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# A cultivation independent, PCR-based protocol for the direct identification of plant pathogens in infected plant material

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**Abstract** A protocol involving PCR, shot-gun cloning and sequencing was developed as a pre-diagnostic screening tool working directly on disease symptoms. The method was used to show the presence of biotrophic and non-biotrophic eukaryotic plant pathogens in leaves and fruits.

**Keywords** *Colletotrichum acutatum* · ITS · *Melampsoridium betulinum* · *Peronospora sparsa* · *Puccinia distincta* · PCR-based detection

The identification of the organisms causing diseases in crop plants is of great importance for determining appropriate control strategies. This is not always an easy task especially when the observer faces unfamiliar diseases in various hosts from different geographical areas. Traditionally, the identification of plant pathogens involves isolation, culturing and identification of the organism. These methods rely on skilled personnel and are often very time consuming (McCartney et al. 2003).

The molecular methods used today are designed to detect specific pathogens, making them unsuitable for the detection of an unknown pathogen (Ward et al. 2004). PCR, using species-specific primers, is widely used, but is useless without prior knowledge of the identity of the possible pathogen(s). One way of identifying unknown eukaryotic organisms is to perform PCR using species non-specific primers which amplify part of the 18S-gene or ITS-sequence of nuclear rDNA genes followed by cloning and sequencing the PCR-product. This approach has worked successfully on pure cultures of a pathogen (Ward and Bateman 1999) or using spores of non-culturable pathogens (Morales et al. 1999). To overcome the difficulties of making pure cultures, PCR can be performed on DNA from a mixture of organisms followed by gel purification of the PCR products prior to cloning. This approach has been used in soil (Smit et al. 1999; Hunt et al. 2004) and plant samples (Zhang et al. 1997; Kernaghan et al. 2003) to study plant and soil-associated fungi. Vandenkoornhuyse et al. (2002) used a similar method, but without gel-purifying the PCR products before cloning, when studying the fungal diversity on grass roots.

Here we show that PCR followed by shot-gun cloning and sequencing can be used to show the presence of plant pathogens from disease symptoms. Instead of using fungus-specific primers, we used the

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eukaryotic ITS primers designed by White et al. (1990), as these primers also facilitate the identification of non-fungal organisms (O'Brien et al. 2005). The method was successfully applied to a diverse range of diseased plant material: blackberry (*Rubus fruticosus*), cherry (*Prunus cerasus*), daisy (*Bellis perennis*) and birch (*Betula* sp.), displaying disease symptoms caused by unknown pathogens.

In the summer 2007, a blackberry grower in southern Jutland, Denmark observed dry berries affecting most of his production. As the disease was unknown to the consultant, the berries were sent for further investigation at Copenhagen University. Danish cherry growers had suffered substantial yield losses in the preceding seasons, probably caused by an unidentified *Colletotrichum* sp. Diseased cherries showing sunken lesions were sent to Copenhagen University from a grower on the island of Funen. In the same summer daisies suffering from severe rust attacks, not seen previously in Denmark were observed in a private garden in Roskilde, and these plants, along with a birch seedling exhibiting severe rust attack, were included in this study.

Plant material displaying disease symptoms was frozen in liquid $N_2$ and ground to a fine powder using a pestle and mortar. Isolation of total genomic DNA from blackberry and cherry was performed using Qiagen DNAeasy (Qiagen, Valencia, CA, USA). From daisy and birch leaves, genomic DNA was extracted using Nucleon Phytopure (Amersham Biosciences, Buckinghamshire, UK). PCR mixtures contained 1× Dynazyme PCR buffer including 1.5 mM $MgCl_2$, 0.15 mM dNTP, 0.5 μM of each primer ITS1 and ITS4 (White et al. 1990) and 1.5 U Dynazyme (Finnzymes, Espoo, Finland). To each reaction, 1 μl of total genomic DNA (10–20 ng) was added. The PCR reactions were made in a total volume of 20 μl and the PCR amplification performed using a MyCycler thermal cycler (Biorad, Hercules, CA, USA). An initial denaturation at 94°C for 5 min was followed by incubation at 30 cycles of: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The PCR was completed with a final extension step at 72°C for 5 min. The PCR products were visualised on an ethidium bromide-stained 1.5% agarose gel run in TBE buffer. Following the manufacturer's instructions, the PCR products were, without any purification, cloned in the pGEM T-easy vector (Promega, Madison, USA) and transformed into competent *Escherichia coli* GC5 cells (Ampliqon, Copenhagen, Denmark). Twenty colonies were picked with a wooden toothpick and lysed by boiling in TE-buffer for 5 min. One microliter of each lysate was used as template in a PCR-reaction using the vector primers Sp6 and T7. The reaction set-up was as described above except for an annealing temperature of 59°C. The PCR-products were separated on an agarose gel as described above and clones yielding different sized PCR-products were chosen for sequencing using the sequencing service from MWG Biotech, Ebersberg, Germany. The resulting sequences were used as query sequences in a BLAST search at the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) in order to identify which organism yielded the cloned ITS-region.

As seen in Fig. 1, the PCR using the primer ITS1 and ITS4 yielded a number of PCR bands. After cloning in the pGem T-easy vector and colony PCR, three to nine colonies from each transformation were chosen for sequencing representing different insert sizes. The BLAST search with the resulting sequences revealed that, besides the host plant sequences, a

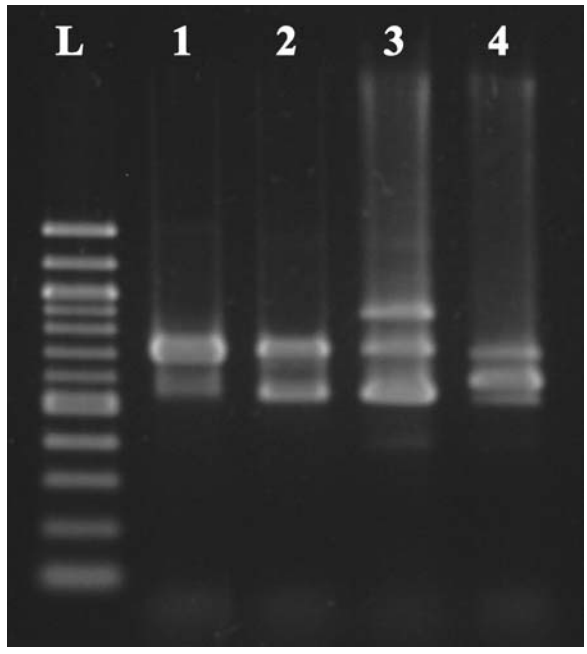


**Fig. 1** PCR amplifications using the primers ITS1 and ITS4 on DNA extracted from diseased plant material. Lanes: *L*, molecular size marker (100 bp, New England Biolabs, Ipswich, MA, USA); *1*, blackberry; *2*, cherry; *3*, birch and *4*, daisy

number of organisms were present in the samples. A few of these were perceived as being possible pathogens and the rest were described as yeasts and fungal epiphytes in the database. The sequence of the sequenced part of the rDNA has been deposited in Genebank (http://www.ncbi.nlm.nih.gov/) under the accession numbers EU391654 to EU391657. In blackberry, *Peronospora sparsa* (EU391654) was found whereas the DNA sample from cherry revealed the presence of *Colletotrichum acutatum* (EU391655). The two rust fungi from daisy and birch were identified as *Puccinia distincta* (EU391656) and *Melampsoridium betulinum* (EU391657), respectively. The protocol presented here proved to be capable of detecting the presence of possible plant pathogens without any prior knowledge of their identity. *Puccinia distincta* on daisy has not previously been identified in Denmark and *P. sparsa* is new to blackberry in Denmark.

One of the biggest problems working with plant-associated fungi is the presence of plant DNA when DNA extraction is made directly on plant parts. To overcome this problem the PCR products can be separated on an agarose gel and purified, or the PCR can be carried out using fungus-specific primers (Zhang et al. 1997; Neubert et al. 2006). In this study we showed that co-amplification of plant DNA is non-problematic as the PCR-products are present in different clones. In many cases plant DNA might be amplified to high levels and the causal pathogen might be overlooked if only present in low amounts. The same problem can be seen if saprophytes or secondary pathogens are present to a great extent. The number of sequenced clones must therefore be chosen with great care. In this study three to nine clones proved to be sufficient to find possible causal agents of the observed symptoms, but a greater number might be required in other cases e.g. if it is a possibility that the disease is caused by a complex of pathogens, there are high levels of secondary infections or the pathogen is present only in small amounts. The amplification of the ITS region using the primers ITS1 and ITS4 yields PCR products of roughly the same size from a number of fungi. Therefore, it is advisable to sequence clones yielding PCR products of the same size. Using this method, we identified two obligate parasites that could cause the observed symptoms. Even though a possible pathogen is found by the described method it cannot be concluded that the observed symptoms are caused by this pathogen, but knowledge of possible pathogens being present in a given plant sample will make a final identification, including isolation, and fulfilment of Koch's Postulates, much easier. The method described here could probably also be used in the same way for bacteria in infected plant material using universal prokaryotic primers (Weisburg et al. 1991; Jensen et al. 1993).

In conclusion, we showed that the easy shot-gun PCR and cloning procedure successfully detected the presence of possible plant pathogens in a range of diseased plant material: blackberry, cherry, daisy and birch and plant parts: fruits and leaves. The method also easily detected basidomycetes, ascomycetes and oomycetes. Furthermore, the procedure has great applicability for routine use at plant health service centres as a pre-diagnostic tool facilitating targeted diagnostics and isolation.

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