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Differentiation of *Seiridium* species associated with virulent cankers on cypress in the Mediterranean region by PCR-SSCP

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A rapid and sensitive method was developed to discriminate between *Seiridium cardinale* and *Seiridium cupressi*, the fungi causing severe cankers on common cypress in the Mediterranean area. The method amplified sequence variants in the ITS2 region of ribosomal DNA using the polymerase chain reaction (PCR), followed by polyacrylamide gel electrophoresis, to reveal single-strand conformation polymorphism (SSCP) between the two species. The greatest separation pattern was obtained with a gel matrix containing 7–10% formamide and 3–5% glycerol under optimized running conditions, which were found to be 30–40 V at 4–5°C for 4–8 h. Sequence homology among isolates within each of the two species caused no mobility shifts, with all isolates displaying the same migration pattern. A few base differences between *S. cardinale* and *S. cupressi* caused markedly different migration patterns, allowing differentiation of the two pathogens. Differences between these fungi at the genetic level are consistent with known data on morphological, physiological and pathogenic characteristics. SSCP analysis constitutes a rapid and easy-to-perform method by which to recognize and distinguish closely related organisms, and has considerable potential for use in diagnosis and taxonomy.

Keywords: cypress canker, ITS2 sequence variation, polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP), *Seiridium cardinale*, *Seiridium cupressi*

Introduction

The common cypress (*Cupressus sempervirens*) is severely under threat from the highly destructive fungal parasite *Seiridium cardinale* (Graniti, 1993). This mitosporic coelomycete, originating from California, USA on native trees of the family Cupressaceae (Wagener, 1939), was reported in Europe around the middle of the 20th century (Barthelet & Vinot, 1944; Grasso, 1951). The presence of highly susceptible hosts and climatic conditions favourable for reproduction of the pathogen facilitated its establishment and spread in the Mediterranean area, where it has caused destructive and recurrent epidemics of canker that have decimated ornamental trees, windbreaks, natural stands and cypress plantations (Solel *et al*., 1983; Graniti, 1986; Raddi *et al*., 1987; Luisi, 1990).

Sanitary measures such as fungicide treatments or the removal and destruction of infected branches and severely infected or dead trees have not resulted in a significant decrease in the current epidemics of cypress canker, the most effective control strategy having been the adoption of cypress-breeding programmes for canker resistance. Four cypress clones have been patented for resistance to cypress canker and are now commercially available (Panconesi & Raddi, 1990, 1991).

A congeneric fungus, identified as *Seiridium cupressi*, is also present in the Mediterranean basin. First reported from Kenya in the 1940s, where it caused heavy losses on Monterey cypress (*Cupressus macrocarpa*; Wimbush, 1944; Nattrass, 1945), it was also recently reported as the cause of a canker disease on the Greek island of Kos in the Aegean (Xenopoulos, 1987). Data on the severity of cankers caused by the disease in the hottest months of the year suggest that it is imperative to avoid any further spread of the pathogen in this area. In particular, comparative studies on the pathogenicity of *S. cardinale* and *S. cupressi* revealed that the latter is capable of causing persistent infections in the warm season, during which the cankers it causes develop more quickly than those caused by *S. cardinale*

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(Xenopoulos, 1991). Furthermore, *S. cupressi* is most active precisely during the period when *S. cardinale* becomes less active. Their pathogenic activity indicates that if these two pathogens were to act jointly on a single tree, as has already been reported from southern Africa (Viljoen *et al*., 1993), they could cause very severe damage.

The resistance of cypress to attacks by *S. cardinale* has been reported to be a nonspecific process, essentially based on the ability of the tree to compartmentalize wounds, and on the invulnerability of the reaction tissues (Ponchet & Andreoli, 1990). The introduction of *S. cupressi* into currently uncontaminated areas and the combined action of these two pathogens could overcome the host defence reaction and be lethal for cypress, frustrating previous and ongoing efforts toward the selection and breeding of cypress genotypes and clones for resistance to *S. cardinale* (Raddi *et al*., 1990).

The threat of the spread of *S. cupressi* in the Mediterranean must be urgently addressed not only on account of the virulence of the pathogen, which appears to be linked to its ability to produce highly toxic metabolites (Sparapano *et al*., 1994), but also because of its epidemiological significance, chiefly on account of the risk of wind dispersal of ascospores produced by the teleomorph, *Lepteutypa cupressi* (A. Graniti, personal communication).

Traditional methods for identifying these fungi are based primarily on cultural and morphological characteristics including growth rate; growth/temperature relations; shape, colour and texture of colonies; density of aerial hyphae; size and morphology of conidia; presence/absence of conidial appendages; and length and morphology of basal and apical conidial appendages. Various studies have shown problems with this approach because of the inconstancy and variability of certain characters such as colour, shape, and the curvature of the conidia and conidial appendages (Nattrass et al., 1963; Swart, 1973; Sutton, 1980; Boesewinkel, 1983; Graniti, 1986; Chou, 1989; Nag Raj, 1994).

An alternative approach is to use a molecular technique such as polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis, with the aim of developing a sensitive and rapid assay that can be employed in routine diagnosis, in place of both ineffective traditional techniques and time-consuming post-PCR processing methods.

The variable internal transcribed spacer 2 (ITS2) region of the ribosomal DNA was selected as a target. To explore the extent of sequence variation existing between the two fungi, and to clarify the composition and distribution of the sequence variants (which, in the SSCP analysis, would presumably have affected intrastrand interaction and altered migration during electrophoresis), this region from one selected, authenticated isolate per species was subjected to prior PCR amplification and sequencing. In addition, this method was compared with postamplification restriction fragment length polymorphism (RFLP) analysis.

Materials and methods

Growth of fungal isolates

A total of 28 isolates found on various hosts of the family Cupressaceae, were used (Table 1). Isolates of *S. cardinale* came from Mediterranean countries (France, Greece, Italy, Portugal, Spain and Turkey), where the disease incidence is currently higher than elsewhere, and originated from geographically distant areas that were fairly representative of the major foci of the disease. To date, *S. cupressi* has been reported only on the small island of Kos in Greece. Two different test isolates were used from this location, one grey and the other rose-coloured in culture (Xenopoulos, 1991). The remaining *S. cupressi* isolates were of diverse geographic origin, and represented the existing type-cultures of this species.

Single-conidium stock cultures were grown using liquid stationary culture in Norkrans medium (Norkrans, 1963) on 9-cm-diameter plastic Petri dishes at a temperature of 24°C. After 1–2 weeks of culturing a dense mycelial mat had covered the surface of the medium. Approximately 15–20 g fresh mycelium was harvested by vacuum filtration through Miracloth (Calbiochem, San Diego, CA, USA). Fungal mycelium was then washed with double-distilled, autoclaved water, dried on absorbent paper, weighed, frozen using liquid N_2, and lyophilized.

DNA preparation

Lyophilized mycelium was ground under liquid N_2 to a fine powder. Genomic DNA was extracted using a modified version of the Raeder & Broda (1985) and Lee & Taylor (1990) protocols. Approximately 30 mg mycelial powder was transferred to a screw-top 1·5 mL Eppendorf tube and suspended in 750 μL extraction buffer (200 mM Tris–HCl pH 8·5, 250 mM NaCl, 25 mM EDTA, 0·5% SDS). The suspension was incubated at 65°C for 1 h and 600 μL of (1 : 1) phenol : chloroform was added. After mixing at 20°C for 15 min the suspension was centrifuged for 30 min at 13 000 *g* (Beckman L8-M ultracentrifuge, Fullerton, CA, USA), and the supernatant removed to another tube where it was incubated for 15 min at 37°C in 100 μL RNase A solution (20 mg mL^{-1}). This solution was again extracted with phenol : chloroform as described above, and the resulting supernatant re-extracted using chloroform : isoamyl alcohol (24 : 1, 600 μL). After centrifugation at 13 000 *g* for a further 10 min, the supernatant was removed and DNA precipitated by the addition of 0·1 vol 3 M sodium acetate and 0·6 vol −20°C absolute ethanol. The DNA was pelleted by centrifugation at 13 000 *g* for 15 min at 4°C. DNA pellets were washed in 80% ice-cold ethanol, centrifuged at 10 000 *g* at 4°C for 5 min to remove salts, and vacuum-dried. The DNA pellets were

Table 1 *Seiridium* isolates used in polymerase chain reaction-single strand conformation polymorphism assays and their sources

Fungus	Code	Original host	Location	Country	Supplier
Seiridium cardinale	2G[a]	*Cupressus goveniana*	Var	France	C. Andreoli
	1Ccl[a]	*Cupressocyparis leylandii*	Drôme	"	"
	Fclus69[b]	*Cupressus lusitanica*	Antibes	"	"
	10S[a]	*Cupressus macrocarpa*	Île St-Honoret	"	"
	30S[a]	*Cupressus dupreziana*	Gard	"	"
	Kar[b]	*Cupressus sempervirens*	Karistos	Greece	S. G. Xenopoulos
	Mouz[b]	"	Mouzaki	"	"
	Sam[b]	"	Samos	"	"
	ATCC 38654	"	Monte Morello, Florence	Italy	A. Panconesi
	Scr[b]	*Cupressus macrocarpa*	Roselle, Grosseto	"	"
	Scj[b]	*Juniperus communis*	Capannuccia, Florence	"	"
	Bsc61[b]	*Cupressus sempervirens*	Putignano, Bari	"	N. Luisi
	B21[b]	*Juniperus communis*	Bernalda, Matera	"	L. Sparapano
	Fsc49[b]	*Cupressus sempervirens*	Lisbon	Portugal	M. F. Caetano
	Scp1[b]	"	Javira, Algarve	"	J. Pinto Gañhao
	Fsc2[b]	"	Terragona	Spain	J. J. Tuset
	Fsc46[b]	"	Diga	"	"
	Fsc38[b]	"	Mugla, Inisdibi	Turkey	P. Raddi
Seiridium cupressi	Bsc55[b]	"	Kos Island	Greece	S. G. Xenopoulos
	Kos[b]	"	Kos Island	"	"
	ATCC 48158	*Cupressocyparis leylandii*	–	New Zealand	H. J. Boesewinkel
	CBS 319·51	*Cupressus* sp.	–	Kenya	R. Ciferri
	CBS 320·51	"	–	"	"
	CBS224·55	*Cupressus macrocarpa*	–	England	D. Rudd Jones
	CBS225·55	*Cupressus forbesii*	–	"	"
	CBS226·55	*Cupressus macrocarpa*	–	"	"
	CBS227·55	"	–	"	"
	BS228·55	*Juniperus procera*	–	"	"

Abbreviations: ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures

[a]Culture code assigned by the supplier.

[b]Culture code assigned at the IPAF (Istituto per la Patologia degli Alberi Forestali, Florence, Italy).

–, Location unknown.

resuspended in 50 μL TE buffer (Tris HCl 10 mM, EDTA 1 mM, pH 8).

PCR amplification

The rDNA ITS2 region was amplified using the general primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), which anneal to conserved portions of the 5·8S and 26S ribosomal genes of several fungal species (White *et al.*, 1990). Stock DNA was quantified using a TKO 100 fluorometer (Hoefer Pharmacia Biotech, San Francisco, CA, USA) and diluted to 10 ng μL^{-1}. Amplification was carried out in 25 μL volumes containing 0·2 μM of each primer; 2·5 μL 10 × Taq DNA polymerase buffer (10 mM Tris HCl pH 8·3, 1·5 mM $MgCl_2$, 50 mM KCl, 0·1 mg gelatin); 200 μM each of dATP, dCTP, dGTP and dTTP; 10 ng template DNA; 0·5 units Taq DNA polymerase (Pharmacia Biotech, Uppsala, Sweden). Incubation was performed in a GeneAmp PCR 9600 thermal cycler (Perkin Elmer Cetus, Applied Biosystem Division, Foster City, CA, USA) using the following cycle parameters: 94°C for 60 s, 50°C for 60 s, and an extension step at 72°C for 120 s initially and increased by 1 s per cycle, using the maximum ramp time between each temperature. The total number of cycles was 35, with an initial denaturation step of 2 min at 94°C and a final extension step of 8 min at 72°C. A negative control with all reagents except DNA was included in all reactions. Results of amplification were checked by electrophoresis on 1% agarose gel (Pharmacia), stained with ethidium bromide, visualized under a UV transilluminator and photographed.

Restriction enzyme digestions and mapping

The amplification products were subjected to restriction enzyme analysis. All restriction enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, MD, USA) and used in accordance with the supplier's instructions. Briefly, 10 μL aliquots of amplified product, a restriction enzyme with its buffer, and water to make up volume were incubated at 37°C for 1–2 h or overnight. Absence or presence and size of bands were scored for the restriction enzymes *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hin*fI, *Mae*II, *Msp*I, *Rsa*I, *Sau*3AI and *Taq*I, and for the combinations *Hin*fI/*Hae*III, *Hha*I/*Dde*I and *Msp*I/*Alu*I. Restriction fragments were separated on 2% agarose gel in 1 × TBE buffer (89 mM Tris–boric acid and 2 mM EDTA) with 0·5 μg mL^{-1}

ethidium bromide, visualized and photographed under UV light.

DNA sequencing

The PCR-amplified ITS2 region of two selected isolates, one of *S. cardinale* (Fclus69) and one of *S. cupressi* (Kos), was sequenced directly after isolation from low-melting agarose gel. PCR products were purified using the Prep-A-Gene kit (BioRad, Richmond, CA, USA), and sequenced by the dideoxy method (Sanger *et al.*, 1977) using a Sequenase 2.0 kit (USB, United States Biochemical Corporation, Cleveland, OH, USA). The two PCR primers ITS3 and ITS4 served as sequencing primers for both strands of the intergenic region (White *et al.*, 1990). PCR products were radiolabelled with ^{35}S following the supplier's instructions. Sequence reactions were run for approximately 3 h on 5% polyacrylamide wedge gels in a TBE buffer. Gels were fixed, vacuum-dried and exposed to Kodak SB X-ray film for varying periods.

SSCP analysis

To denature the double strands prior to electrophoresis, 3 μL aliquots of the PCR product were mixed with 10 μL deionized formamide and 7 μL loading buffer (50% sucrose, 60 mM EDTA, 0·25% bromophenol blue). The mixture was incubated for 3 min at 95°C, then immediately cooled in an ice bath to avoid renaturation of the individual strands. Ten μL of the mixture was then loaded on 15 × 15 cm vertical nondenaturing polyacrylamide gels (acrylamide : bis-acrylamide 89 : 1) (Hoefer Pharmacia Biotech, San Francisco, CA, USA), in a 1 × TBE buffer (89 mM Tris–boric acid, 2 mM EDTA). To evaluate whether experimental conditions affected the pattern obtained, electrophoretic migration was tested at a number of temperatures (from 22°C to 4°C), voltages (20–100 V) and gel running times (from 1·5 h to overnight). To find the optimal conditions for separation of single DNA strands, migration was also tested in gels containing varying amounts of formamide (3–30%) and glycerol (1–10%). The strands were visualized by silver staining (Bassam *et al.*, 1991).

Results

PCR amplification

The DNA concentration of all extractions was determined using a DyNA Quant 200 (Hoefer) fluorometer and adjusted to 10 ng μL^{-1} for PCR amplification. All DNAs proved to be amplifiable under the PCR conditions employed. Migration of the resulting PCR products on 1% agarose gel revealed that amplification of the ITS2 region with the primers ITS3–4 produced a fragment of roughly 340 bp in all isolates investigated. The amplified fragment included the entire ITS2 region, the two primer annealing sites, and a portion of the 5·8S and 26S ribosomal genes (Fig. 1).

Restriction data analysis

RFLP analysis proved completely ineffective for discriminating between the two fungi. Most restriction endonucleases (*Hae*III, *Hha*I, *Mae*II, *Msp*I, *Rsa*I and *Taq*I) failed to produce any band in the small ITS2 fragment. The RFLP patterns observed in *S. cardinale* digests did not differ from those observed in *S. cupressi* for the enzymes *Alu*I, *Dde*I, *Hin*fI and *Sau*3AI, with all isolates displaying identical restriction profiles (Fig. 2). The ITS2 region was therefore shown to be highly conserved for all isolates analysed.

Sequencing the ITS2 region

The sequence of the ITS2 region was determined in the amplification products of the two selected isolates of *S. cardinale* and *S. cupressi*. PCR products were found to vary between the species in both length and nucleotide sequence, while isolates within each species had identical sequences. Length was also constant within each species: the ITS2 region was 159 bp long in samples of *S. cardinale* and 158 bp long in samples of *S. cupressi*. The *S. cupressi* sequence differed from the consensus *S. cardinale* sequence at three positions: 5, 84 and 158, with loss of A residues at position 5, G→C transversions at position 84, and G→T transversions at residue 158 (Fig. 2).

Detection of DNA polymorphism by SSCP analysis

Migration of the PCR samples as single DNA strands on polyacrylamide gel caused a change in mobility of the isolates of the two pathogens. However, the migration pattern of the single DNA strands provided no informative results until the optimal conditions for separation were determined. Formamide and glycerol in combination had a strong effect on the migration of single DNA strands. The use of only one of these substances always produced less distinct separation patterns. The addition of both substances at low concentrations (0–7% formamide, 0–3% glycerol) had a negative effect on migration; the SSCP patterns were indistinct, with all isolates displaying an identical migration pattern represented by only one band due to a lack of separation or to a minimal and undetectable separation distance between individual strands. High concentrations (>20% formamide, >7–8% glycerol), caused a decrease in the separation distance, thus reducing the amount of information supplied by the migration patterns; no difference in mobility of single strands was observed between *S. cardinale* and *S. cupressi* isolates at these concentrations. Higher concentrations failed to distinguish between the species, as no variation in migration was detected. Furthermore, inadequate concentrations of glycerol and formamide

changed not only the separation distance between strands, but also the separation patterns between isolates, reducing information from the SSCP analysis. The best results were obtained with the addition of 10% formamide and 5% glycerol. These concentrations gave a clear separation between strands without affecting the separation pattern of the species. Under room temperature conditions (20–22°C) the SSCP pattern for all strains was identical, showing two closely migrating bands. The use of lower running temperatures did not improve migration of single nucleic acid fragments in the gels until temperatures of approximately 5–8°C were reached. Optimal separation of individual strands, with the clearest and most distinct mobility shifts, was obtained with a gel matrix containing 7–10% formamide and 3–5% glycerol under optimized running parameters, which for this DNA fragment were found to be 30–40 V at 4–5°C for 4–8 h. Under these conditions the isolates of *S. cardinale* and *S. cupressi* differed markedly in migration pattern. All *S. cardinale* isolates had a unique migration pattern consisting of two well separated single strands, while the *S. cupressi* isolates comigrated to produce two less separated strands (Fig. 3). No difference in mobility between isolates of *S. cardinale* from the different countries was observed, indicating a substantial sequence homology in the populations of this mitosporic fungus. Variation in electrophoretic conditions did not substantially alter the migration pattern of isolates of the two pathogens. Electrophoresis for 8 h at 40 V or overnight running at 20 V did not cause a detectable increase or decrease in the separation distance between strands.

Discussion

SSCP is a mutation detection method based on the ability to detect sequence variation in short PCR-amplified DNA fragments through the conformation that such fragments assume under electrophoresis as single-stranded molecules in nondenaturing polyacrylamide gels. Under these conditions, the single strands fold back on themselves in a sequence-dependent manner. One or a few nucleotide differences among the scanned DNA segments may cause subtle changes in conformation and thus different degrees of mobility in the gel, allowing their differentiation. Application of this technique to a 340 bp fragment from the rDNA ITS2 region was shown to be an efficient method for differentiating between *S. cardinale* and *S. cupressi*, the two major fungi associated with cypress canker diseases.

The relatedness of *Seiridium* species pathogenic on cypress has already been investigated at a molecular level (Viljoen *et al*., 1993). In a study conducted with 12 *Seiridium* isolates and based on sequence comparison of a small fragment from the ITS1 region, the amount of variation found did not permit differentiation of isolates, and led these authors to conclude that cypress canker is caused by a single species of *Seiridium* with

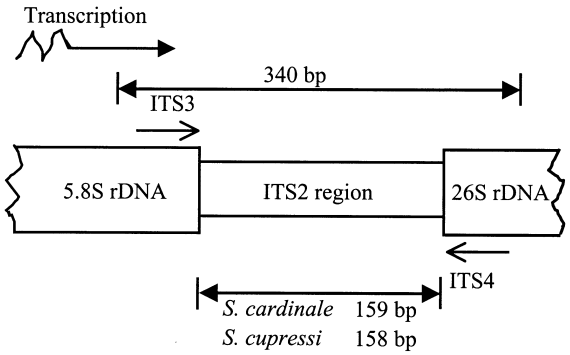

Figure 1 Schematic representation of the 3′ end of the 5·8S coding region, internal transcribed spacer (ITS2), and 5′ end of the 26S subunit. The two primers ITS3–4 produced a fragment of approximately 340 bp in *Seiridium* isolates. The amplified ITS2 region showed interspecific size polymorphism (distances are given in base pairs).

extensive variation in morphological features. However, there is reason to suspect that the limited number of isolates examined, the unsatisfactory documentation of distinctive characteristics, and the lack or poor quality of type material in at least some cases led to a questionable specific attribution of the isolates. As a consequence, the data presented appear to be insufficient to infer identity at the species level. In reality, the fungi that cause cypress canker are distinct, genuine biological entities and can be recognized by a number of morphological, cultural, physiological and pathogenic characteristics (Graniti, 1998). The present study confirms separation of these taxa.

SSCP analysis is being increasingly adopted in various fields of applied biology. Attempts to transfer this methodology to forest pathology have already given good results, discriminating among intersterility groups of the root-rot pathogen of conifers *Heterobasidion annosum*, and allowing accurate differentiation of the heteroecious pine rust fungus *Cronartium flaccidum* from the related, autoecious rust *Peridermium pini* (Kasuga & Mitchelson, 1994; Moricca & Ragazzi, 1998).

The SSCP technique was found to be more effective and sensitive than restriction analysis of amplified ribosomal DNA. With the RFLP technique, all isolates tested shared identical restriction sites and fragment lengths. The monomorphic profiles obtained were due to the conservation of many restriction sites, a result that indicates a high degree of relatedness between these fungi. Such closeness was confirmed by DNA sequencing of representative isolates, with only three base differences found in the fragment analysed between the two species.

A comparison of the two methods was undertaken because most laboratories do not have equipment for SSCP analysis, but RFLP is more commonly available. However, with growing demands for high resolution and accuracy, in conjunction with the need for cost and time saving, the rationalization of genetic analysis methodologies is desirable.

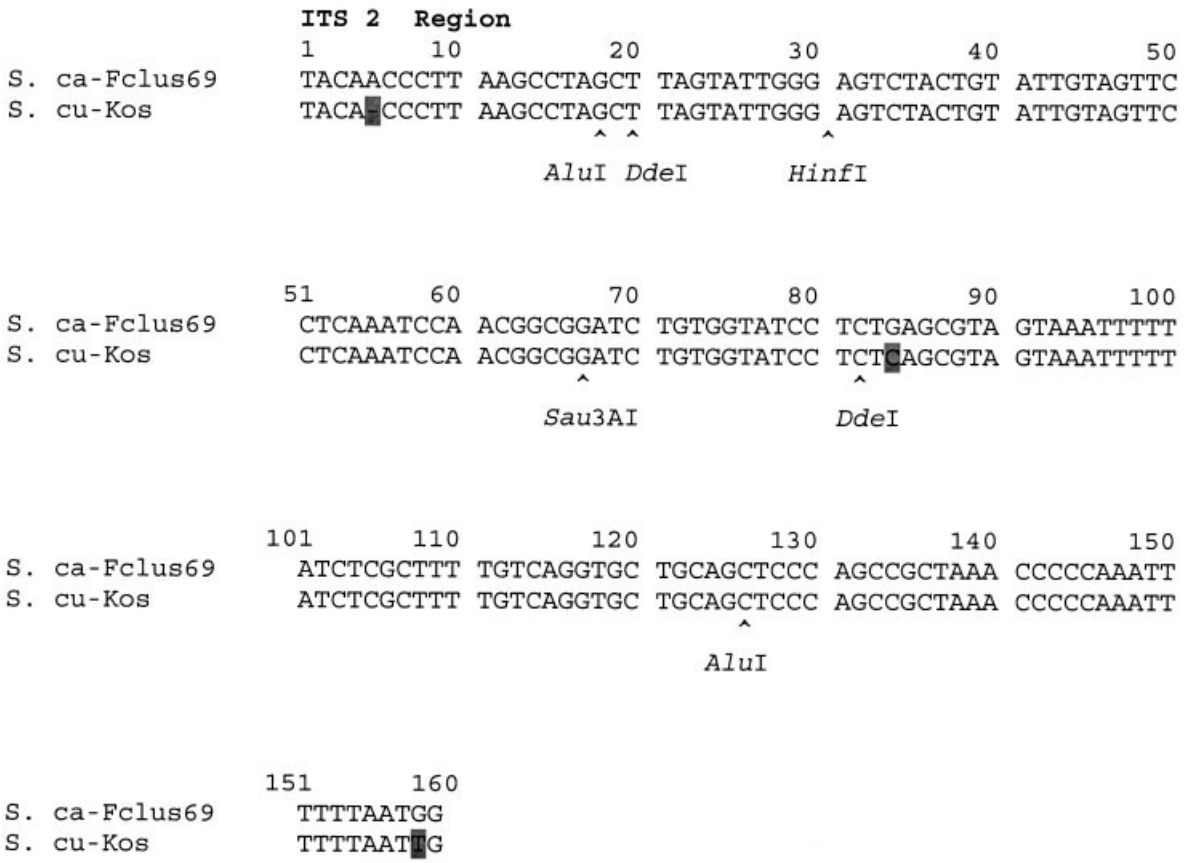

Figure 2 Aligned nucleotide sequences of the antisense strand of the ribosomal DNA internal transcribed spacer 2 (ITS2) region of *S. cardinale* and *S. cupressi*, with restriction site map for four enzymes. Shaded portions indicate positions in which variant nucleotides occurred, with a dash indicating a gap.

The rationale for applying SSCP to explore isolates of the two pathogens resides in the current state of knowledge on the origin and reproductive biology of these related fungi and their genetic diversity. Both introduced species (*S. cardinale* from western USA and *S. cupressi* from Kenya) lack sexual reproduction (completely unknown for *S. cardinale*, while the teleomorph of *S. cupressi*, *Lepteutypa cupressi*, is exceptionally rare). In the absence of sexual reproduction, genomes were suspected to be homogeneous, with a limited range of variation at the intraspecific level.

Although the PCR-SSCP technique distinguished the two species, it was necessary to optimize the experimental conditions: type of gel matrix (acrylamide : bisacrylamide ratio); electrophoretic conditions (especially running temperature, running time, formamide and glycerol concentrations); size of amplicons to be scanned; dimensions of the gel; overloading of the gel, etc. The electrophoretic mobility of single DNA strands was found to be strongly dependent on the environmental conditions. When uniform conditions were applied, informative and reproducible migration patterns were obtained. All isolates within each species comigrated to give the same separation patterns. These identical migration patterns reflected the sequence homology existing at the intraspecific level, as seen by sequence inspection. A marked difference was observed between the electrophoretic mobility of isolates identified as *S. cardinale* and those identified as *S. cupressi*. Because electrophoretic behaviour directly reflects differences in nucleotide composition of the fragments screened, the assay provided conclusive evidence of polymorphism among the taxonomic entities investigated; a single-base deletion and two base substitutions in the ITS2 region

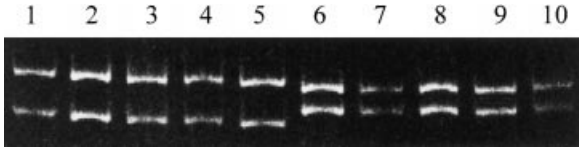

Figure 3 PAGE-SSCP patterns of PCR-generated ITS2 products of the *S. cardinale* isolates Fsc2, ATCC 38654, 10S, Scj and Mouz (lanes 1–5) and the *S. cupressi* isolates ATCC 48158, CBS227·55, CBS320·51, CBS225·55 and Bsc55 (lanes 6–10) under optimized running conditions: migration at 4°C for 5 h at 40 V in a gel matrix containing 10% formamide and 5% glycerol. All *S. cardinale* isolates had a unique profile of two well separated, comigrating single DNA strands and were clearly differentiated from the *S. cupressi* isolates which displayed an identical profile of two less-separated, comigrating single DNA strands.

were sufficient to alter strand mobility in the polyacrylamide gel, thus differentiating *S. cardinale* from *S. cupressi*.

The development of an assay enabling rapid differentiation of the two microorganisms is important because both pathogens are well adapted to the environmental conditions predominant in the Mediterranean basin, and a combination of concurrent factors could transform the diseases caused by these fungi into severe and widespread epidemics in this area. These factors include: (i) favourable climatic conditions; (ii) extensive presence of susceptible hosts; (iii) the density and contiguity of cypress stands and plantations; (iv) the presence of a number of vectors such as insects (corkborers, *Phloeosinus* spp. and a seed-bug, *Orsillus maculatus*) and birds; (v) frost and wind damage which cause small bark lesions through which infections may penetrate (Panconesi, 1990); and (vi) atmospheric conditions that allow maturation and discharge of conidia repeatedly throughout the year.

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