Microsatellites in the mitochondrial genome of *Phytophthora cinnamomi* failed to provide highly polymorphic markers for population genetics

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Abstract

Microsatellites were evaluated as genetic markers for the mitochondrial genome (mtDNA) of *Phytophthora cinnamomi* for population studies. Two (A)n microsatellite loci were cloned from the mtDNA of *P. cinnamomi*. Amplification products from these loci showed little polymorphism among *Phytophthora* isolates due to their location in coding regions of mtDNA. A further three (A)n microsatellite loci obtained from the complete mtDNA sequence of *P. infestans* were also not highly polymorphic, although located in non-coding mtDNA. The presence of the (A)n microsatellites was not conserved in the genus *Phytophthora*. Unlike those of the chloroplast genome of plants, (A)n microsatellite loci of mtDNA do not have potential as highly polymorphic markers in *Phytophthora*. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Microsatellite; Mononucleotide repeat; *Phytophthora cinnamomi*; Oomycete genetics

1. Introduction

Microsatellites are used extensively as genetic markers in population studies because they are highly length polymorphic, codominantly expressed and scattered in abundance throughout the genome in eukaryotes [1]. They consist of tandem repeats of very short nucleotide motifs [2]. A microsatellite locus is detected by polymerase chain reaction (PCR) amplification with primers that anneal to the unique flanking sequences of the microsatellite [3]. Because these flanking sequences and the microsatellite location are often conserved, primers are often transferable among related species [4].

Microsatellites are known to occur in the chloroplast genome (cpDNA) of plants. Those with the repeating motif of (dA)n-(dT)n (termed (A)n microsatellites hereafter) of cpDNA have been developed into useful highly polymorphic genetic markers of pine and soybean populations [5,6]. It is not known whether (A)n microsatellites are present in mitochondrial genomes (mtDNA) and have similar potential as highly polymorphic genetic markers. We considered such markers in our search for mtDNA...
markers to study the breeding systems and population genetics of the oomycete *Phytophthora cinnamomi* Rands.

*P. cinnamomi* is a root rot pathogen causing disease in thousands of plant species in natural vegetation, as well as forestry and crop plants worldwide [7]. In the investigation reported in this paper, we searched for and characterised (A)_n microsatellites of the mtDNA of *P. cinnamomi* and assessed their potential as highly polymorphic genetic markers.

2. Materials and methods

2.1. Isolates, DNA extraction and manipulations

*Phytophthora* isolates were maintained and mycelium grown as described previously [8]. Genomic DNA was extracted by a method modified from Stewart and Via [9] and Graham et al. [10]. Twenty-five mg of lyophilised mycelium was ground in 600 µl of extraction buffer [10] (with 2% polyvinylpyrrolidone-40) in a 1.5-ml microfuge tube and incubated for 1 h at 70°C. RNase A was added at 500 µg ml⁻¹ and incubated at 37°C for 20 min. The mixture was extracted with chloroform/isoamyl alcohol (24:1), DNA was precipitated with isopropanol, then dried and resuspended in TE buffer [9]. MtDNA was separated from a scaled up genomic DNA extract by equilibrium centrifugation in 1.1 g ml⁻¹ CsCl, 100 µg ml⁻¹ bisbenzimide in a vertical rotor at 20°C and 105,000×g for 40 h [11]. The mtDNA was removed, extracted three times with isopropanol saturated by 5 M NaCl in TE buffer, then three volumes of TE buffer were added and DNA was ethanol precipitated [12]. Restriction digests of mtDNA, agarose gel electrophoresis and Southern blotting to nylon membrane were performed by standard protocols [12].

2.2. Cloning and sequencing

DNA fragments excised from agarose gels were purified using a silica matrix DNA purification kit (Bresaclean, Bresatec, Australia). Fragments to be cloned were ligated into *BamH1* digested pUC18 [12] and transformed into E. coli DH5αMCR (Life Technologies) by the method of Inoue et al. [13]. Subcloning to a size suitable for DNA sequencing was by standard protocols [12]. Both forward and reverse strands were sequenced using an automated DNA sequencer (Applied Biosystems 373) and fluorescent cycle sequencing technology. PCR products to be sequenced were treated similarly.

2.3. PCR and electrophoresis

Primers were designed using the program PRIMER (version 0.5, Whitehead Institute for Biomedical Research). One primer of each pair was synthesised with either the fluorescent dye 6-carboxyfluorescein (6-FAM) or 5-carboxyfluorescein (FAM) attached to the 5’ end for use with the GeneScan® system (Applied Biosystems). PCR amplifications were done in 10-µl volumes as described previously [14]. Thermocycling was performed with the temperature controlled by a thermistor within a reaction tube. A touchdown PCR thermocycle program was used [15]. For primer pairs mt2–7 and mt4–12 a two step program was used: one initial cycle of 94°C 1 min, then denaturation at 94°C 10 s and annealing at 57°C 0 s (58°C for mt2–7), reducing the annealing temperature by 1°C after every 3 cycles until 50°C annealing (51°C for mt2–7) was reached, then continuing for 30 cycles. An extension step at 72°C 20 s was included in each cycle for the other primer pairs. For primer pairs which did not amplify a product in some isolates, lower initial annealing temperature (up to 3°C lower) and varying MgCl₂ concentrations (between 3 and 0.5 mM) were tested in attempts to obtain an amplification product. A final extension step at 72°C 90 min was used in all programs to ensure non-templated addition of one nucleotide to the 3’ end of all amplification products [16], thus enabling precise size determination.

The fluorescently labeled amplification products were separated by 6% denaturing polyacrylamide gel electrophoresis using the GeneScan® system. Size differences of one base pair could be accurately distinguished by reference to a standard within each lane.
3. Results and discussion

3.1. Cloning and characterisation of \((A)_n\) microsatellites from P. cinnamomi

*Nde*II digested mtDNA of *P. cinnamomi* isolate A2400 gave a distinctive pattern when separated by agarose gel electrophoresis (Fig. 1A) that was partially evident in Southern hybridisation with the (dT)\(_{20}\) probe (Fig. 1B). This allowed us to directly excise individual probe-positive DNA fragments from a parallel agarose gel for subsequent cloning. Two clones sequenced had \((A)_n\) microsatellites of suitable length (Fig. 2); longer microsatellites are more likely to be polymorphic [17]. We designed primers and amplified the \((A)_n\) microsatellites of loci mt2-7 and mt4-12 in 25 *P. cinnamomi* isolates and 15 isolates of 8 other *Phytophthora* spp.

No length polymorphism was found at the mt2-7 or mt4-12 loci among 25 *P. cinnamomi* isolates from a wide geographic range, nor within the other *Phytophthora* spp. (Table 1). However, there was length polymorphism among species, but only of three base pairs difference (one codon length). From this result we hypothesised that these \((A)_n\) microsatellites may be located within conserved coding sequences of the mitochondrial genome. This would explain their lack of polymorphism compared to \((A)_n\) microsatellites of chloroplasts [5,6] as variations of one repeat unit in the microsatellite (i.e. one base pair) would result in frame shift mutations in the coding sequence. Our hypothesis is supported by an almost perfect sequence alignment of clones mt2-7 and mt4-12 with coding regions of the recently available complete mtDNA sequence of *P. infestans* (Fig. 2) ([18], B.F. Lang, unpublished).

![Fig. 1](image1.png)

**Fig. 1.** A: Agarose gel electrophoresis of *Nde*II digested mtDNA of *P. cinnamomi* isolate A2400 with adjacent sizes given in base pairs. B: Southern hybridisation of this DNA with (dT)\(_{20}\). Southern blots were prehybridised for 1 h and probed with 1 pmol ml\(^{-1}\) biotinylated (dT)\(_{20}\) oligonucleotide in hybridisation buffer (5 \times SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% purified casein) at 40°C (the \(T_m\) of a (dA)\(_{15}\)(dT)\(_{15}\) DNA duplex with the hybridisation buffer’s Na\(^+\) concentration [12]) for 1 h. Stringency washes were 3 \times 1 min at 40°C in 5 \times SSC, 0.1% SDS. The hybridised probe was detected by chemiluminescence as described by the kit manufacturer (Lightning\textsuperscript{TM} system, Bresatec).

![Fig. 2](image2.png)

**Fig. 2.** Nucleotide sequences of clones mt2-7 and mt4-12 containing \((A)_n\) microsatellites (GenBank accession numbers AF051358 and AF051359, respectively). Primer annealing sites are underlined and the boxed regions identify regions homologous to *P. infestans* mtDNA.
3.2. Development of (A)n microsatellite markers from P. infestans mtDNA sequence

To identify additional polymorphic microsatellites, we searched the as yet unpublished (B.F. Lang) complete mtDNA sequence of P. infestans and found three different (A)n microsatellites for which we designed primers. Our rationale for using P. infestans sequence data was that in general microsatellite locations and their flanking sequences are conserved among related species [4] and in particular (A)n microsatellite locations in chloroplasts are conserved among species of a genus [5,6]. The (A)n microsatellites were located in non-coding DNA between the genes: nad6 and nad4L, an (A)10 microsatellite, with primers mtnad6F 5’-AAAGCTATTTTTATGT-TAATTCAC-3’ and mtnad6R 5’-ATTGTCTTTCTT-TAAATTATATGGG-3’; cox2 and cox1, an (A)12 microsatellite, with primers mtcox1F 5’-TAGAAGATTATTTAATTGG-3’ and mtcox1R 5’-TGTTGACCATTTATTTATTTT-3’; and atp9 and nad9, an (A)12 microsatellite, with primers mtatp9F 5’-TTTATTCTGTTTAATGGC-3’ and mtatp9R 5’-CAGCAACAAATTCTAC-3’. The primers were intentionally located within coding DNA flanking the non-coding DNA to be amplified, and where possible designed with the primer’s 3’ base not in the wobble position of a codon. This maximised the probability that the primers would anneal to and amplify the same loci in mtDNA of Phytophthora spp. other than P. infestans. These three loci were amplified in the 40 Phytophthora isolates used previously as well as six P. infestans isolates.

All three loci were polymorphic but mtnad6 was unable to be amplified in isolates from many species.

Table 1
Phytophthora isolates used, their origin and the size of amplified products of mtDNA loci

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>Origina</th>
<th>mtDNA loci sizes (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mt2–7</td>
</tr>
<tr>
<td>P. cinnamomi</td>
<td>15</td>
<td>Australiaa</td>
<td>88</td>
</tr>
<tr>
<td>P. cinnamomi</td>
<td>6</td>
<td>Papua New Guinea</td>
<td>88</td>
</tr>
<tr>
<td>P. cinnamomi</td>
<td>1</td>
<td>Papua New Guinea</td>
<td>88</td>
</tr>
<tr>
<td>P. cinnamomi</td>
<td>1</td>
<td>USA</td>
<td>88</td>
</tr>
<tr>
<td>P. cinnamomi</td>
<td>2</td>
<td>Japan</td>
<td>88</td>
</tr>
<tr>
<td>P. cactorum</td>
<td>1</td>
<td>Australia</td>
<td>88</td>
</tr>
<tr>
<td>P. cactorum</td>
<td>1</td>
<td>Australia</td>
<td>88</td>
</tr>
<tr>
<td>P. cambivora</td>
<td>2</td>
<td>Australia</td>
<td>88</td>
</tr>
<tr>
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<td>1</td>
<td>Australia</td>
<td>88</td>
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<tr>
<td>P. citrophthora</td>
<td>1</td>
<td>Australia</td>
<td>88</td>
</tr>
<tr>
<td>P. cryptogea/drechsleri</td>
<td>2</td>
<td>Australia</td>
<td>88</td>
</tr>
<tr>
<td>P. cryptogea/drechsleri</td>
<td>1</td>
<td>Australia</td>
<td>88</td>
</tr>
<tr>
<td>P. infestans</td>
<td>5</td>
<td>Wales, UK</td>
<td>n.t.</td>
</tr>
<tr>
<td>P. infestans</td>
<td>1</td>
<td>Australia</td>
<td>n.t.</td>
</tr>
<tr>
<td>P. nicotianae</td>
<td>1</td>
<td>Australia</td>
<td>85</td>
</tr>
<tr>
<td>P. parasitica</td>
<td>2</td>
<td>Australia</td>
<td>85</td>
</tr>
<tr>
<td>P. sojae</td>
<td>1</td>
<td>Australia</td>
<td>88</td>
</tr>
<tr>
<td>P. sojae</td>
<td>1</td>
<td>Australia</td>
<td>88</td>
</tr>
</tbody>
</table>

aAll isolates obtained from M. Dudzinski and K. Old, CSIRO Forestry and Forest Products, Canberra, Australia, except for Welsh P. infestans DNA from N. Pipe, University of Wales, Bangor, UK, and Australian P. infestans from A. Hardham and B. Grant. Repetition of PCR and electrophoresis for each isolate gave identically sized products.
bIsolates were obtained from a broad range of host plants, of natural and agricultural ecosystems.
cAustralian isolates were from diverse geographical locations across five states (New South Wales, Queensland, South Australia, Tasmania and Western Australia).
dNo product amplified with these primers.
eIsolates belong to this species complex.
fIsolate not tested with these primers.

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and mtatp9 in a few isolates, even with non-stringent PCR conditions (Table 1). We suggest that this is caused by mitochondrial gene order differences among the Phytophthora spp. and isolates. For example such order differences are found among P. megasperma isolates [19]. The primers were designed to anneal to mitochondrial genes in the order found in P. infestans, an order which may not be conserved in the entire genus.

In contrast to cpDNA (A)n microsatellites [5] these three loci were not highly polymorphic in Phytophthora, for example the mtatp9 locus gave three length variants amongst 25 P. cinnamomi isolates from diverse geographic locations. In addition, stutter bands were absent from the amplifications of the loci mtnad6, mtcox1 and mtatp9 (including in P. infestans isolates), but present in the amplifications of the loci mt2–7 and mt4–12, in all isolates tested (Fig. 3). Stutter bands are indicative of microsatellite amplification [3] including (A)n microsatellite amplification [5]. We suggest that (A)n microsatellites are absent from the mtnad6, mtcox1 and mtatp9 loci of the isolates tested, including from the six P. infestans isolates tested. This contrasts with the presence of (A)n microsatellites in the P. infestans isolate used for the complete mtDNA sequence.

3.3. Sequencing of mtatp9 length variants

Sequencing of the P. cinnamomi length variants of locus mtatp9 confirmed the absence of an (A)n microsatellite greater than five base pairs in length. The length variants were the result of deletions or additions of base pairs at no particular position in the non-coding region amplified. The flanking coding regions of the length variants from P. cinnamomi were identical to that of the P. infestans mtDNA sequence used for primer design, confirming the amplification of the desired region of the P. cinnamomi mitochondrial genome.

Although we were able to clone (A)n microsatellites from P. cinnamomi, they were not highly polymorphic due to their location in conserved coding regions of the mitochondrial genome. The presence of (A)n microsatellites is not conserved in non-coding mtDNA within the genus Phytophthora, in contrast to the conserved presence of (A)n microsatellites in cpDNA of plant genera [5,6]. We conclude that mitochondrial (A)n microsatellite loci do not have potential as highly polymorphic markers for Phytophthora spp. or perhaps related oomycetes. Their potential in other organisms would require further investigation.
Acknowledgments

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References