

A duplex PCR method for the simultaneous identification of *Phytophthora ramorum* and *Phytophthora kernoviae*

A. Schlenzig

Science and Advice for Scottish Agriculture (SASA), Plant Health, Roddinglaw Road, Edinburgh EH12 9FJ (GB);
e-mail: Alexandra.Schlenzig@sasa.gsi.gov.uk

Phytophthora ramorum and *Phytophthora kernoviae* are two fungus-like organisms affecting a wide range of hardy ornamental plants and trees. Emergency measures are implemented in the European Union for *P. ramorum* and aim to eradicate, or at least prevent the further spread of this harmful pathogen. *Phytophthora kernoviae* has so far been found only in New Zealand, the UK and Ireland, and is regulated on a UK level using the same measures as for *P. ramorum*. Both *Phytophthora* species have a similar host range and can be diagnosed using similar methods. Therefore a duplex PCR detection, based on the internal transcribed spacer (ITS) regions of the ribosomal DNA, was developed to enable simultaneous testing to reduce diagnostic times. The method was tested for its specificity and sensitivity, and on plant samples, and was shown to be reliable for identification of the two organisms.

Introduction

Phytophthora ramorum was described in 2001 (Werres *et al.*, 2001), initially as causing leaf lesions and dieback on *Rhododendron* and *Viburnum* in nurseries in the Netherlands and Germany. Subsequently, it was also found to be the cause of a disease named 'sudden oak death', which has devastated coastal forests on the US Pacific coast since the mid-1990s (Rizzo *et al.*, 2002). *Phytophthora kernoviae* was found in the UK in 2003 during the *P. ramorum* surveys in woodland in Cornwall, concurrently on European beech (*Fagus sylvatica*) and *Rhododendron ponticum* (Brasier *et al.*, 2005). So far, *P. kernoviae* is not as widespread as *P. ramorum* and has not been reported from the European mainland.

Both pathogens are subject to official plant health controls requiring host plants moving in trade or established plants in public gardens, parks and forests to be monitored by inspectors. On observation of possible symptoms, samples are sent to government laboratories for diagnosis. In Scotland, this diagnostic service is provided by Science and Advice for Scottish Agriculture (SASA). Although, in April 2002, Scotland was one of the first countries in Europe to find *P. ramorum* (Schlenzig, 2006), it was not until December 2007 that *P. kernoviae* was detected there. By the end of 2009 there were 37 ongoing outbreak sites in Scotland, 22 infected with *P. ramorum*, 12 with *P. kernoviae* and three sites with both pathogens present. With the increased number of outbreaks, a steep increase in submitted samples made a PCR method able to detect both pathogens in one test desirable.

Materials and methods

The European and Mediterranean Plant Protection Organization (OEPP, 2006) published the diagnostic protocol PM 7/66 (1)

Phytophthora ramorum, in which several methods for the detection of this pest are described, including a conventional PCR method according to Lane *et al.* (2003). This method was extended into a duplex PCR by incorporating primers for the detection of *P. kernoviae* described below.

DNA extraction

DNA was extracted using the Nucleo Spin Plant DNA extraction kit (Macherey & Nagel, Düren, Germany). Mycelium from fungal cultures or from two leaf discs (diameter 5 mm) were transferred into 1.5 mL microcentrifuge tubes with 400 µL C1 extraction buffer (supplied with the kit), and were ground using micro-pestles with sterile sand as grinding agent. Further extraction was performed in accordance with the manufacturer's instructions.

Primer design

For *P. ramorum* detection, primers PramF1 and PramR1, as described in the above-mentioned EPPO protocol, were used. For *P. kernoviae* detection, forward primer Pkern60F (K. Hughes, Food and Environment Research Agency, UK, pers. comm. 2008) was modified in one base pair (5'-TCC TCG TTG GCA GTT TCG AC-3'), and reverse primer PkernR1 (5'-CAC AAC TAC ATT CTG CAC AGC-3') was designed. Ribosomal RNA gene sequences from three isolates of *P. kernoviae* (GenBank accession numbers EU442181.1, EU732594.1 and AY940661.1) were aligned with the sequences of 12 other *Phytophthora* species with similar host plant range (*P. ramorum*, *P. syringae*, *P. cinnamomi*, *P. cactorum*, *P. lateralis*, *P. nicotianae*, *P. citricola*, *P. citrophthora*, *P. gonapodyides*, *P. heveae*, *P. cryptogea*, *P. drechsleri*), and the reverse primer was designed in a region

where the sequence was unique for *P. kernoviae*. Consideration was given to the expected resulting PCR product (approximately 470 bp) being distinctly different in size from the product of the primers for *P. ramorum* (approximately 690 bp). Specificity of the primer was first assessed using Basic Local Alignment Search Tool (BLAST) analysis.

PCR

For simplex PCR, 20 µL reaction mixtures were set up containing 2 µL 10× PCR buffer (including 15 mM MgCl₂), 0.125 mM of each dNTP, 0.2 µM of each primer (Pkern60F/PkernR1 or PramF1/PramR1), 0.65 U *Taq* DNA polymerase (Sigma-Aldrich, Poole, UK) and 2 µL template DNA. Thermocycler conditions were set at 2 min denaturation at 94°C followed by 30 cycles at 94°C for 30 s, 57°C for 30 s, 72°C for 30 s, and a final elongation step of 5 min at 72°C.

For the duplex PCR, reaction mixtures were set up as above, but with 0.2 µM each of primers Pkern60F, PkernR1, PramF1 and PramR1, and were processed under the same thermocycler settings as above.

The detection limit of the primer set for *P. kernoviae* was determined by adding dilutions of known amounts of template DNA (from 20 fg to 200 ng) to the reaction.

Results and discussion

Specificity of the *P. kernoviae* primers was tested in a simplex PCR with 15 other *Phytophthora* species (Fig. 1). None of the other *Phytophthora* species produced a PCR product.

The lowest detectable amount of pure *P. kernoviae* DNA was 2 pg (data not shown). The same dilutions of *P. kernoviae* were then tested in a duplex PCR. The detection limit was again 2 pg (Fig. 2). The detection limit of *P. ramorum* DNA for the simplex and duplex PCR as above was established as 20 pg (Fig. 3). When the same dilutions of *P. kernoviae* were mixed with increasing amounts of *P. ramorum* DNA and tested in the duplex PCR, the detection limit for *P. kernoviae* stayed at 2 pg. However, in the presence of high amounts of *P. kernoviae* DNA (>200 pg), the detection limit of *P. ramorum* decreased to 200 pg (Fig. 4).

The duplex PCR described here has been used in Scotland as an identification and confirmation method after plating samples onto semi-selective V8 medium (Jung *et al.*, 1996) for 1261 samples (as at March 2010). Over this time, 295 samples tested positive for *P. ramorum* (170), *P. kernoviae* (123) or both (2). The PCR never

gave a positive result for any of the other *Phytophthora* species detected by the plate test, nor did it fail to confirm the cases in which *P. ramorum* or/and *P. kernoviae* had been detected on the plates. The observed decline in the detection limit for *P. ramorum* in the presence of high concentrations of *P. kernoviae* should not lead to problems in practical diagnosis, as the presence of both species in the same sample is a very rare event. In Scotland, it has been observed only twice so far and both samples were identified correctly by the duplex PCR.

Although this is not the method followed by SASA, the duplex PCR was also tested directly on infected rhododendron leaves. Over 4 weeks, 30 submitted samples were tested in parallel with plating and extracting DNA from two leaf discs (diameter 5 mm) from the edge of lesions. No discrepancies occurred between the results of both methods: 12 samples were negative and 18 samples were positive. Considering the limited number of samples, more validation is recommended for any laboratory wishing to use the described duplex PCR directly on plant samples.

Acknowledgements

The author would like to thank M. Kowalewska and R. Gray for excellent technical assistance.

Une méthode de PCR duplex pour l'identification simultanée de *Phytophthora ramorum* et *Phytophthora kernoviae*

Phytophthora ramorum et *Phytophthora kernoviae* sont deux agents pathogènes affectant une large gamme de plantes ligneuses ornementales et d'arbres. Des mesures d'urgence ont été mises en œuvre dans l'Union européenne contre *P. ramorum* et vise à éradiquer ou au moins à éviter toute autre dissémination de ce dangereux pathogène. *P. kernoviae* n'a pour l'instant été trouvé qu'au Royaume-Uni et en Irlande et est réglementé au niveau national en utilisant les mêmes mesures que pour *P. ramorum*. Les deux espèces de *Phytophthora* ont une gamme d'hôtes similaire et peuvent être diagnostiquées en utilisant des méthodes similaires. Par conséquent, une détection par PCR duplex, basée sur la région *ITS* (*Internal Transcribed Spacer*) de l'ADN ribosomal a été développée pour permettre des tests simultanés et réduire le temps nécessaire au diagnostic. La méthode a été testée pour sa spécificité et sa sensibilité sur des échantillons végétaux et s'est avérée fiable pour l'identification des deux organismes.



Fig. 1 Specificity of primer pair Pkern60F/PkernR1 for the detection of *P. kernoviae*. Lane 1: *P. citrophthora*, 2: *P. heveae*, 3: *P. citricola*, 4: *P. nicotianae*, 5: *P. cryptogea*, 6: *P. gonapodyides*, 7: *P. cactorum*, 8: *P. drechsleri*, 9: *P. syringae*, 10: *P. idaei*, 11: *P. cambivora*, 12: *P. cinnamomi*, 13: *P. infestans*, 14: *P. rubi*, 15: *P. kernoviae*, 16: *P. ramorum*, 17: negative control, 18: PCR marker.

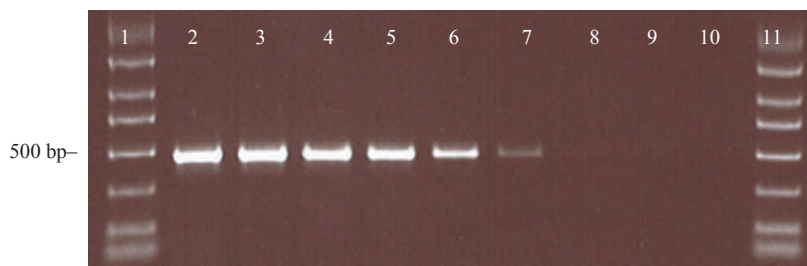


Fig. 2 Sensitivity of primer pair Pkern60F/PkernR1 for the detection of *P. kernoviae* in the duplex PCR. Lanes 1 and 11: PCR markers; lane 2: 200 ng DNA, 3: 20 ng DNA, 4: 2 ng DNA, 5: 200 pg DNA, 6: 20 pg DNA, 7: 2 pg DNA, 8: 200 fg DNA, 9: 20 fg DNA, 10: negative control.

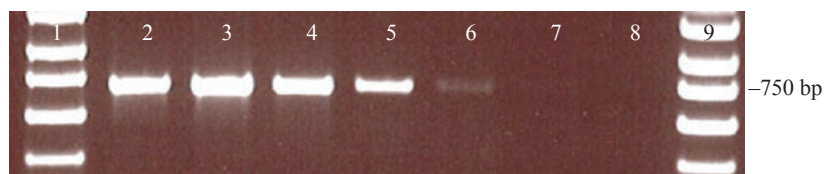


Fig. 3 Sensitivity of the duplex PCR for the detection of *P. ramorum*. Lanes 1 and 9: PCR markers; lane 2: 200 ng DNA, 3: 20 ng DNA, 4: 2 ng DNA, 5: 200 pg DNA, 6: 20 pg DNA, 7: 2 pg DNA, 8: negative control.

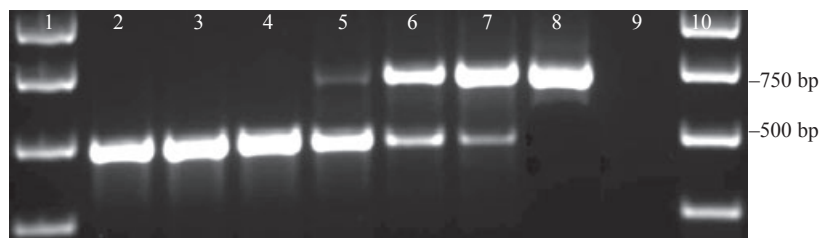


Fig. 4 Sensitivity of duplex PCR with *P. kernoviae*/*P. ramorum* DNA mixtures. Lanes 1 and 10: PCR markers; lane 2: 40 ng *P. kernoviae* DNA, 3: 20 ng *P. kernoviae* DNA + 2 pg *P. ramorum* DNA, 4: 2 ng *P. kernoviae* DNA + 20 pg *P. ramorum* DNA, 5: 200 pg *P. kernoviae* DNA + 200 pg *P. ramorum* DNA, 6: 20 pg *P. kernoviae* DNA + 2 ng *P. ramorum* DNA, 7: 2 pg *P. kernoviae* DNA + 20 ng *P. ramorum* DNA, 8: 40 ng *P. ramorum* DNA, 9: negative control.

Метод двойной PCR для одновременного выявления *Phytophthora ramorum* и *Phytophthora kernoviae*

Phytophthora ramorum и *Phytophthora kernoviae* - два грибных организма, поражающих широкий спектр зимостойких декоративных растений и деревьев. В Европейском Союзе проводятся чрезвычайные меры в отношении *P. ramorum* с тем, чтобы ликвидировать этот вредный патоген или, по крайней мере, предотвратить его дальнейшее распространение. *P. kernoviae* до сих пор встречался только в Великобритании и Ирландии и подвергается регулированию на национальном уровне, с использованием тех же мер, что и в отношении *P. ramorum*. Оба вида *Phytophthora* имеют аналогичный диапазон растений-хозяев и могут быть диагностированы с помощью сходных методов. Поэтому обнаружение с помощью двойной PCR, основанное на внутреннем транскрибированном спейсере (ITS) районов рибосомной ДНК, было разработано для одновременного анализа и сокращения времени диагностики. Этот метод был проверен на специфичность и чувствительность на пробах растений и показал свою надежность для идентификации этих двух организмов.

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