LACK OF SPECIFICITY OF THE MOLECULAR DIAGNOSTIC METHOD FOR IDENTIFICATION OF *APHANOMYCES ASTACI*

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Reçu le 7 décembre 2006 *Received December 7, 2006*
Accepté le 15 mai 2007 *Accepted May 15, 2007*

ABSTRACT

A recent PCR-test developed for identification of *Aphanomyces astaci,* the organism responsible for crayfish plague, provided false positives for *Aphanomyces frigidophilus*, *Aphanomyces repetans,* and some *Saprolegnia* spp. Real-time PCR showed that with the designed primers, *A. astaci* and *A. frigidophilus* cannot be distinguished. The results of this study show that this particular crayfish plague PCR-test ought to be improved and that molecular-based techniques need to be contrasted to histological evidences and disease history.

Key-words: Oomycetes, crayfish plague, ITS, crayfish, Conservation, invasive species.

MANQUE DE SPÉCIFITÉ DE LA MÉTHODE DE DIAGNOSTIC MOLÉCULAIRE POUR L'IDENTIFICATION D'*APHANOMYCES ASTACI*

RÉSUMÉ

Un test PCR récemment développé pour identifier *Aphanomyces astaci*, organisme responsable de la peste de l'écrevisse, a donné des faux positifs pour *Aphanomyces frigidophilus*, *Aphanomyces repetans*, et pour quelques *Saprolegnia* spp. La PCR en temps réel montre, qu'avec les "primers" redessinés, *A. astaci* et *A. frigidophilus* ne peuvent pas être distingués. Les résultats de cette étude montrent que ce test PCR, conçu pour identifier la peste de l'écrevisse, doit être amélioré et que les techniques moléculaires doivent être confortées par des évidences histologiques et en fonction de l´histoire de la maladie.

Mots-clés : Oomycètes, peste de l'écrevisse, ITS, écrevisse, conservation, espèces invasives.

Article available at http://www.kmae-journal.org or http://dx.doi.org/10.1051/kmae:2007002

INTRODUCTION

The heterokont *Aphanomyces astaci* (*Saprolegniales, Oomycetes*) is responsible for the crayfish plague and is amongst the 100 worst invasive species (DIÉGUEZ-URIBEONDO *et al.,* 2006). This organism devastated the indigenous European crayfish and represents a threat for the Australasian crayfish species (UNESTAM, 1972). Recent molecular diagnosis method based on species-specific PCR primers for ITS sequences (OIDTMANN *et al.,* 2004) has allowed approaching the diagnosis of crayfish plague on clinical samples without carrying out tedious isolations and virulence experiments (CERENIUS *et al.,* 1988). However, this test has been only applied to a limited number of *Aphanomyces* species. Two new *Aphanomyces* species were recently isolated from crayfish and crayfish plague cases i.e. *Aphanomyces frigidophilus* (BALLESTEROS *et al.,* 2006; KITANCHAROEN and HATAI, 1997), and *A. repetans* (ROYO *et al.,* 2004). The objective of this study was to confront the existing crayfish plague PCR-diagnostic test to recently described *Aphanomyces* species, and to evaluate the specificity of the primers designed by using real-time PCR. Real-time PCR allow detection of PCR amplification during the early phases of the reaction, and therefore quantify the degree of specificity for primers, which represents a distinct advantage over traditional PCR detection (LIVAK *et al.,* 1995).

MATERIALS AND METHODS

To test the application of this method both clinical samples and pure cultures of isolates were used. Clinical samples consisted of cuticles preserved in 70% ethanol from crayfish plague cases that occurred in Spain during 2004 and 2005. Sample 1, was from a crayfish plague-like case from which *A. frigidophilus* was isolated (BALLESTEROS *et al.,* 2006). Samples 2 and 4, belonged to crayfish plague cases, and sample 3, were from *Saprolegnia parasitica* infected crayfish. Pure cultures of isolates of *A. frigidophilus* SAP233, *A. repetans* SAP60, *A. astaci* strains L1, Kv1 and T1, *S. parasitica* SAP210, *Saprolegnia australis* SAP211 were used in the experiments. Pure cultures were grown as drop cultures (CERENIUS and SÖDERHÄLL, 1985), and genomic DNA was extracted using an E.Z.N.A.-Fungi DNA miniprepkit kit (Omega Biotek, Doraville, GA). For cuticle samples, total genomic DNA was extracted using a DNeasy kit for animal tissue (Qiagen, Valencia, CA). Genomic DNA was amplified with primers 525 and 640 as described in (OIDTMANN *et al.,* 2004) based on the ITS region surrounding the 5.8S rDNA gene from *A. astaci* strain FDL 457 (OIDTMANN *et al.,* 2004). PCR amplifications were carried out using PCR Beads (Amersham Biosciences, Piscataway, NJ) in 25 µl containing 0.4 µM of each primer and 150 ng of pure culture DNA and 1 µg of infected cuticle DNA. Cycling conditions were as described by (OIDTMANN *et al.,* 2004). Amplified products were detected by 2% agarose gel electrophoresis in TE buffer, stained with ethidium bromide and visualized under UV light. Real-time PCR was performed in a LigthCycler (Roche Molecular Biochemicals, Indianapolis, IN). In each capillary, 20 µl reactions were pipetted. The reactions contained 2 µl DNA template, 1 µM of each primer (525 and 640), 2 µl of the SYBR Green PCR Master Mix (Roche Molecular Biochemicals), 2.4 µl of 25 mM $MgCl_2$ and 11.6 µl of sterile water provided by the manufacturer. After a preincubation stage of 95°C for 30 s, amplifications were performed for 25 cycles of 95°C for 10 s, 52°C for 5 s, 62°C for 5 s and 72°C for 10 s. After amplification, a melting curve was generated by holding the reaction at 65°C for 15 s, and then heating to 95°C with a ramp rate of $0.1°C.s^{-1}$. To assign a melting temperature for each sample, the fluorescence signal was plotted against temperature. Aliquots of some DNA templates were used to repeat the assays in the ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). In each well, 25 µl reactions were pipetted containing 3 µl of DNA template, 0.2 µM of each primer, 12.5 µl of the SYBR® Green PCR Master Mix (Applied Biosystems) and 8.5 µl of sterile Milli-Q water. Controls, DNA-free, were run for each experiment. Amplification conditions were: (a) incubation step at 95°C for 10 min; (b) DNA amplification for 40 cycles of 95°C for 15 s, 55°C for 30 s and 72°C for 30 s. A melting curve temperature profile was obtained by 95°C for 1 min, 60°C for 1 min and heating to 95°C in 20 min. Data were analyzed using the software version SDS 1.9.1 and Dissociation Curves 1.0. (Applied Biosystems)

RESULTS AND DISCUSSION

From both diagnostic samples infected with *A. frigidophilus* or *S. parasitica* (Figure 1A), and in pure cultures from all *Aphanomyces* and *Saprolegnia* species tested, amplicons of the same length as for *A. astaci* were obtained. Similar results were obtained after increasing annealing temperature and reducing the number of cycles (Figure 1B). Because the species *A. frigidophilus* and *A. repetans*, occur in crayfish species (BALLESTEROS *et al.,* 2006; ROYO *et al.,* 2004), and *Saprolegnia* spp. are ubiquitous with a worldwide and distribution, a great number of false positive is expected when applying the primers used in current crayfish plague PCR-test.

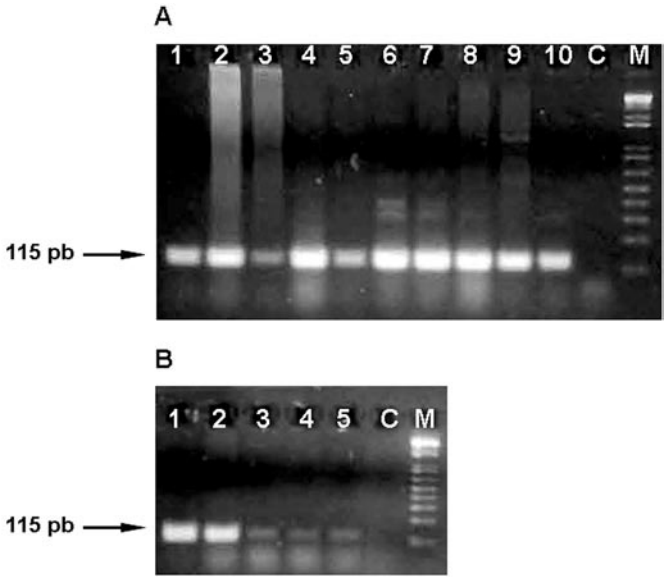

Figure 1

PCR products from cuticle samples and pure cultures.
(A) DNA amplification using an annealing temperature of 54°C.
Lane 1: cuticle from where *Aphanomyces frigidophilus* was isolated; lane 2: cuticle infected with *Aphanomyces astaci*; lane 3: cuticle infected with *Saprolegnia parasitica*, lane 4: *A. frigidophilus* SAP233; lane 5: *Aphanomyces repetans* SAP60; lane 6: *A. astaci* L1; lane 7: *A. astaci* Kv1; lane 8: *A. astaci* Ti1; lane 9: *S. parasitica* SAP210; lane 10: *Saprolegnia australis* SAP211; lane C: DNA-free control; M: 100-pb marker.
(B) DNA amplification using an annealing temperature of 58°C.
Lane 1: *A. astaci* Ti1; lane 2: *A. frigidophilus* SAP233; lane 3: *A. repetans* SAP60; lane 4: *S. parasitica* SAP210; lane 5: *S. australis* SAP211.

Figure 1

Produits PCR à partir d'échantillons de cuticule et de cultures pures.
(A) Amplification de l'ADN utilisant une température d'annealing de 54° C.
Ligne 1 : cuticule à partir de laquelle *Aphanomyces frigidophilus* a été isolée ; ligne 2 : cuticule infectée par *Aphanomyces astaci* ; ligne 3 : cuticule infectée par *Saprolegnia parasitica* ; ligne 4 : *A. frigidophilus* SAP233 ; ligne 5 : *Aphanomyces repetans* SAP60; ligne 6 : *A. astaci* L1; ligne 7 : *A. astaci* Kv1 ; ligne 8 : *A. astaci* Ti1 ; ligne 9 : *S. parasitica* SAP210 ; ligne 10 : *Saprolegnia australis* SAP211 ; ligne C : contrôle sans ADN ; M : marqueur 100-pb.
(B) Amplification de l'ADN utilisant une température d'annealing de 58° C. Ligne 1 : *A. astaci* Ti1 ; ligne 2 : *A. frigidophilus* SAP233 ; ligne 3 : *A. repetans* SAP60 ; ligne 4 : *S. parasitica* SAP210 ; ligne 5 : *S. australis* SAP211.

However, with real-time PCR a strong fluorescence signal was detected in DNA amplified from clinical samples infected with *A. frigidophilus* and *A. astaci,* but not in the sample infected with *S. parasitica* (Figure 2). In pure cultures, amplification signal was detected for the three *A. astaci* isolates tested (Ti, Kv1 and L1) and for *A. frigidophilus*. However, only a weak signal was obtained with DNA from *A. repetans* and *S. parasitica* (Figure 3). Both species, *A. frigidophilus* and *A. astaci* have a high ITS sequence similarity (BALLESTEROS *et al.,* 2006) and, therefore, the target fragment amplified with this test only differed in 8 out of 115 nucleotides (Figure 4). Thus, the application of real time PCR using these primers to distinguish *A. astaci* from closely related *A. frigidophilus*, appears to be difficult because: (a) the nucleotide variation in the target sequences is not sufficient to discriminate the melting peaks of these two species, since they only differ in 0.5°C; (b) in clinical samples, the high efficiency of amplification in *A. astaci* can be reduced by the presence of other *Aphanomyces* species in the same sample, and the amplification signal modify the melting peak.

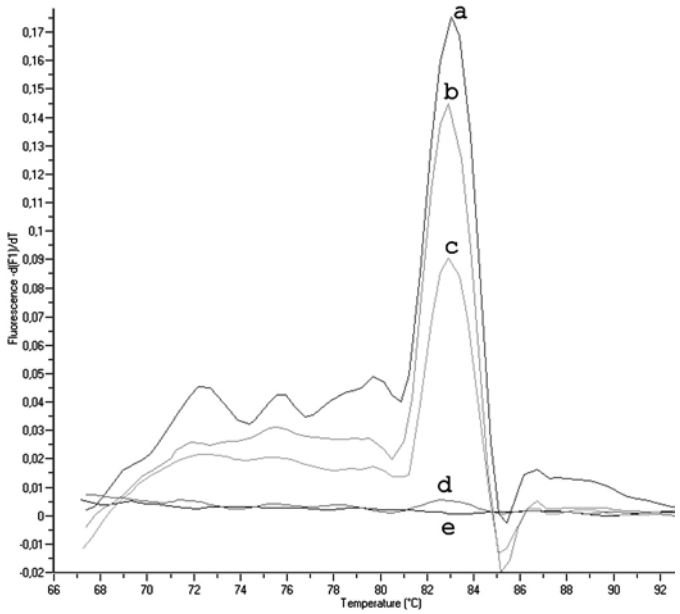

Figure 2

Real-time PCR melting curves for cuticle samples. (a and b), cuticles from infected with *Aphanomyces astaci* from two crayfish plague cases; (c), cuticle from where *Aphanomyces frigidophilus* was isolated; (d), cuticle infected with *Saprolegnia parasitica*; and (e) DNA-free control. Melting shows a single peak around 82.6°C.

Figure 2

Courbes de PCR en temps réel pour des échantillons de cuticule. (a et b), cuticules infectées avec *Aphanomyces astaci* à partir de deux cas de peste ; (c), cuticule à partir de laquelle *Aphanomyces frigidophilus* a été isolée ; (d), cuticule infectée avec *Saprolegnia parasitica* ; et (e) contrôle sans ADN. Le point de fusion correspond à un pic unique d'environ 82,6° C.

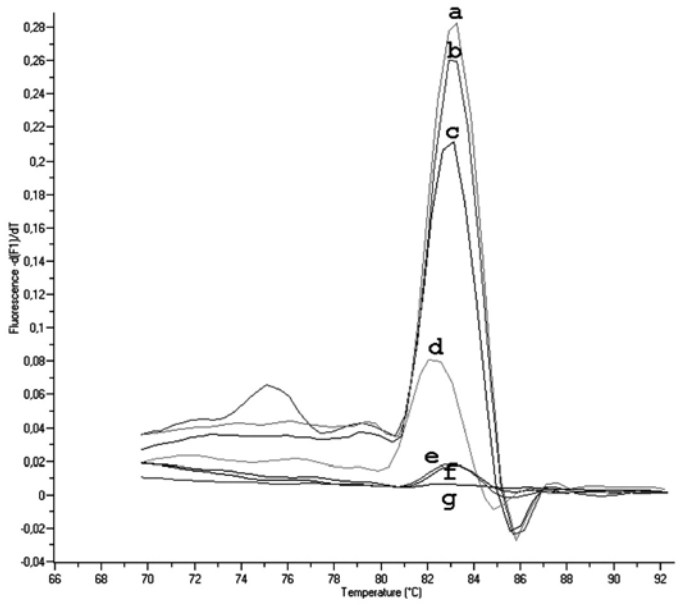

Figure 3

Real-time PCR melting curves for pure cultures. (a), *Aphanomyces astaci* Kv1; (b), *A. astaci* L1; (c), *A. astaci* Ti1; (d), *Aphanomyces frigidophilus* SAP233; (e), *Aphanomyces repetans* SAP60; (f), *Saprolegnia parasitica* SAP210; and (g), DNA-free control. Melting shows a peak at 82.4°C for *A. frigidophilus* and at 83.2°C for *A. astaci* strains.

Figure 3

Courbes de PCR en temps réel pour des cultures pures. (a), *Aphanomyces astaci* Kv1 ; (b), *A. astaci* L1 ; (c), *A. astaci* Ti1 ; (d), *Aphanomyces frigidophilus* SAP233 ; (e), *Aphanomyces repetans* SAP60 ; (f), *Saprolegnia parasitica* SAP210 ; et (g), contrôle sans ADN. Le point de fusion correspond à un pic de 82,4° C pour *A. frigidophilus* et de 83,2° C pour les souches d'*A. astaci*.

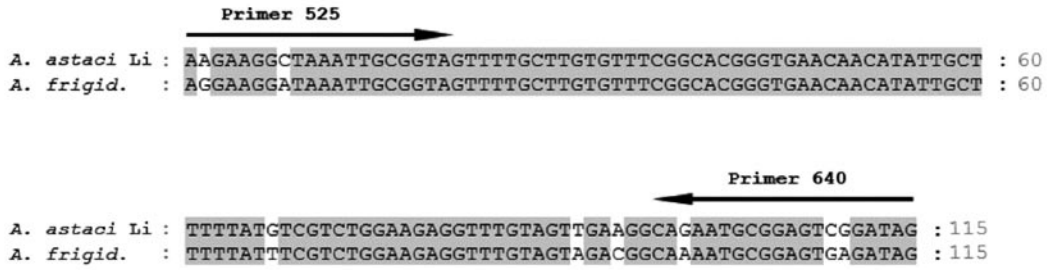

Figure 4

Alignment of the sequence amplified from *Aphanomyces astaci* and *Aphanomyces frigidophilus* showing the location of primers 525 and 640. Nucleotides matching the sequence are shaded.

Figure 4

Alignement de la séquence amplifiée à partir d'*Aphanomyces astaci* et d'*Aphanomyces frigidophilus* montrant l'emplacement des primers 525 et 640. Les nucléotides correspondant à la séquence sont ombrés.

Thus, to accurately identify the presence of *A. astaci*, it is necessary to carry out new approaches based on other sequences of ITS regions or other genes. A PCR-based test for *A. invadans* was successfully and allowed distinguishing this species from *A. astaci* and *A. frigidophilus* (VANDERSEA *et al.,* 2006). In *A. astaci*, a new PCR-based method has been recently developed to detect this parasite in North American crayfish (OIDTMANN *et al.,* 2006). New primers were designed to specifically amplify parts of the internal transcribed spacer (ITS) regions and the 5.8 rRNA gene of *A. astaci* and its specificity was tested against several closely related *Aphanomyces* species, that might be found in or on crayfish. However, the recent increase of studies on ITS sequence of Oomycetes species shown the existence of new *Aphanomyces* ITS sequences, probably representing new *Aphanomyces* spp. and, some of which are closely related to *A. astaci* (Diéguez Uribeondo and Kozubikova, unpublished) that need to be *Aphanomyces* characterized.

Thus, on the light of the results presented and the new few studies carried out on the diversity of *Aphanomyces* spp., our results emphasized the need of always contrasting molecular data with classical microscopy evidences and disease history (CERENIUS *et al.,* 1988) in order to confirm the presence of *A. astaci*. Development of a more accurate molecular technique and characterization of new *Aphanomyces* spp. will facilitate a more accurate and rapid identification of the presence of this parasite in diagnostic samples, water, or potential vectors, which is crucial to implement appropriate management measures for the endangered indigenous European species of crayfish.

ACKNOWLEDGEMENTS

This work was supported by grants Flora Micológica Ibérica (REN2002-04068-C02-01GLO), Carl Tryggers Stiftelse and Swedish Research Council for Environmental, Agriculture Sciences and Spatial Planning (FORMAS). We thank Dr. Pilar San Pio and Dr. Catherine Souty-Grosset for revising the French text.

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