RESEARCH LETTER

DNA-based method for rapid identification of the pine pathogen, *Phytophthora pinifolia*

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Abstract

Phytophthora pinifolia causes a needle and shoot disease in *Pinus radiata*, referred to as 'Daño Foliar del Pino'. This newly discovered disease requires intensive research efforts that necessitate the processing of large numbers of samples for which accurate identification, often by people not experienced in *Phytophthora* taxonomy, is required. The aim of this study was, therefore, to develop species-specific primers for *P. pinifolia* that amplify the internal transcribed spacer region of the ribosomal operon and the nuclear *Ypt*1 gene, respectively. The primers were tested over several *Phytophthora* spp., as well as fungi isolated from *P. radiata*. In all cases, only *P. pinifolia* was amplified. In addition to the species-specific primers, a PCR-restriction fragment length polymorphism protocol using available *Phytophthora* genus-specific primers was also used to generate a species-specific profile for *P. pinifolia*. This provided a characteristic profile that allows the identification of *P. pinifolia*, and it could also discriminate between 27 different species of *Phytophthora*. Both techniques reported in this study make it possible to identify large numbers of *P. pinifolia* cultures accurately and efficiently, which will be important for both quarantine work and biological research on this important new pathogen.

Introduction

Phytophthora pinifolia Durán, Gryzenh. & M.J. Wingf. is the causal agent of a recently discovered needle and shoot disease on *Pinus radiata* (Durán *et al.*, 2008). The disease is known as 'Daño Foliar del Pino', and subsequent to its first detection in 2004, extensive research was conducted to determine its cause. The disease increased rapidly between 2004 and 2006 when the largest area was affected. The affected area has decreased significantly during 2007 and 2008, and was confined to plantations close to the coast, in most cases.

In adult trees, symptoms start in the lower crown and gradually spread from the central to the distal part of the foliage. The infection may result in almost complete defoliation of the trees during the winter and the spring seasons. In seedlings, damage is characterized by the rapid death of the growing terminal shoots due to girdling cankers that develop on the young stems, which can ultimately lead to death of the entire plant. A characteristic symptom in all the age groups is black bands on the needles that represent one of the earliest symptoms of infection (Durán *et al.*, 2008).

Phytophthora pinifolia resides in Clade 6 of the most contemporary phylogeny for *Phytophthora* spp. (Cooke *et al.*, 2000a; Kroon *et al.*, 2004). This placement is unusual, because it is the only species of the Clade 6 without a known soil-borne phase and it also lacks nested or extended sporangium proliferation. Furthermore, it is the first *Phytophthora* sp. to be described to cause a pine needle and shoot disease.

The discovery of a new pine needle and shoot disease of *P. radiata* requires urgent and intensive research in order to understand the biology of the pathogen and to develop effective management procedures. Such studies will generate very large numbers of samples where the presence or absence of *P. pinifolia* will need to be accurately determined.

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In this regard, *Phytophthora* spp. are difficult to identify with certainty and accurate diagnoses typically require experienced taxonomists (Cooke *et al.*, 2000b; Martin *et al.*, 2000; Duncan & Cooke, 2002). In order to facilitate the accurate identification of *P. pinifolia* in large numbers of samples, an urgent need has arisen to have robust procedures available for rapid and accurate diagnoses, including those by non-specialists.

Contemporary identification of *Phytophthora* spp. generally includes molecular methods and, particularly, DNA sequence comparisons (Schena & Cooke, 2006). These methods have also led to the discovery of many new *Phytophthora* spp. that would not have been easily recognized using classical morphology-based techniques (Cooke *et al.*, 1999; Schubert *et al.*, 1999). DNA-based methods have, furthermore, given rise to protocols for rapid identification of some of the most important *Phytophthora* spp. (Martin & Tooley, 2004; Drenth *et al.*, 2006; Schena *et al.*, 2006). These protocols have made it possible to identify species from large number of samples generated from surveys, which are focussed on epidemiology and quarantine (Hayden *et al.*, 2004; Cooke *et al.*, 2007; Williams *et al.*, 2009).

The aim of this study was to develop species-specific primers for PCR identification of *P. pinifolia*. Furthermore, a PCR-restriction fragment length polymorphism (RFLP) protocol developed by Drenth *et al.* (2006), which can detect and differentiate between 27 *Phytophthora* spp., was applied to *P. pinifolia* to determine whether this test would be useful to distinguish this species from the other important *Phytophthora* spp. that may be isolated during field surveys.

Materials and methods

Isolates

Cultures of 50 *Phytophthora* spp. related to *P. pinifolia*, as well as various fungi commonly isolated from *P. radiata* needles, were used in this study (Supporting Information, Table S1). The *Phytophthora* spp. were grown on carrot agar (Erwin & Ribeiro, 1996), amended with β-sitosterol ($0.02\,g\,L^{-1}$) at 25 °C for 10 days. Isolates of fungi were grown on potato dextrose agar ($20\,g\,L^{-1}$) (Biolab, Merck, Midrand, South Africa) at 20 °C for 7–10 days. All the isolates are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

Primer development

DNA extraction

For all the samples used in this study, DNA was extracted from the mycelium scraped off the surface of the agar plates using PrepMan Ultra sample preparation reagent (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. The concentration of isolated DNA for each culture was determined with a NanoDrop ND 1000 spectrophotometer and NANODROP 3.2.1 software (NanoDrop Technologies Inc., Rockland, DE) and adjusted to $50\,ng\,\mu L^{-1}$ with sterile water.

DNA sequencing

Twenty isolates of *P. pinifolia*, including the ex-type culture (Table S1), collected in June 2007, were used for DNA analysis.

The internal transcribed spacer (ITS) region of the rRNA gene was amplified using the primers ITS1 and ITS4 (White *et al.*, 1990). The ras-related protein gene *Ypt*1 of the nuclear DNA was amplified using the primers Yph1F and Yph2R (Schena *et al.*, 2006). The PCR reaction mixtures and reaction conditions were the same as those described by Durán *et al.* (2008). Successful amplification was confirmed by gel electrophoresis (1% agarose gel stained with ethidium bromide and visualized under UV light). PCR products were purified through Sephadex G-50 (Sigma-Aldrich, St. Louis, MO) in Centri-Sep spin columns (Princeton Separations, Adelphia, NJ) following the manufacturer's instructions to remove excess primers and nucleotides.

PCR products were sequenced with the forward and reverse primers used in the amplification reactions. Reactions were performed using an ABI PRISM™ Big Dye terminator sequencing reaction kit following the manufacturer's instructions (Perkin-Elmer, Applied Biosystems). Sequencing was carried out using an ABI 3100™ automated DNA sequencer and the sequences obtained were verified using the software programme, MEGA 3.0 (Kumar *et al.*, 2004). Individual sequences generated in this study were deposited in GenBank (Table S2).

Primer design

For both the ITS region and *Ypt*1 gene, a data matrix was compiled using sequences obtained in this study for *P. pinifolia* and those retrieved from GenBank and published for other *Phytophthora* spp. (Table S2). For the ITS region, the sequences generated in this study were added to the sequence data from Durán *et al.* (2008) and for the *Ypt*1 gene, to the sequence data from Schena *et al.* (2006).

Sequences were aligned using the software MEGA 3.0 (Kumar *et al.*, 2004) and the alignment of all sequences was also checked visually. The *P. pinifolia*-specific PCR primers were developed using the software PRIMER3 web v. 0.3.0 (Rozen & Skaletsky, 2000). The primer sequences were checked for possible sequence homology with other DNA

sequences using a BLAST search in GenBank (NCBI, Bethesda, MD).

Primer testing

The primers were tested using PCR reactions with *P. pinifolia* DNA extracted from the same 20 isolates from which the rRNA gene sequences were used for primer design, as well as for 30 additional *P. pinifolia* isolates that had been identified separately using rRNA gene sequence data. Species that are phylogenetically most closely related to *P. pinifolia*, namely *Phytophthora humicola*, *Phytophthora gonapodyides*, *Phytophthora megasperma* and *Phytophthora inundata*, and other *Phytophthora* spp. available in the culture collection (CMW) of the FABI (Table S1), were also tested. Fungal species isolated from *P. radiata* needles as well as other fungal species isolated from conifers and present in CMW were also included (Table S1).

To assess the sensitivity of the PCR reaction to detect *P. pinifolia*, DNA extracted from pure cultures was serially diluted with sterile water to yield final concentrations ranging from 10 ng μL^{-1} to 1 fg μL^{-1} of DNA and amplified with both sets of primers. Sterile water replaced template DNA to provide a negative control. The PCR product was visualized using gel electrophoresis.

PCR-RFLP

Twenty isolates of *P. pinifolia* (Table S1) were analysed using the RFLP identification protocol described by Drenth *et al.* (2006). In addition, 15 *Phytophthora* spp., including the species that are phylogenetically most closely related to *P. pinifolia* (Table S1), were tested using this procedure. The PCR reactions and the digestions with the enzymes MspI, RsaI and TaqI were performed in an iCycler thermocycler (Bio-Rad). The digested DNA was size fractionated using 3% agarose gels containing ethidium bromide at 120 V for 90 min and visualized under UV light.

Results

Primer development

Amplification of the ITS regions of the *P. pinifolia* isolates generated fragments of 811 bp and amplification of the *Ypt*1 gene yielded fragments of 568 bp. The sequences for both the ITS region and *Ypt*1 were identical in all the isolates tested. Based on the sequences obtained, the following species-specific primers for *P. pinifolia* were identified: Pfoli1F (5′-GCTCTATCGCGAGCGTTT-3′) and Pfoli1R (5′-CGCAAATGACTGAAAAAGCA-3′) for the ITS region, and Yfoli1F (5′-CAGGCTGGACTCTGCTCTTC-3′) and Yfoli1R (5′-CCCACTACACAAGAGAGTTAGTTTT-3′) for the *Ypt*1 gene. BLAST searches with these primer sequences against the GenBank database showed that no organisms had sequence motifs identical to those of the primers designed for *P. pinifolia*.

PCR amplification from DNA of *P. pinifolia* isolates was successful using both sets of primers (Pfoli1F–Pfoli1R) and (Yfoli1F–Yfoli1R). Primer set Pfoli1F–Pfoli1R produced a fragment of 557 bp, and primer set Yfoli1F–Yfoli1R produced a fragment of 216 bp. Sequencing of these fragments confirmed that the region amplified was that of the targeted locus. No amplification was observed in the negative control, with DNA of other *Phytophthora* spp. (Fig. 1) or the fungi tested (data not shown). The sensitivity test showed that the Pfoli1F and Pfoli1R primer set produced a visually identifiable amplicon from 2.5 pg μL^{-1} of DNA, while the Yfoli1F and Yfoli1R primer set produced a visually identifiable amplicon from 100 pg μL^{-1} of DNA.

PCR-RFLP

Amplification of *P. pinifolia* with the primers A2F and I2R, produced a fragment of 813 bp, with no fragment visible in the negative control. In the RFLP profile (Fig. 2), fragments for the *P. pinifolia* isolates digested with the enzymes MspI, RsaI and TaqI were distinct from those of the other *Phytophthora* spp. described by Drenth *et al.* (2006) and those that are phylogenetically most closely related to *P. pinifolia* (Table S3).

Discussion

In this study, two sets of primers were developed that will provide an accurate and rapid identification of *P. pinifolia* from cultures. Both the ITS rRNA gene region and *Ypt*1 gene were targeted to produce these species-specific primers, while the combined assays increased the reliability of the identification. Both sets of primers were able to discriminate *P. pinifolia* from the *Phytophthora* spp. known to be phylogenetically most closely related to it and residing in the phylogenetic Clade 6 of Brasier *et al.* (2003). They also distinguished *P. pinifolia* from a number of other *Phytophthora* spp. isolated from forest environments.

The primer set Pfoli1F and Pfoli1R, designed from the ITS region, was able to detect the *P. pinifolia* DNA from very small samples (2.5 pg μL^{-1}). This level of detection sensitivity compares favourably with that reported previously for *Phytophthora citricola* (Schubert *et al.*, 1999) and *Phytophthora cinnamomi* (Drenth *et al.*, 2006). The amount of DNA corresponded to the DNA content of approximately two nuclei based on the approximate size of the *Phytophthora* genome (Drenth *et al.*, 2006).

The choice of the ITS region to develop species-specific PCR primers for *P. pinifolia* was based on the fact that this region has been used previously to develop specific detection methods for *Phytophthora quercina*, *P. citricola*,

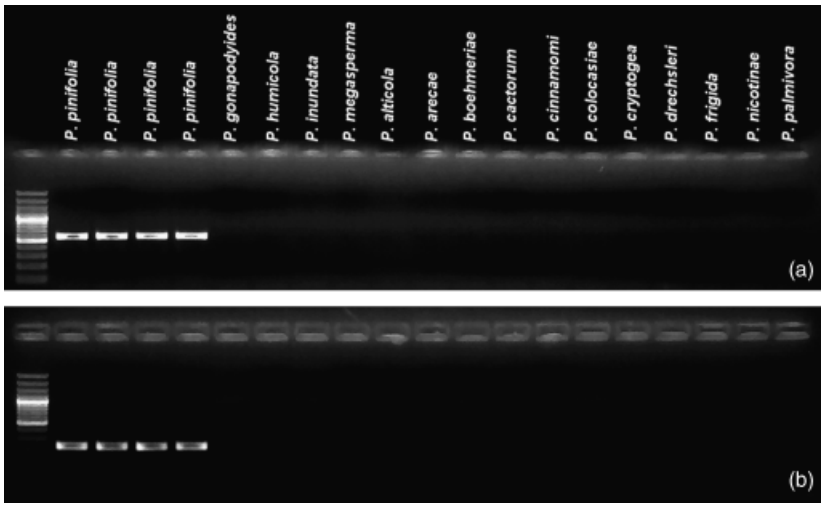

Fig. 1. Agarose gel stained with ethidium bromide and visualized under UV light, showing the PCR product of *Phytophthora pinifolia* and several other *Phytophthora* spp. with the species-specific primer sets Pfoli1F and Pfoli1R (a) and Yfoli1F and Yfoli1R (b).

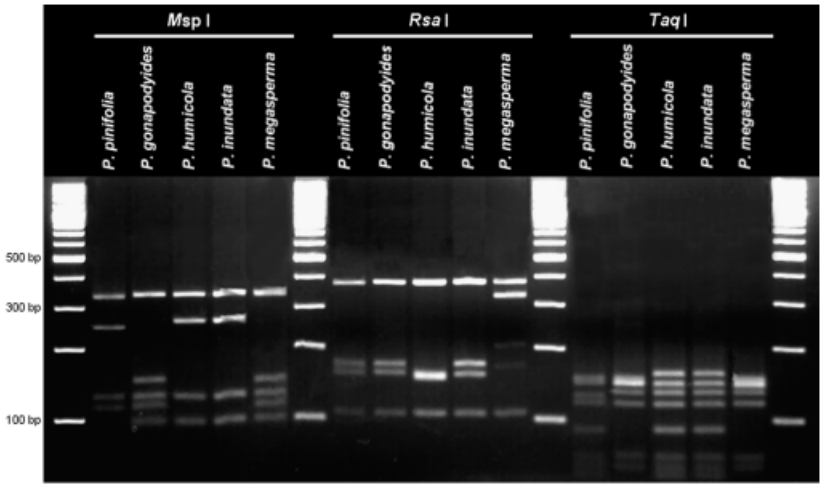

Fig. 2. Agarose gel showing ITS amplicons of *Phytophthora* spp. belonging to Clade 6 amplified using *Phytophthora* genus-specific primer A2F and I2R (Drenth *et al.*, 2006) and digested with restriction enzymes MspI, RsaI and TaqI, after PCR. Lane 1, 7, 13 and 19 – 100-bp ladder.

Phytophthora cambivora, (Schubert *et al.*, 1999), *Phytophthora ramorum* (Hayden *et al.*, 2004), *Phytophthora nicotinae* (Grote *et al.*, 2002) and *P. megasperma* (Nigro *et al.*, 2005). A shortcoming of the ITS locus is that in some circumstances, it fails to discriminate among closely related taxa (Schena & Cooke, 2006; Bowman *et al.*, 2007) although there was no evidence of this problem in the case of *P. pinifolia*.

Using the primer set Yfoli1F and Yfoli1R designed for the ras-related protein gene *Ypt*1, DNA could be amplified from *P. pinifolia* samples as small as 100 pg μL^{-1}. This level of sensitivity is 40 times lower than that needed for detection using the Pfoli1F and Pfoli1R primers based on the ITS region. This lower level of sensitivity may either be due to the fact that the *Ypt*1 gene is present only as a single copy in the genome (Chen & Roxby, 1996), compared with the multiple copies of the rRNA gene locus and/or the design of the oligonucleotide primers for the assay. The detection sensitivity for the primers Yfoli1F and Yfoli1R was lower than that described for *P. inundata* and *P. megasperma* using the species-specific primers developed for these species (Schena *et al.*, 2008), but similar to the detection limit for species-specific primers designed by Schena *et al.* (2008) for *P. cambivora*, *P. cinnamomi*, *Phytophthora lateralis*, *Phytophthora nemorosa* and *Phytophthora psychrosphila*.

The PCR-RFLP profile for *P. pinifolia*, obtained using primers A2F and I2R developed by Drenth *et al.* (2006), was unique compared with those described for the *Phytophthora* spp. used in their development. These primers can thus be used together with those developed in this study to provide an added base for identification of *P. pinifolia* isolates. The fact that we were able to produce profiles for *P. gonapodyides*, *P. megasperma*, *Phytophthora drechsleri*, *Phytophthora palmivora* and *P. cinnamomi* identical to those produced by Drenth *et al.* (2006) also emphasizes the reliability of this method.

The diagnostic procedures described in this study allow for the screening and reliable identification of large numbers of *P. pinifolia* of isolates in a short period of time. The level of specificity observed with our assays for *P. pinifolia* makes additional sequencing steps unnecessary for reliable species identifications. These techniques should be valuable in augmenting research on the biology and epidemiology of this important new pathogen, for which very little knowledge is currently available, as well for quarantine procedures.

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References

Bowman KD, Albrecht U, Graham JH & Bright DB (2007) Detection of *Phytophthora nicotianae* and *P. palmivora* in citrus roots using PCR-RFLP in comparison with other methods. *Eur J Plant Pathol* **119**: 143–158.

Brasier CM, Sanchez-Hernandez E & Kirk SA (2003) *Phytophthora inundata* sp. nov., a part heterothallic pathogen of trees and shrubs in wet or flooded soils. *Mycol Res* **107**: 477–484.

Chen Y & Roxby R (1996) Characterization of a *Phytophthora infestans* gene involved in the vesicle transport. *Gene* **181**: 89–94.

Cooke DEL, Jung T, Williams NA, Schubert R, Bahnweg G, Osswald W & Duncan JM (1999) Molecular evidence supports *Phytophthora quercina* as a distinct species. *Mycol Res* **99**: 799–804.

Cooke DEL, Drenth A, Duncan JM, Wagels G & Brasier CM (2000a) A molecular phylogeny of *Phytophthora* and related Oomycetes. *Fungal Genet Biol* **30**: 17–32.

Cooke DEL, Duncan JM, Williams NA, Hagenaar-de Weerdt M & Bonants PJM (2000b) Identification of *Phytophthora* species on the basis of restriction enzyme fragment analysis of the internal transcribed spacer regions of ribosomal RNA. *Bull OEPP* **30**: 519–523.

Cooke DEL, Schena L & Cacciola SO (2007) Tools to detect, identify and monitor *Phytophthora* species in natural ecosystems. *J Plant Pathol* **89**: 13–28.

Drenth A, Wagels G, Smith B, Sendall B, O'Dwyer C, Irvine G & Irwin JAG (2006) Development of a DNA-based method for detection and identification of *Phytophthora* species. *Australas Plant Path* **35**: 147–159.

Duncan JM & Cooke DEL (2002) Identifying, diagnosing and detecting *Phytophthora* by molecular methods. *Mycologist* **16**: 59–66.

Durán A, Gryzenhout M, Slippers B, Ahumada R, Rotella A, Flores F, Wingfield BD & Wingfield MJ (2008) *Phytophthora pinifolia* sp. nov. associated with a serious needle disease of *Pinus radiata* in Chile. *Plant Pathol* **57**: 715–727.

Erwin DC & Ribeiro OK (1996) Culture, physiology, and genetics of *Phytophthora* species. *Phytophthora Diseases Worldwide* (Erwin DC & Ribeiro OK, eds), pp. 42–95. APS Press, St. Paul, MN.

Grote D, Olmos A, Kofoet A, Tuset JJ, Bertolini E & Cambra M (2002) Specific and sensitive detection of *Phytophthora nicotianae* by simple and nested PCR. *Eur J Plant Pathol* **108**: 197–207.

Hayden KJ, Rizzo D, Tse J & Garbelotto M (2004) Detection and quantification of *Phytophthora ramorum* from California forests using a real-time polymerase chain reaction assay. *Phytopathology* **94**: 1075–1083.

Kroon LPNM, Bakker FT, van den Bosch GBM, Bonants PJM & Flier WG (2004) Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genet Biol* **41**: 766–782.

Kumar S, Tamura K & Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**: 150–163.

Martin FN & Tooley PW (2004) Identification of *Phytophthora* isolates to species level using restriction fragment length polymorphism analysis of polymerase chain reaction-amplified region of mitochondrial DNA. *Phytopathology* **94**: 983–991.

Martin RR, James D & Lévesque CA (2000) Impacts of molecular diagnostic technologies on plant disease management. *Annu Rev Phytopathol* **38**: 207–239.

Nigro F, Yaseen T, Schena L, Ippolito A & Cooke DEL (2005) Specific PCR detection of *Phytophthora megasperma* using the intergenic spacer region of ribosomal DNA. *J Plant Pathol* **87**: 300.

Rozen S & Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (Krawetz S & Misener S, eds), pp. 365–386. Humana Press, Totowa, NJ.

Schena L & Cooke DEL (2006) Assessing the potential of regions of the nuclear and mitochondrial genome to develop a "molecular tool box" for the detection and characterization of *Phytophthora* species. *J Microbiol Meth* **67**: 70–85.

Schena L, Hughes KJD & Cooke DEL (2006) Detection and quantification of *Phytophthora ramorum*, *P. kernoviae*, *P. citricola* and *P. quercina* in symptomatic leaves by multiplex real-time PCR. *Mol Plant Pathol* **7**: 365–379.

Schena L, Duncan JM & Cooke DEL (2008) Development and application of a PCR-based 'molecular tool box' for the identification of *Phytophthora* species damaging forests and natural ecosystems. *Plant Pathol* **57**: 64–75.

Schubert R, Bahnweg G, Nechwatal J, Jung T, Cooke DEL, Duncan JM, Müller–Starck G, Langebartels C, Sandermann H Jr & Oβwald WF (1999) Detection and quantification of

Phytophthora species which are associated with root-rot diseases in European deciduous forests by species-specific polymerase chain reaction. *Forest Pathol* **29**: 169–188.

White TJ, Bruns T, Lee S & Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *PCR Protocols: A Guide to Methods and Applications* (Innis MA, Gelfand D, Sninsky J & White T, eds), pp. 315–322. Academic Press, San Diego, CA.

Williams N, Hardy GEJ St & O'Brien PA (2009) Analysis of the distribution of *Phytophthora cinnamomi* in soil at a disease site in Western Australia using nested PCR. *Forest Pathol* **39**: 95–109.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Cultures of *Phytophthora* spp. and various fungal species associated with pines that were used to test the specificity of the species-specific primer sets Pfoli1F and Pfoli1R, and Yfoli1F and Yfoli1R.

Table S2. GenBank accession numbers of *Phyphthora* spp. sequences used to design specific primers.

Table S3. Amplicon sizes after amplification with genus specific primers A2F and I2R, and fragment sizes (bp) of different *Phytophthora* species after restriction digests with three different restriction enzymes.

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