Virus Genes (2010) 40:130–134
DOI 10.1007/s11262-009-0421-7

Incidence of endornaviruses in *Phytophthora* taxon douglasfir and *Phytophthora ramorum*

Zisis Kozlakidis · Neil A. Brown · Atif Jamal · Xiyu Phoon · Robert H. A. Coutts

Received: 10 July 2009 / Accepted: 31 October 2009 / Published online: 14 November 2009

Abstract In this investigation, we show that four *Phytophthora* taxon douglasfir isolates from the USA, irrespective of their geographical location or host plant, and 20% of a representative cohort of *Phytophthora ramorum* isolates contain endornavirus dsRNAs. Three endornavirus-specific RT–PCR amplicons were generated by RT–PCR using dsRNA isolated from the four *Phytophthora* taxon douglasfir isolates and one representative *Phytophthora ramorum* isolate as template with oligonucleotide primers designed from the sequence of Phytophthora endornavirus 1. The amplified segments showed a very high degree of sequence similarity suggesting that the virus has gone through a population bottleneck during its emergence.

Keywords Endornavirus · dsRNA · *Phytophthora* spp.

The genus *Endornavirus* contains large, double-stranded (ds) RNA viruses which are known to infect plants, fungi, and oomycetes [1] and apart from *Helicobasidium mompa* endornavirus 1-670 [2] are not associated with disease symptoms. Endornaviruses resemble members of the family *Hypoviridae* because no true virions have been identified and both consist of a linear dsRNA genome with a single open reading frame (ORF) with recognisable helicase and polymerase domains [1, 3]. The complete sequence of the 13.9 kb genome of an endornavirus from an oomycete, *Phytophthora* isolate P441 from Douglas fir was described by Hacker et al. [4] and named Phytophthora endornavirus 1 (PEV1). The single ORF encoded by PEV1 contained conserved motifs for an RNA helicase, a putative UDP glycosyltransferase (UGT) and an RNA-dependent RNA polymerase (RdRP) [4; Fig. 1a]. These proteins are presumed to be processed by virus-encoded proteinases although evidence of proteinase activity is lacking.

No systematic surveys of different isolates of Phytophthora endornaviruses have been carried out although it is known that some isolates of *P. infestans*, the causative agent of potato late blight, do contain large dsRNAs [5] but major Phytophthora pathogens of forest trees such as *P. ramorum*, the causative agent of sudden oak death, have not been investigated. In this study, we have analysed the genetic structure of *P.* taxon douglasfir endornavirus populations in the USA and for the first time *P. ramorum* endornavirus populations in the UK, USA and Europe by analysing the nucleotide sequences of three regions of the endornavirus genome.

Collections of the informally designated *P.* taxon douglasfir, a yet unnamed *Phytophthora* species, were isolated from Douglas fir (*Pseudotsuga menziesii* subsp. *menziesii*) in Oregon, USA and from carrot (*Daucus carota*) and white cockle (*Silene latifolia*) in New York, USA by E. M. Hansen and P. B. Hamm, Oregon State University, Oregon, USA (Table 1). The isolates were named P440, P441, P458 and P475, respectively. Isolate P441 is known to contain a dsRNA which was classified as the first non-plant member of the genus *Endornavirus* and named PEV1 [4]. *P.* taxon

Zisis Kozlakidis and Neil A. Brown contributed equally to this study.

The nucleotide sequence data reported in this article has been assigned the accession numbers AM 941193, AM941200, AM941404; AM941194, AM941202, AM941402; AM941195, AM941201, AM941403; AM941196, AM941197, AM941410 for isolates P440, P458, P475, and 3543/04, respectively.

Z. Kozlakidis · N. A. Brown · A. Jamal · X. Phoon · R. H. A. Coutts (✉)
Division of Biology, Faculty of Natural Sciences, Sir Alexander Fleming Building, Imperial College London, Imperial College Road, London SW7 2AZ, UK
e-mail: r.coutts@imperial.ac.uk

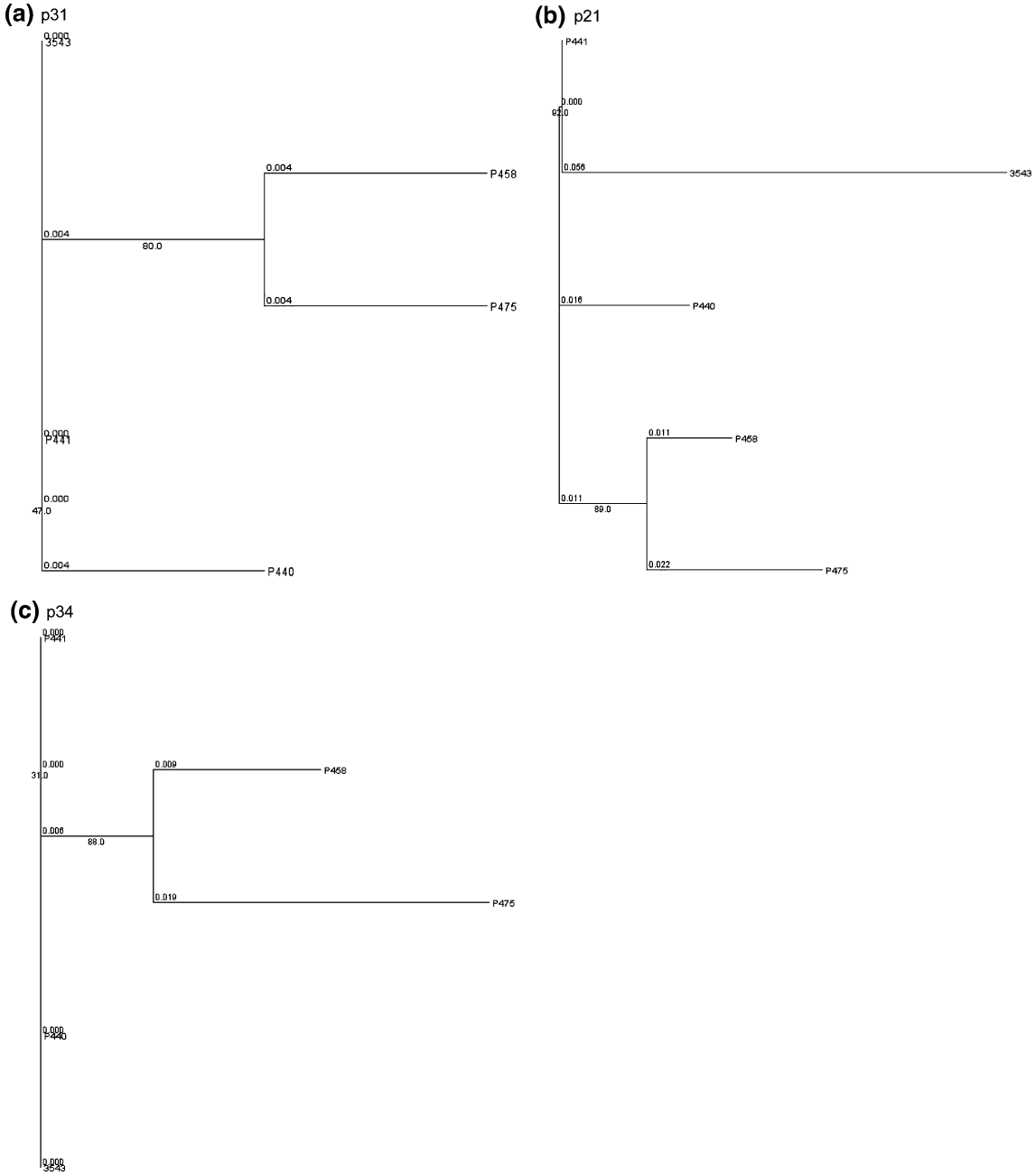

Fig. 1 Phylogenetic trees for the *P.* taxon douglasfir and *P. ramorum* isolates shown to contain endornavirus dsRNAs, based on three regions of the Phytophthora endornavirus 1 genome. **a, b** and **c** Unrooted trees generated by the neighbour-joining method using the MAFFT program with amino acid sequences of endornavirus polypeptides p31, p21 and p34, respectively; numbers indicate percentage of bootstrap support from 1,000 replicates with branch lengths indicated

douglasfir isolates were cultured on pea broth [6] at 20°C for 10–14 days. Thirty-five isolates of *P. ramorum* were taken from a number of diseased hosts in the UK, the USA and various locations in Europe (Table 1). All isolates of *P. ramorum* were confirmed as being *bona fide* specimens following isolation on selective media using EPPO recommended protocols and PCR using *P. ramorum* specific primers for nuclear and mitochondrial genes [7], the products of which were cloned and sequenced (results not shown). All *P. ramorum* isolates were cultured in carrot broth for 14–35 days as described previously [8].

Infection frequencies with endornavirus dsRNAs were determined by isolating total nucleic acid extracts from *Phytophthora* species mycelia and fractionating dsRNA by a lithium chloride procedure, removing traces of DNA and single stranded RNA with DNase1 and S1 nuclease,

Table 1 Location of *Phytophthora* species isolates, isolate code and mating type, host plant, infection frequency of isolates with endornavirus dsRNA obtained from *Phytophthora* species isolates

Location: Country of origin,town, state (Oregon [OR]; California [CA];New York [NY]; county(where known)	Isolate code and mating type (where known)	Host plant	Endornavirus DsRNA presence
USA, OR	P440*	*Pseudotsuga menziesii*	+
USA, OR	P441 (PEV1)*	*Pseudotsuga menziesii*	+
USA, NY	P458*	*Daucus carota*	+
USA, NY	P475*	*Silene latifolia*	+
UK, Wormley, Surrey	1091/05	*Rhododendron sp.*	+
UK, West Horsley, Surrey	3543/04	*Viburnum tinus*	+
UK, Chiddingfold, Surrey	7981/04	*Viburnum bodnantense*	+
UK, Northwood, Middlesex	10743/04	*Viburnum bodnantense*	+
UK, Charfield, Gloucestershire	16466/03	*Viburnum sp.*	+
Netherlands	18009/03	*Viburnum sp.*	+
Netherlands	18010/03	*Rhododendron sp.*	+
USA, Pacific west coast (Nursery isolates)	RHCC-1-A2	All unknown	−
	RHCC-4-A2		−
	RHCC-33-A2		−
	WSDA 3403-A2		−
	WSDA 3765-A2		−
USA, Marin Water district, CA	P1347	*Lithocarpus densiflorus*	−
USA, Marin Water district, CA	P1348-A2	*Quercus agrifolia*	−
USA, Marin China Camp, CA	P1370	*Arbutus sp.*	−
USA, Sonoma Fairfield Osborn, CA	P1371	*Umbellularia californica*	−
USA, OR	P1404-A2	*Lithocarpus densiflorus*	−
Poland	P1410-A1	*Rhododendron sp.*	−
USA, Marin County, CA	P1419	*Quercus agrifolia*	−
USA, Marin County, CA	P1420sz.2	*Quercus agrifolia*	−
Unknown	P1426	*Quercus agrifolia*	−
UK, unknown	P1446-A1	*Viburnum bodnantense*	−
UK, unknown	P1452-A1	*Rhododendron sp.*	−
UK, unknown	P1453-A1	*Viburnum bodnantense*	−
UK, unknown	P1457-A1	*Rhododendron sp.*	−
UK, unknown	P1463-A1	*Rhododendron sp.*	−
UK, unknown	P1467-A1	*Rhododendron grandiflora*	−
Mallorca	P1492-A1	*Rhododendron sp.*	−
France	P1493-A1	*Rhododendron sp.*	−
Belgium	P1500-A2	Unknown	−
USA, CA	P1507-A2	*Laurus nobilis*	−
USA, CA	P1510-A2	*Laurus nobilis*	−
USA, CA	P1511-A2	*Laurus nobilis*	−
Germany	P1577-A1	*Rhododendron catabiense*	−
UK, Cornwall	P1616-A1	*Nothofagus cunninghamii*	−

All *Phytophthora* species isolates were identified as *Phytophthora ramorum* apart from P440, P441, P458 and P475 which were isolates of *Phytophthora* taxon douglasfir

+ Positive; − negative

respectively, followed by electrophoresis through 1% agarose gels in TAE (40 mM Tris–acetate, 1 mM EDTA, pH 8.0) buffer and visualisation by staining with ethidium bromide (0.5 μg/ml) as described by Coutts et al. [9]. Occasionally, total RNA extracts were extracted from mycelia for RT–PCR using the RNeasy® plant mini kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions for fungal mycelia. Approximately,

100 mg of mycelia, which had been frozen in liquid nitrogen and reduced to a powdered state, was processed in each extraction.

Oligonucleotide primers were designed to amplify specific regions of the PEV1 polyprotein gene sequence which included an internal region of the putative UGT gene encoding a polypeptide fragment of ca. 21 kDa termed p21, and the regions immediately 3′- and 5′- of this gene towards, respectively, the helicase-like and the RdRP regions of the genome [4] encoding fragments of the virus polyprotein ca. 31 and 34 kDa in size, termed p31 and p34. The internal region of the UGT gene primers were 5′-GTAGTTAA ATTTATCAACAAAATCAAGCATG-3′ and 5′-AAGAT TCAATCTCTACAACCACCCCCTC-3′ positioned at 8,546 and 9,311 bp in the genome, respectively. Primers for the region 5′- of the UGT gene, towards the helicase-like region, were 5′-GTCAGAGCCACTTTCTCGCG-3′ and 5′-CTCCTGCGTAGGTGGAGTAGG-3′, positioned at 7,181 and 8,121 bp, respectively, encoding p31. Primers for the region 3′- of the UGT gene, towards the RdRP region, were 5′-GTCCCTGATCTAGAGTCAAGAG-3′ and 5′-GC TGGAATGTGGTCACCAC-3′, positioned at 9,890 and 11,000 bp, respectively, encoding p34.

Denaturation of 8–10 μg of dsRNA or total RNA as template, reverse transcription, PCR amplification, cloning and sequencing of amplicons were performed as described previously [9, 10]. The sequences of cloned amplicons generated in three separate experiments for each positive sample were compared for accuracy. Edited sequences were used to construct unrooted phylogeneitc trees using the neighbour-joining method with a bootstrap of 1,000 which were aligned using the Fast Fourier Transform MAFFT programme [11] and the trees visualised on the web (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/).

Total dsRNA extracts from the four *P.* taxon douglasfir isolates and 35 *P. ramorum* isolates were assessed for the presence of a 13.9 kb genomic dsRNA. We found that all four *P.* taxon douglasfir isolates of USA origin harboured an endornavirus and seven of the 35 *P. ramorum* isolates surveyed harboured an endornavirus of a similar size to PEV1 (Table 1). Additionally, three of the *P.* taxon douglasfir isolates contained smaller dsRNA elements which were not investigated further. None of the 15 *P. ramorum* isolates from the USA harboured an endornavirus and of the seven isolates that did contain an endornavirus five were isolated in the UK and two isolated in the Netherlands (Table 1).

Primers designed from the sequence of PEV1 to amplify three different genomic regions produced single amplicons of the predicted sizes for all four *P.* taxon douglasfir endornaviruses and all of the *P. ramorum* endornaviruses tested. The combined size of the three amplicons represented >16% of the anticipated genome size of the endornaviruses sampled. The data were analysed to discover the most parsimonious trees for the three genomic regions and all three trees (Fig. 1a–c) show a separation of the New York and Oregon *P.* taxon douglasfir isolates into two clades, the former of which contained the one representative isolate examined in detail by RT–PCR from *P. ramorum* discovered in West Horsley, Surrey, UK 3543 on *Viburnum* sp. (Table 1). This data illustrated that the intra-species genetic diversity of the endornaviruses sampled was low over the genomic regions examined as has been found with many other members of the alpha-like super group of single-stranded RNA viruses [2] from which the common ancestor of the endornaviruses is believed to have arisen [4]. In order to calculate the selection pressure exerted on the entire population of Phytophthora endornaviruses, the pairwise ratio of non-synonymous to synonymous mutations (dN/dS) in the genomic sequences were calculated and pooled. Notwithstanding some polymorphisms between the sequences analysed most of the substitutions were silent, encoding the same amino acid (results not shown), demonstrating strong purifying selection.

Previously, it was hypothesised that Phytophthora endornaviruses were distributed throughout Phytophthora species [4] and here we show all *P.* taxon douglasfir isolates sampled from the USA, irrespective of their geographical location or host plant contain such elements and that 20% of a representative cohort of *P. ramorum* isolates from Europe and the USA also harbour endornaviruses. The endornaviruses we identified demonstrated a very high degree of sequence similarity which suggests that the virus has gone through a population bottleneck during its emergence. Therefore, the Phytophthora endornavirus genome has remained highly conserved, as demonstrated by the purifying selection pressure exerted on the genome. It has been suggested that most if not all *P. ramorum* isolates in Europe were imported in nursery stock during the 1990s [12] and that the isolates are near clonal so the reason for their absence from some, but not all European isolates is unclear but might indicate that endornavirus dsRNAs can be eliminated in some situations.

One of the most important findings from this investigation is the demonstration for the first time of the presence of endornaviruses in *P. ramorum*. Whether the presence of endornaviruses in *Phytophthora* species has effects on fungal growth or pathogenicity is unknown but since *Helicobasidium mompa* endornavirus 1-670 is a hypovirulence factor, reducing virulence of the host fungus [2] this may well be worth exploring in the future.

Acknowledgements Z. Kozlakidis would like to thank The Wellcome Foundation for a VIP fellowship. A. Jamal thanks the Higher Education Commission of Pakistan for a PhD fellowship. All studies carried out on *Phytophthora* taxon douglasfir was under licence number PHL 189B/5412(06/2006) amended (10/2006) issued by

DEFRA. We should like to thank Clive Brasier at the Forest Research Agency, Alice Holt Lodge, Farnham, Surrey, UK, and Beatrice Henricot at RHS Garden Wisley, Woking, Surrey, UK for supplying nitrogen-powdered mycelia of the licensed *Phytophthora ramorum* isolates examined.

References

1. M. Gibbs, P. Pfeiffer, T. Fukuhara, Endornavirus, in *Virus Taxonomy*, *Eighth Report of the International Committee on Taxonomy of Viruses*, ed. by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, L. A. Ball (Elsevier Academic Press, London, 2005), pp. 603–605
2. H. Osaki, H. Nakamura, A. Sasaki, N. Matsumoto, K. Yoshida, Virus Res. **118**, 143–149 (2006)
3. D.L. Nuss, B.I. Hillman, D. Riglin, N. Suzuki, in *Hypoviridae* in *Virus Taxonomy*, *Eighth Report of the International Committee on Taxonomy of Viruses*, ed. by C. M. Fauquet, M. A. Mayo, J. Maniloff, U. Desselberger, L. A. Ball (Elsevier Academic Press, London, 2005), pp. 597–601
4. C.V. Hacker, C.M. Brasier, K.W. Buck, J. Gen. Virol. **86**, 1561–1570 (2005)
5. G. Cai, W.E. Fry, B.I. Hillman, K. Myers, Phytopathol. **99**(Suppl), S18 (2009) (abstract)
6. J.B. Ristaino, M. Madritch, C.L. Trout, G. Parra, Appl. Environ. Microbiol. **64**, 948–954 (1998)
7. L. Schena, D.E.L. Cooke, J. Microbiol. Methods **67**, 70–85 (2006)
8. C.M. Brasier, S.A. Kirk, Mycol. Res. **108**, 823–827 (2001)
9. R.H.A. Coutts, L. Covelli, F. Di Serio, A. Citir, S. Açikgöz, C. Hernández, D. Alioto, A. Ragozzino, R. Flores, J. Gen. Virol. **85**, 3399–3403 (2004)
10. R.H.A. Coutts, I.C. Livieratos, J. Phytopathol. **151**, 525–527 (2003)
11. K. Katoh, K. Kuma, H. Toh, T. Miyata, Nucl. Acids Res. **33**, 511–518 (2005)
12. C.M. Brasier, Plant Pathol. **5**, 702–808 (2008)