to the PCR reaction. Sensitivity was tested additionally by using a 10-fold serial dilution of the DNA solution obtained from the DNA extraction of cuticle spiked with 10 000 spores.

Analysis of PCR reactions. After completion of a PCR reaction, 6 µl from each tube were analysed by electrophoresis in 1.3 % agarose gel.

Testing cuticle from various areas of crayfish body. In order to investigate in which area on the crayfish body cuticle would most frequently be infected with *Aphanomyces astaci* (as indicated by a positive PCR result), cuticle was sampled from 6 different regions: soft abdominal cuticle, telson, proximal walking leg, dorsal abdomen, cephalothorax and eyestalks. Sampling sites are indicated in Fig. 1.

We tested 51 animals (26 *Orconectes limosus*, 16 *Pacifastacus leniusculus* and 9 *Procambarus clarkii*) for all 6 sources of crayfish cuticle using the semi-nested assay. In 30 of these 51 crayfish, the analysis was repeated using the single round assay (15 *O. limosus* and 15 *P. leniusculus* respectively). Using the semi-nested assay, the soft abdominal cuticle and telson were examined in an additinoal 88 individuals (23 *O. limosus* and 65 *P. leniusculus*). The crayfish originated from sites in Germany, Switzerland, Sweden, and England.

Transmission experiments. We placed 5 Noble crayfish *Astacus astacus* in plastic mesh cages (26 × 14 × 14 cm), in 70 l aquaria (1 cage per aquarium) with 10

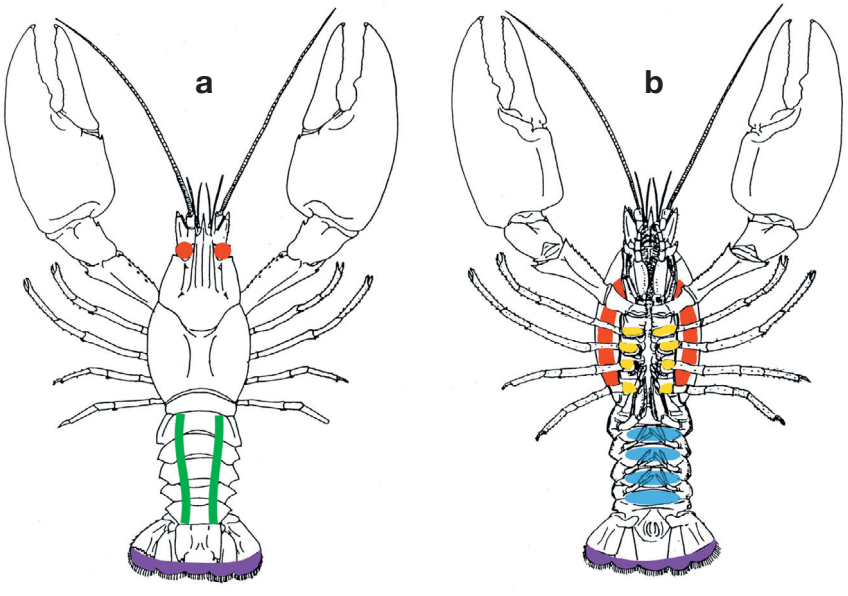

Fig. 1. Sampling sites of crayfish cuticle. (a) Dorsal view of crayfish: red, eyestalks; green and lilac, sampling areas on dorsal abdomen and telson respectively. (b) Ventral view: blue, red and yellow, sampling areas on soft abdominal cuticle, cephalothorax and walking legs respectively

uncaged North American crayfish (either *Orconectes limosus* or *Pacifastacus leniusculus*) in each aquarium. To prevent constant loss of *Aphanomyces astaci* spores, no flow-through system was used. Instead, water was changed every 2 wk. The aquaria were equipped with an oxygen disperser and the water temperature was 19 ± 1°C). We set up 8 aquaria in this way to test whether *O. limosus* and *P. leniusculus* from 8 different populations (7 *O. limosus* populations, 1 *P. leniusculus* population) would transmit *A. astaci* to the Noble crayfish. Prior to being placed in the aquaria, the North American crayfish were immuno-suppressed by withdrawing 2% weight volume haemolymph in relation to body weight. Melanisations were recorded in North American crayfish. Any Noble crayfish found dead were removed and examined by light microscopy for the presence of hyphae in their soft abdominal cuticle. Material for DNA extraction was collected from some North American crayfish that died during the study. The cuticle sampled was preferentially from areas with melanisations. The experiments were terminated after an observation period of just over 9 mo (278 d).

RESULTS

Choice of primers to detect *Aphanomyces astaci*

The positions of Primers 42, 525 (both forward primers) and 640 (reverse primer) in relation to the *Aphanomyces astaci* sequence and the sequences of other *Aphanomyces* species are shown in Fig. 2. Primer 42 diverges in the target area by at least 7 bp from the closely related Oomycetes, and Primers 525 and 640 by at least 2 and 3 bp respectively.

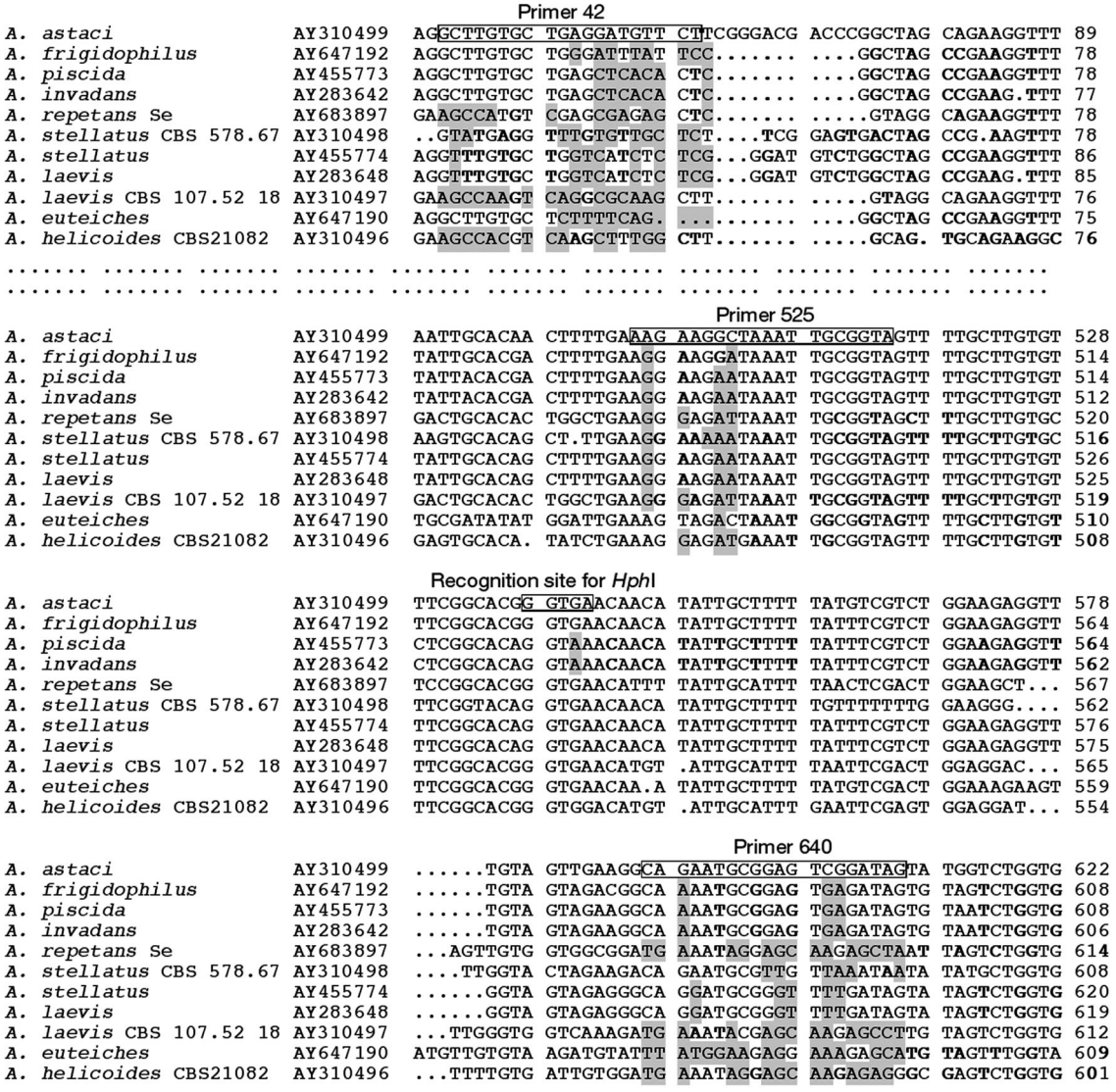

Fig. 2. *Aphanomyces* spp. sequence alignment of partial internal transcribed spacers 1 and 2 showing position of Primers 42, 525 and 640 and restriction enzyme recognition site. GenBank accession nos. of sequences provided. Nucleotides deviating from primer sequences are shaded in grey

Specificity of Primers 42 and 640

Using Primers 42 and 640, an amplicon of the expected length of 569 bp was obtained with all *Aphanomyces astaci* strains included in this study (Fig. 3). No PCR product was observed when submitting DNA of any of the other organisms listed in Table 1 to the PCR. Results for *A. invadans* and *A. frigidophilus* are presented in Fig. 4. To ensure that the lack of a product was not due to insufficient amounts of DNA being submitted to the PCR, a positive control was run. Amplicons were obtained with all DNA except for *Aeromonas hydrophila*.

Specificity of Primers 525 and 640

We had shown in a previous study that Primers 525 and 640 discriminate *Aphanomyces astaci* from a large number of other organisms (Oidtmann et al. 2004). In the process of developing the assay to detect *A. astaci* in carrier crayfish, we also tested the specificity of this primer set against *A. invadans* and *A. frigidophilus*. PCR assays of large amounts of DNA extracted from pure cultures of these Oomycetes obtained products of the size expected for *A. astaci* (Fig. 4).

Detection threshold of PCR

The detection thresholds for both the single round and the semi-nested PCR assay were the same. For cellular template DNA purified from *Aphanomyces astaci* mycelium, the detection limit was 100 fg. PCR assays of DNA extracted from *A. astaci* spores still obtained a product when 1 spore had been submitted to the initial DNA extraction. The same detection limit was found for spiked samples. The results for the single round PCR assays are shown in Figs. 5 to 7.

Testing cuticle from various areas of crayfish body

Crayfish from 3 different species were tested using the semi-nested assay (n = 51). Overall a positive PCR result was most frequently obtained using either soft abdominal cuticle (27 individuals positive) or telson (26 individuals). Proximal walking leg and dorsal abdomen were less frequently positive (21 and 18 individuals respectively), whereas a positive result from either cephalothorax or eyestalk was rare (4 and 1 individuals respectively).

Differences between crayfish species were noticeable. The soft abdominal cuticle was most frequently positive

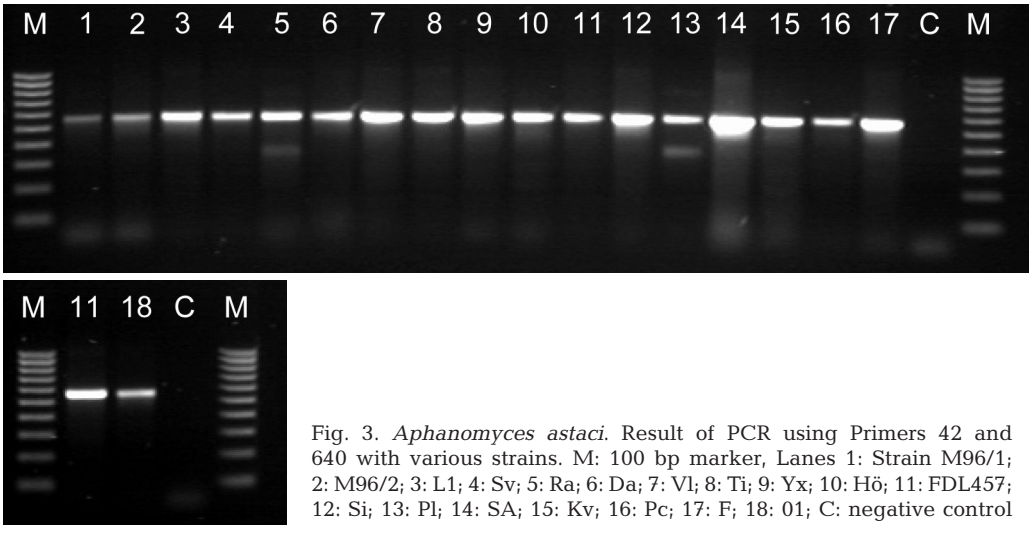

Fig. 3. *Aphanomyces astaci*. Result of PCR using Primers 42 and 640 with various strains. M: 100 bp marker, Lanes 1: Strain M96/1; 2: M96/2; 3: L1; 4: Sv; 5: Ra; 6: Da; 7: Vl; 8: Ti; 9: Yx; 10: Hö; 11: FDL457; 12: Si; 13: Pl; 14: SA; 15: Kv; 16: Pc; 17: F; 18: 01; C: negative control

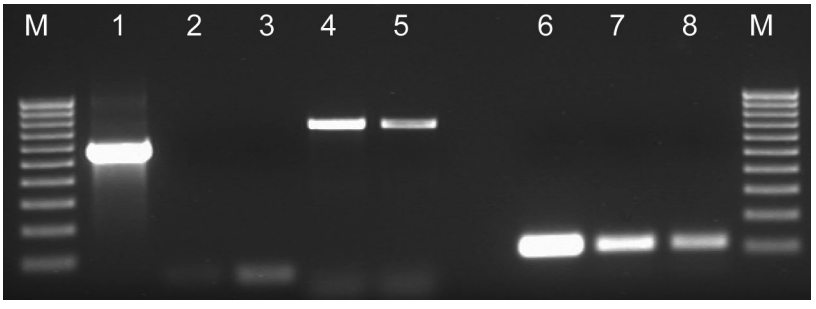

Fig. 4. *Aphanomyces* spp. Single round and semi-nested PCR with *A. frigidophilus* and *A. invadans* DNA. M: 100 bp marker; Lanes 1 to 3: PCR products using Primers 42 and 640; 1: *A. astaci* DNA (1 ng); 2: *A. frigidophilus* (100 ng); 3: *A. invadans* (100 ng); Lanes 4 and 5: positive controls for *A. frigidophilus* and *A. invadans* using primers ITS 1 and 4; 4: *A. frigidophilus* (100 ng); 5: *A. invadans* (100 ng); Lanes 6 to 8: PCR products using semi-nested assay; 6: *A. astaci* DNA (1 ng); 7: *A. frigidophilus* (100 ng); 8: *A. invadans* (100 ng)

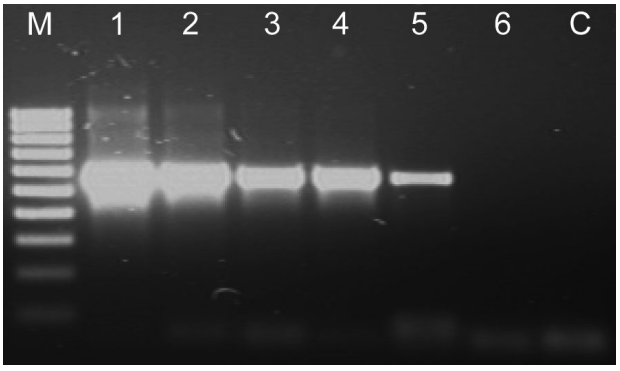

Fig. 5. Sensitivity of single round PCR assay using different amounts of genomic *Aphanomyces astaci* DNA. M: 100 bp marker; Lanes 1: 1 ng; 2: 100 pg, 3: 10 pg, 4: 1 pg, 5: 100 fg, 6: 10 fg; C: negative control

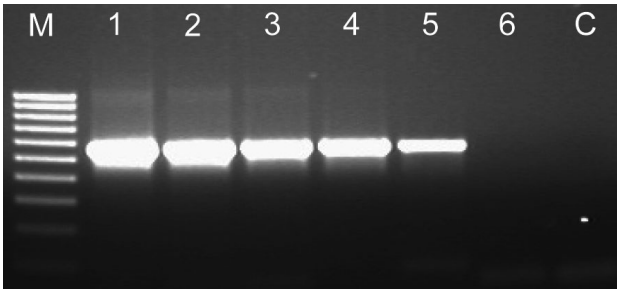

Fig. 6. Sensitivity of single round PCR using DNA extracted from *Aphanomyces astaci* spores. M: 100 bp marker; Lanes 1: 10 000 spores submitted to DNA extraction; 2: 1000 spores; 3: 100 spores; 4: 10 spores; 5: 1 spore; 6: 0.1 spore; C: negative control. Note: Spore numbers indicate number of spores submitted to DNA extraction; however, only 10 µl of the ca. 100 µl DNA extract were entered into the PCR reaction

in *Orconectes limosus*, whereas in *Pacifastacus leniusculus* the telson scored highest. In *Procambarus clarkii* only 1 of 9 individuals tested positive for both the soft abdominal cuticle and proximal walking leg (Table 2).

Comparison of results using single round and semi-nested assay

DNA extracts, which had been positive using the semi-nested assay, were retested using the single round assay 2 yr after the initial semi-nested PCRs had been run. Of 68 tissue extracts which had previously been positive using the semi-nested assay, 10 were now negative in the single round PCR (5× dorsal abdomen, 3× cephalothorax, 1× eyestalks and 1×

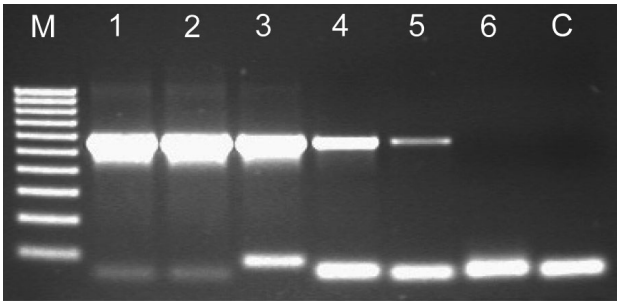

Fig. 7. Sensitivity of single round PCR after DNA extraction of 50 mg soft abdominal *Astacus astacus* cuticle spiked with dilution series of *Aphanomyces astaci* spores. M: 100 bp marker; Lanes 1: cuticle spiked with 10 000 spores; 2: 1000 spores; 3: 100 spores; 4: 10 spores; 5: 1 spore; 6: 0.1 spore; C = negative control

Table 2. *Orconectes limosus*, *Pacifastacus leniusculus* and *Procambarus clarkii*. Results (no. of tests positive) of semi-nested PCR assay comparing 6 different tissues. n: no. of individuals tested

Species	n	Soft abdominal cuticle (SAC)	Telson (T)	Proximal walking leg	Dorsal abdomen	Cephalo-thorax	Eye-stalk	Animals diagnosed as infected	Animals diagnosed as infected based on T and SAC only
O. limosus									
Population 1	15	13	12	11	11	3	0	15	15
Population 2	5	4	4	3	2	0	0	4	4
Population 3	5	5	3	4	2	0	0	5	5
Population 4	1	0	0	0	0	0	0	0	0
Total	26	22	19	18	15	3	0	24	24
P. leniusculus									
Population 1	15	4	7	2	3	1	1	10	10
Population 2	1	0	0	0	0	0	0	0	0
Total	16	4	7	2	3	1	1	10	10
P. clarkii									
Population 1	1	1	0	1	0	0	0	1	1
Population 2	3	0	0	0	0	0	0	0	0
Population 3	5	0	0	0	0	0	0	0	0
Total	9	1	0	1	0	0	0	1	1
Total all crayfish	51	27	26	21	18	4	1	35	35

Table 3. *Orconectes limosus* and *Pacifastacus leniusculus*. Comparison of PCR results (no. of tests positive) for various sampling areas of crayfish exoskeleton using single round and semi-nested assay

Species	Soft abdominal cuticle (SAC)	Telson (T)	Proximal walking leg	Dorsal abdomen	Cephalo-thorax	Eye-stalk	Animals diagnosed as infected	Animals diagnosed as infected based on T and SAC only
O. limosus (n = 15)								
Semi-nested	13	12	11	11	3	0	15	15
Semi-nested after 2 yr	13	12	11	11	3	0	15	15
Single round after 2 yr	13	12	10	8	1	0	15	15
P. leniusculus (n = 15)								
Semi-nested	4	7	2	3	1	1	10	10
Semi-nested after 2 yr	4	8	2	1	1	0	10	10
Single round after 2 yr	4	8	2	1	0	0	10	10

proximal walking leg). The results for soft abdominal cuticle remained the same, whereas 1 additional sample of telson was identified as positive using the single round assay. The DNA was also retested with the semi-nested assay; 3 previously positive samples were now (2 yr after the initial PCR) negative (Table 3).

Comparison of soft abdominal cuticle versus telson

Soft abdominal cuticle and telson DNA extracts from 139 individuals were analysed. The telson was positive slightly more frequently than the soft abdominal cuticle (Table 4).

Table 4. *Orconectes limosus*, *Pacifastacus leniusculus* and *Procambarus clarkii*. PCR results (no. of tests positive) for soft abdominal cuticle and telson. Abbreviations and further details as in legend to Table 2. *: Results obtained using single round PCR assay

Species	n	Soft abdominal cuticle (SAC)	Telson (T)	Animals diagnosed as infected based on SAC and T
O. limosus				
Population 1*	15	13	12	15
Population 2	5	4	4	4
Population 3	5	5	3	5
Population 4	1	0	0	0
Population 5	13	3	1	4
Population 6	10	0	8	8
Total	49	25	28	36
P. leniusculus				
Population 1*	15	4	8	10
Population 2	1	0	0	0
Population 3	30	10	16	20
Population 4	15	8	3	9
Population 5*	20	0	0	0
Total	81	22	26	39
P. clarkii				
Population 1	1	1	0	1
Population 2	3	0	0	0
Population 3	5	0	0	0
Total	9	1	0	1
Total all crayfish	139	48	55	75

Transmission experiments

In all experimental groups, mortalities were observed both among North American and Noble crayfish. Mortalities among the North American crayfish were noted early in all groups: between 1 and 3 individuals had died by Day 4 in each group. After these initial mortalities, no mortalities occurred for at least 2 or 4 mo in 2 groups respectively. However, in 1 group (the only *Pacifastacus leniusculus* group), all signal crayfish had died by Day 12. The mortality rate throughout the course of the experiment was high. Only 9 out of 80 North American crayfish, spread across 4 groups, were still alive when the experiment was terminated (Day 278). The mean survival time of the North American crayfish (not taking into account individuals that were euthanised on termination of the experiment) was 118.5 d (median 115.5 d, SD 97.77 d). The mean survival time of the Noble crayfish (not taking into account individuals that were killed on termination of the experiment) was 95 d (median 62 d, SD 87.65 d). Only 1 Noble crayfish was still alive by the end of the experiments.

To investigate whether transmission of *Aphanomyces astaci* had occurred, Noble crayfish that died were examined for the presence of hyphae. Based on the investigation of cohabitant Noble crayfish, 2 groups of North American crayfish could be identified as carriers of *A. astaci*. Strong hyphal infestation of the soft abdominal cuticle was observed in 2 and 3 out of the 5 Noble crayfish from these groups respectively. *A. astaci* was confirmed by PCR. The first mortality in Noble crayfish due to transmission of crayfish plague occurred 10 d after first contact. In 2 further groups, hyphal infestation was seen at a very low level, but was not confirmed as *A. astaci* by PCR. In all other groups, no hyphae were noted.

We also tested 44 North American crayfish from the 8 populations using the semi-nested PCR assay. Of these, 37 tested positive. The 2 populations shown to be infected by transmission experiments to Noble cray-

fish were confirmed to be infected by direct PCR from North American crayfish but, importantly, 4 additional populations were also identified as infected. Only 7 individuals tested negative; among these were 4 individuals from the 2 populations in which *Aphanomyces astaci* infection could not be shown.

Cuticle melanisations had been recorded in the North American crayfish before the start of the experiments. Melanisations were noted in all North American crayfish groups. In 1 of the 2 groups in which *Aphanomyces astaci* was not found, only 1 out of 10 individuals showed melanisations (*Pacifastacus leniusculus* group). In the other *A. astaci*-negative group, 7 out of 10 *Orconectes limosus* showed melanisations. In the 6 groups identified as infected by PCR, frequency of individuals with melanisations varied between 7 and 10 out of 10 crayfish.

DISCUSSION

Of the 6 sites of the crayfish exoskeleton sampled, the soft abdominal cuticle and telson tested positive most frequently for *Aphanomyces astaci*. The 51 crayfish investigated for this part of the study consisted of individuals from 3 different North American crayfish species from 9 different populations. The species tested represent those found most frequently across Europe. In order to avoid bias, we included more than one population per species.

The best data are for *Orconectes limosus*, in which, of 26 individuals tested, 24 were infected. Positive individuals originated from 3 different populations. The soft abdominal cuticle tested positive most frequently, closely followed by the telson and proximal walking leg.

In *Pacifastacus leniusculus*, 10 out of 16 individuals, derived from 2 populations, tested positive. All positive individuals came from 1 of the 2 populations tested. The telson was most frequently infested. Since the data on the distribution of infestation sites originate from a single population only, they may not be representative for other populations. Therefore more individuals from a variety of populations should be tested to substantiate those results.

Since only a single specimen of *Procambarus clarkii* tested positive, no conclusions could be drawn on preferred infestation sites for this species.

Based on the findings from 6 tissue sampling sites, we focused on the soft abdominal cuticle and telson and analysed further crayfish, examining these 2 areas only. Combined with the results from the 6 different sampling sites, we eventually had sufficient data for the soft abdominal cuticle and telson of 139 individuals. As before, the soft abdominal cuticle and telson tested positive with similar frequencies; there was no clear advantage of testing one tissue over the other. However, it was found that testing both tissues did have a clear advantage: instead of 48 or 55 individuals that would have been recognised as infected using either soft abdominal cuticle or telson, analysis of both tissues identified 75 individuals as carriers. Testing any of the other tissues in addition to the soft abdominal cuticle or telson never identified an additional individual as positive. Therefore, our results suggest that (1) the soft abdominal cuticle and telson are the best tissues to sample, and (2) analysing both substantially increases the test sensitivity at the individual level.

The detection threshold of both single round (50 cycles) and semi-nested (20/35 cycles) assay was 100 fg of cellular DNA extracted from *Aphanomyces astaci* mycelium. In relation to data provided on the genome size of other Oomycetes (Kamoun 2003), 100 fg would correspond to 0.4 to 4.5 genomic units.

PCR assays of DNA extracted from *Aphanomyces astaci* spores still obtained a product when the equivalent of 1 spore had been submitted to the initial DNA extraction. This equals the theoretical amount of DNA from 0.1 spore, since only 10 µl of the 100 µl DNA suspension obtained by DNA extraction was used in the PCR.

These detection limits correspond to those found in a previous assay for the detection of *Aphanomyces astaci* (Oidtmann et al. 2004) and are slightly lower than those reported by Phadee et al. (2004) for the detection of *A. invadans*, i.e. 1 pg (ca. 4 to 40 genomic units) of genomic *A. invadans* DNA in unspiked samples and 250 pg (ca. 1000 to 10 000 genomic units) in spiked samples. In the assay presented herein, the effect of using spiked samples was negligible, the most likely explanation being that cuticle is virtually free of DNA unless infected with pathogens, which means that no other DNA interferes in the PCR reaction.

Using the semi-nested PCR assay, a PCR product of the size expected for *Aphanomyces astaci* was obtained with 100 ng of *A. invadans* or *A. frigidophilus* DNA. The product was faint when 10 ng of DNA were used, and disappeared with 1 ng. According to the above calculations, 10 ng correspond to 4×10^4 to 10^5 genomic units. This indicates that a sequence difference of 2 or 3 bp between the template DNA and each of Primers 525 and 640 was insufficient to suppress non-specific amplification at high DNA concentrations under the PCR conditions applied. In contrast, Primer 42 differs from *A. invadans* by 7 bp and from *A. frigidophilus* by 8 bp, and was therefore expected to be more specific. This was supported by experimental results, as we did not obtain a product when running the single round PCR assay.

The semi-nested assay probably could have been improved by increasing the annealing temperature in

the second round of the PCR. However, in order to obtain a high level of sensitivity combined with a high level of specificity, the single round assay using Primer 42 in combination with Primer 640 appeared most convenient.

Most of the data obtained from testing North American crayfish for their carrier status were obtained using the semi-nested assay; 2 yr after some of the semi-nested PCRs had been run, we re-tested several DNA extracts using both single round and semi-nested assay. Most results were confirmed, but in a few cases, the PCR was no longer positive. This was more frequently the case using the single round assay compared to the semi-nested assay. The most likely explanation is that DNA degradation had occurred in the samples. This is to be expected when samples are stored for longer periods of time. This comprises one of the advantages of amplifying shorter PCR products, and is beneficial (for instance) when historical samples or crayfish in poor condition (frozen material, autolytic specimens, etc.) are investigated.

It could also be speculated that the PCR products were due to the presence of *Aphanomyces invadans* or *A. frigidophilus* DNA in the cuticle samples. However, the fact that *A. invadans* has not yet been found in Europe makes its presence highly unlikely. In addition, we excluded the presence of *A. invadans* using the *Hph* I restriction enzyme digest (*A. frigidophilus* cannot be distinguished with restriction enzyme *Hph* I). Furthermore, taking into account the infestation levels required to generate a PCR product (40 000 to 400 000 genomic units in a 30 to 50 mg cuticle sample), this appears highly unlikely.

It appears that the single round PCR has some advantages over the semi-nested assay. Apart from avoiding the small chance of cross-reactions, the former produces a longer PCR product, which is more useful for sequencing. Sequencing of PCR products is generally recommend to confirm the result. The semi-nested assay could be very useful, however, when the single round PCR does not yield a product and degraded DNA could be the cause. Interpretation of a positive result should then be done with care, bearing in mind the (low) chance of positive PCR reactions due to closely related Oomycetes.

Thus far we have isolated *Aphanomyces frigidophilus* only from a single signal crayfish population. In view of the presence of this new *Aphanomyces* strain in Europe, it would be interesting to investigate its prevalence among both North American crayfish species and crayfish susceptible to crayfish plague. There is potential for unexpected changes in the susceptibility of European crayfish species to *A. astaci* since an infection with the closely related *A. frigidophilus* might increase their prophenoloxidase levels, providing some protection against *A. astaci* without causing lethal infection.

In the past, caged sentinel crayfish have been used to determine whether watercourses were enzootic for *Aphanomyces astaci*. However, there are several reasons why such indicator crayfish might not develop crayfish plague: The cage could be placed in a position where crayfish would not come into contact with spores; or the North American crayfish may not have gone through a moulting cycle during the exposure period, which would make release of spores highly unlikely. The results of our small scale contact experiment confirm that the 'cage in the river' method is not very reliable. Using the contact method, only 2 out of 8 populations were identified as carriers, whereas the PCR assay detected 6 populations. In each of the 2 populations in which no crayfish was recognised as infected by PCR, only 2 individuals had been tested by PCR, and thus lack of detection may have been due to small sample size.

Considering that the conditions in the laboratory experiments were designed to enhance transmission of crayfish plague, the value of field experiments would appear even more limited. The crayfish were kept in close proximity, facilitating *Aphanomyces astaci* spores to find a new crayfish host by chemotaxis. Additionally, haemolymph had been withdrawn from the North American crayfish, and this was expected to compromise their ability to control a potentially latent *A. astaci* infection. In a previous (unpublished) study, we found that signal crayfish have significantly higher concentrations of granular haemocytes in their haemolymph than Noble crayfish. Granules of this haemocyte type contain important components of the crayfish immune system, including the precursor of phenoloxidase. Phenoloxidase plays an important role in the melanisation of invading pathogens, including *A. astaci* (Söderhäll & Ajaxon 1982, Cerenius & Söderhäll 2004). The higher concentration of granular haemocytes (and as a consequence a bigger reservoir of important enzymes) may partially explain the higher resistance of signal crayfish to crayfish plague compared to Noble crayfish.

Persson et al. (1987) have previously shown that acute crayfish plague in signal crayfish can be triggered by inducing a reduction of haemocyte numbers. Whereas Persson et al. (1987) achieved a decline of circulating haemocytes by administering zymosan, an ingredient of yeast cell walls, we chose to withdraw haemolymph. The proportion of mortality due to an activated *Aphanomyces astaci* infection in our experiments is not known: at least some mortalities appeared to be due to moulting and intraspecific competition.

Wider applications of PCR method

The method presented allows detection of North American crayfish as carriers of *Aphanomyces astaci*. Information gained from analysing crayfish could be used for various wider purposes: Knowledge of the carrier status of an individual North American crayfish population would allow assessment of the risk originating from it with respect to spread of the pathogen. A higher risk would be expected from a population with high prevalence than from a population with very low prevalence. Such information could (for instance) be used for decisions by the relevant authorities on live fish movements (e.g. for stocking purposes into the wild or transports between farms), which have been shown to be a potential pathway for the transmission of crayfish plague (Oidtmann et al. 2002). Furthermore, if eradication of North American crayfish populations is considered, priority could be given to populations with the highest infection levels.

Another application could be screening of crayfish imports and certification prior to dispatch. This would be of particular interest to those countries which decide to run programmes for the eradication of crayfish plague.

Ideally, any *Aphanomyces astaci* detected should also be strain typed. This would allow epidemiological data to be collected on the spread of the pathogen and possibly allow differentiation between isolates of different physiological properties, including virulence. The area targeted by the PCR assay is conserved between the various *A. astaci* isolates sequenced to date, and is therefore unsuitable for this purpose. Present strain typing methods for *A. astaci* rely on pure cultures (Huang et al. 1994). However, since the cultivation of *A. astaci* is difficult, possibilities for strain typing are extremely limited. Future research may eventually result in a suitable molecular method, targeting a different area of the genome, that is more suitable for this purpose.

Outlook

It has become clear that developing a diagnostic test for *Aphanomyces astaci* is a continuing process. During the development of the methods presented, 2 new *Aphanomyces* species were described. This led us to rearrange the protocol in order to ensure specificity.

Other closely related Oomycetes may still be identified. This emphasises the need to isolate and culture suspected Oomycetes from crayfish to provide comparative material for testing specificity of diagnostic methods, including PCR, and to identify new species. This will help to reveal new species and revise the present methods if needed.

It will also remain relevant to occasionally isolate *Aphanomyces astaci* from susceptible crayfish during plague outbreaks. Primer 42 is located in an area of high divergence, which makes it a good tool to distinguish *A. astaci* from other closely related *Aphanomyces* spp. However, if mutations were to occur in *A. astaci*, then this area could be a preferred site. This seems not to have happened so far, since sequence data from the various genetic groups do not diverge in the area in question.

The distribution of *Aphanomyces astaci* infection across the exoskeleton needs to be analysed in any new North American crayfish species for which this test is intended. This also applies to *Procambarus clarkii*, for which the data collected during this study were insufficient to make general predictions about suitable sampling sites.

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