# Comparison of *Sphaeropsis sapinea* and *Sphaeropsis sapinea* f. sp. *cupressi*

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Seven isolates of *Sphaeropsis sapinea*, and three of a fungus reported to represent a physiological form of *S. sapinea*, i.e. *S. sapinea* f. sp. *cupressi*, were compared on the basis of various morphological and cultural criteria. All isolates lacked conidiophores and conidia were generally produced through holoblastic ontogeny and percurrent proliferation. Conidial lengths of *S. sapinea* were significantly ($P = 0{\cdot}001$) longer than those of *S. sapinea* f. sp. *cupressi* but widths did not differ significantly. All isolates of *S. sapinea* grew significantly faster ($P = 0{\cdot}01$) than any single isolate of *S. sapinea* f. sp. *cupressi* on 5 of 6 culture media and at 10, 20, 25 and 30 °C. *Sphaeropsis sapinea*, *S. sapinea* f. sp. *cupressi* and three closely related taxa, namely *Lasiodiplodia theobromae*, *Diplodia mutila*, and *Botryosphaeria dothidea* were compared using isozyme analysis. Of the 16 alleles that occurred in *S. sapinea* f. sp. *cupressi*, 3 were unique to this taxon. With the exception of *Amy* (160) and *Gpi* (107), which occurred only in *S. sapinea* and *S. sapinea* f. sp. *cupressi*, 11 other alleles common to these two taxa were also common with 1, or more, of the remaining 3 taxa. Cluster analysis of the genetic distances between the five taxa revealed that *S. sapinea* was more closely related to *D. mutila* than to *S. sapinea* f. sp. *cupressi*. The results of this study, therefore, provide substantial evidence for the lack of a close relationship between *S. sapinea* f. sp. *cupressi* and *S. sapinea*.

Stem and branch canker of Italian cypress trees (*Cupressus sempervirens* L.) has recently been described by Solel *et al.* (1987) in Israel. Isolations from the diseased cambial tissue revealed a fungus said to resemble the pine pathogen, *Sphaeropsis sapinea* (Fr.: Fr.) Dyko & Sutton [syn.: *Diplodia pinea* (Desm.) Kickx]. The fungus was considered to be a subpopulation of *S. sapinea* and was thus referred to as *Sphaeropsis sapinea* f. sp. *cupressi* (Solel *et al.*, 1987; Madar, Solel & Kimchi, 1989). *S. sapinea* f. sp. *cupressi* differed from *S. sapinea* by not being pathogenic on pine, and by having slightly smaller spores and no ornamentation on the inner conidium wall.

The nomenclature of *S. sapinea* has been the subject of considerable confusion. The fungus has, over the past 150 years, acquired at least 23 synonyms of which *Diplodia pinea* (Desm.) Kickx was the most popular (Waterman, 1943; Punithalingam & Waterston, 1970; Sutton, 1980; Sutton & Dyko, 1989). Confusion in the synonymy of *S. sapinea* was attributed by Grove (1919) to variation in the maturity of spore-bearing material examined by collectors. However, this complex situation is probably more attributable to the wide coniferous host range, extensive geographical distribution, and the large variety of disease symptoms with which *S. sapinea* has been associated (Punithalingam & Waterston, 1970; Gibson, 1979; Swart, Knox-Davies & Wingfield, 1985). Other contributory factors are undoubtedly the considerable variation within the species regarding cultural and morphological characteristics, and pathogenicity (Wang *et al.*, 1985; Palmer, Stewart & Wingfield, 1987; Swart *et al.*, 1991).

Examination of *S. sapinea* f. sp. *cupressi* on culture media revealed several significant cultural and morphological characteristics which were totally inconsistent with numerous isolates of *S. sapinea* collected from many parts of the world. This led us to question whether the fungus associated with canker of *C. sempervirens* in Israel was indeed a specialized form (forma specialis) of *S. sapinea*. By definition, a forma specialis would be characterized only by differences in physiological adaptation to a particular host plant (Hawksworth, 1974). This study was, therefore, aimed at elucidating the taxonomic relationship between *S. sapinea* and its reputed forma specialis *cupressi*.

## MATERIALS AND METHODS

***Comparison of conidia and conidial ontogeny.*** A single isolate of *S. sapinea* f. sp. *cupressi* was obtained from Dr Z. Solel, The Volcani Center, Bet Dagan, Israel during 1987. This isolate was allowed to sporulate on malt extract agar (MEA; Difco Laboratories, Detroit, MI) on which pieces of autoclaved cypress bark had been placed and several monoconidial isolates were subsequently made from this culture. Three monoconidial isolates of *S. sapinea* f. sp. *cupressi* (CPP 1, CPP 10 and CPP 12) were compared with seven isolates of *S. sapinea*. The latter included five South African isolates with

**Table 1.** Fungal isolates used for starch-gel electrophoresis

| | Host species | Geographical location | No. of isolates |
|---|---|---|---|
| *Sphaeropsis sapinea* | *Pinus radiata* D. Don | South Africa | 8 |
| | | Chile | 2 |
| | | New Zealand | 2 |
| | | Australia | 2 |
| | *Pinus taeda* L. | South Africa | 2 |
| | *P. patula* Schlecht. & Cham. | South Africa | 2 |
| | *P. halepensis* Mill. | South Africa | 1 |
| | *P. elliottii* Engelm. | South Africa | 1 |
| | *P. virginiana* Mill. | South Africa | 1 |
| | *P. kesiya* Royle ex Gordon | China | 1 |
| | *P. muricata* D. Don | New Zealand | 1 |
| | | U.K. | 2 |
| | *P. nigra* Arnold | U.K. | 4 |
| | | U.S.A. | 1 |
| | *P. oocarpa* Schiede | Honduras | 1 |
| | | Zambia | 1 |
| | *P. sylvestris* L. | U.K. | 1 |
| | *P. banksiana* Lamb. | Michigan, U.S.A. | 1 |
| | *P. resinosa* Ait. | Michigan, U.S.A. | 5 |
| | *Cedrus atlantica* (Endl.) Carr. | Spain | 1 |
| *Diplodia mutila* | *Malus* sp. | South Africa | 1 |
| *Botryosphaeria dothidea* | *Protea* sp. | South Africa | 1 |
| *Lasiodiplodia theobromae* | *P. radiata* | South Africa | 1 |
| *S. sapinea* f. sp. *cupressi* | *C. sempervirens* | Israel | 1 |

**Table 2.** Enzyme and buffer systems used to compare *Sphaeropsis sapinea* and *S. sapinea* f. sp. *cupressi* using starch-gel electrophoresis

| | Commission no. | Enzyme locus | Buffer system* |
|---|---|---|---|
| Acid phosphatase | 3.1.3.2 | *Acp-1* | A |
| | | *Acp-2* | A |
| Amylase† | 3.2.1.1 | *Amy* | A, B |
| α-Esterase | 3.1.1.1 | *Est-1* | A |
| | | *Est-2* | A |
| | | *Est-3* | A |
| | | *Est-4* | A |
| | | *Est-5* | A |
| β-Glucosidase | 3.2.1.21 | *β-Glu* | A |
| Guanine deaminase | 3.5.4.3 | *Gda* | B |
| Glucose phosphate isomerase | 5.3.1.9 | *Gpi* | A |
| Malate dehydrogenase | 1.1.1.37 | *Mdh* | A, C |
| Peptidase | | | |
| Gly-Leu substrate | 3.4.13.2 | *Pep-gl* | B |
| Phe-Pho substrate | 3.4.13.9 | *Pep-ph* | B |

* A = Buffer according to Ridgway, Sherburne & Lewis (1970). Electrode buffer, 0·06 M lithium hydroxide, boric acid 0·3 M, pH 8·1. Gel buffer, 0·03 M Tris, 0·005 M citric acid, 0·0006 M LiOH, 0·003 M boric acid. Adjust pH to 8·5 with NaOH. Run at 250 V and 50 mA for 3 h. B = Buffer according to Markert & Faulhaber (1965). Electrode buffer, 0·18 M Tris, 0·1 M boric acid, 0·004 M NaEDTA, pH 8·7. Gel buffer, 1:4 dilution of electrode buffer, pH 8·7. Run at 250 V and 50 mA for 4 h. C = Buffer according to Whitt (1970). Electrode buffer, 0·15 M Tris, 0·05 M citric acid. Gel buffer, 1:14 dilution of electrode buffer. Run at 150 V and 50 mA for 3 h.

† Opaque bands which appeared against the translucent starch gel after approximately 3 h incubation at 45° were interpreted to reflect amylase activity.

marked differences in cultural appearance (CWS 1, CWS 5, CWS 8, CWS 10 and CWS 33) and two from the north central United States (CWS 59 and CWS 60).

All isolates were grown under continuous black-light at 18 °C on 2% WA with autoclaved pine needles on the agar surface until pycnidia had formed after approximately 14 d. Conidial development in *S. sapinea* f. sp. *cupressi* and *S. sapinea* was compared using differential interference contrast microscopy. For each isolate, the length and width of 50 conidia was measured and a one-way analysis of variance (ANOVA) was performed on conidial measurements. Tukey's HSD procedure was used to compare mean conidial width and length.

***Comparison of growth on different media.*** Isolates were grown on the following media: potato–dextrose agar (PDA; Difco), MEA, Czapek–Dox agar (CD; Merck), water agar (WA), or corn-meal agar (CMA; Difco). A 5 mm diam. plug of each isolate from an actively growing MEA culture was placed fungus-side down on each of three 90 mm diam. Petri dishes containing 25 ml of medium. Dishes were sealed with Parafilm (American National Can, Greenwich, CT) and incubated in the dark at 25°. Colony diameters were measured along two perpendicular lines after 96 h. The experiment was arranged as a randomized complete block design and conducted three times. Variances among trials were tested for homogeneity via Bartlett's test, and a two-way ANOVA was performed on the pooled data. Tukey's HSD procedure for comparison of means was applied where the ANOVA showed significant variation. Observations of culture morphology were made 10 d after isolates were transferred to the test media.

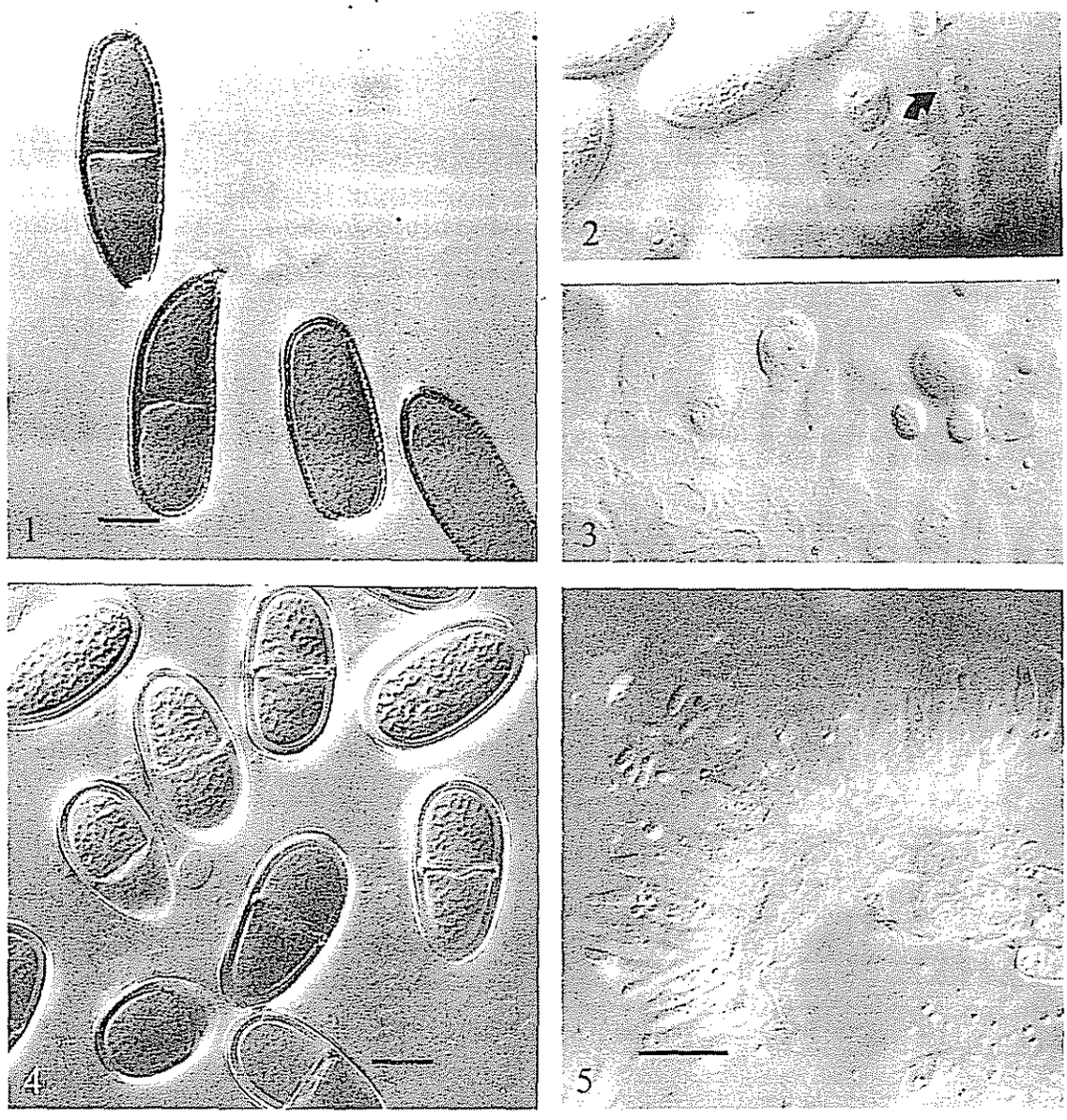


Fig. 1. Conidia of *Sphaeropsis sapinea* (bar, 10 μm). Fig. 2. Holoblastic conidial ontogeny of *S. sapinea* f. sp. *cupressi* with collarette (arrow). Fig. 3. Conidiogenous cells of *S. sapinea* f. sp. *cupressi* with percurrent proliferation. Fig. 4. Mature and immature (non-septate) conidia of *S. sapinea* f. sp. *cupressi* (bar, 10 μm). Fig. 5. Spermatia and spermatiophores of *S. sapinea* f. sp. *cupressi* (bar, 5 μm).

***Comparison of growth at different temperatures.*** Isolates were transferred as above to 90 mm diam. Petri dishes containing 20 ml MEA. Three plates of each isolate were incubated for 96 h at 10, 15, 25, and 30° after which colony diameters were measured. The experiment was arranged as a randomized complete block design and conducted three times. Variances among trials were tested for homogeneity, and a two-way ANOVA was performed on the pooled data. Tukey's HSD procedure was applied for comparison of means.

***Electrophoretic comparison.*** Isolates used for starch-gel electrophoresis included 41 monoconidial isolates of *S. sapinea* from various countries, one isolate of each of *Lasiodiplodia theobromae* (Pat.) Griff. & Maubl., *Diplodia mutila* Fr. (syn. *Sphaeropsis malorum* Berk.) and *Botryosphaeria dothidea* (Mougeot: Fr.) Cesati & de Notaris from South Africa and the original isolate of *S. sapinea* f. sp. *cupressi* obtained from Israel (Table 1). Mycelium was produced by culturing the isolates in Petri plates on 2% MEA that had been covered with a cellophane membrane (Visking tubing) in order to avoid agar contamination. Dishes were incubated at 25° for 3 d whereafter the mycelium was scraped off the cellophane with a scalpel. Approximately 3 $cm^3$ of mycelium was ground with a chilled mortar and pestle together with 0·5 M Tris-HCl buffer (pH 8·0) containing 0·001 M pyrodoxal-5-phosphate (tissue:buffer, 1:1) and *ca* 200 mg of polyvinylpurrolidone (PVP–MW 360000). The homogenate was centrifuged at about 5000 *g* for 5 min and the clear supernatant was absorbed onto Whatman filter-paper wicks (2 × 10 mm). Forty-five wicks, each representing a different isolate, were inserted into a horizontal starch gel (Sigma Chemical Co., St Louis, MO) and subjected to electrophoresis as described by O'Malley, Wheeler & Guries (1980), Harris & Hopkinson (1976) and Shaw & Prasad (1970).

After completion of electrophoresis, 1·5 mm slices of gel

**Table 3.** Conidial dimensions of isolates of *Sphaeropsis sapinea* and *S. sapinea* f. sp. *cupressi*

| Isolate number* | Conidial dimensions (µm)† Mean length | Mean width |
|---|---|---|
| CWS 1 | 39·9 bc | 15·6 abc |
| CWS 5 | 39·8 bc | 16·0 ab |
| CWS 8 | 39·8 bc | 16·2 ab |
| CWS 10 | 40·9 b | 14·9 abc |
| CWS 33 | 44·9 a | 17·8 a |
| CWS 59 | 34·6 d | 12·3 c |
| CWS 60 | 36·6 cd | 13·5 bc |
| CPP 1 | 25·1 e | 12·1 c |
| CPP 10 | 24·7 e | 12·8 bc |
| CPP 12 | 25·3 e | 12·4 c |

* Numbers having a CWS and CPP prefix, respectively, represent isolates of *S. sapinea* and *S. sapinea* f. sp. *cupressi* that are maintained at the Department of Plant Pathology, University of the Orange Free State, Bloemfontein, South Africa.

† Mean of 50 conidia. Values followed by different letters are significantly different ($P = 0·01$) according to Tukey's HSD procedure. Mean length s.e. = 0·7, mean width s.e. = 0·3.

**Table 5.** Growth of isolates of *Sphaeropsis sapinea* and *S. sapinea* f. sp. *cupressi* on malt extract agar at five temperatures

| Isolate number* | Colony diameter after 3 d (mm)† 10° | 15° | 20° | 25° | 30° |
|---|---|---|---|---|---|
| CWS 1 | 19·6 mnop | 34·2 ghi | 49·1 e | 64·4 ab | 30·8 ij |
| CWS 5 | 20·0 mno | 36·9 fg | 51·3 de | 66·3 a | 37·2 fg |
| CWS 8 | 18·7 nop | 38·2 fg | 47·3 e | 60·8 bc | 33·3 ghi |
| CWS 10 | 19·3 mnop | 39·9 f | 51·4 de | 68·3 a | 27·8 jk |
| CWS 33 | 21·6 lmno | 33·2 ghij | 51·2 de | 59·8 bc | 34·2 ghi |
| CWS 59 | 17·3 op | 23·6 klmn | 31·4 hij | 56·4 cd | 17·3 op |
| CWS 60 | 18·7 nop | 29·7 ij | 36·7 fgh | 58·1 c | 31·0 hij |
| CPP 1 | 7·4 r | 18·5 op | 22·4 klmno | 24·0 klmn | 14·3 pq |
| CPP 10 | 7·5 r | 20·9 lmno | 23·3 klmn | 25·5 kl | 14·2 pq |
| CPP 12 | 7·3 r | 19·1 mnop | 23·2 klmn | 24·3 klm | 11·0 qr |

* Numbers having a CWS and CPP prefix represent isolates of *S. sapinea* and *S. sapinea* f. sp. *cupressi*, respectively.

† Values are means of three replications. Interaction values followed by the same letter are not significantly different ($P = 0·01$) according to Tukey's HSD procedure.

were stained with specific enzyme stains as described by O'Malley *et al.* (1980), Harris & Hopkinson (1976) and Shaw & Prasad (1970). Three electrophoretic buffer systems and 25 enzyme stains were tested initially for resolution and stainability; from these, nine enzyme systems encoded by 14 loci producing resolvable banding patterns were selected for analysis (Table 2).

Because the isolates used either do not have a teleomorph or did not form it in pure culture, the genetic basis of the isozyme bands, or genotype, could not be unequivocally determined. Therefore, a conservative approach to the interpretation of observed isozyme phenotypes was taken by assuming mobility differences, or electromorphs, are allelic variants within a given isozyme locus. Subsequent references to alleles or allele frequencies are used in this sense. In some instances asymmetric banding intensities were observed for a phenotype in an individual. Since this rarely occurred, dosage effects were ignored and equal weight was given to each band to calculate allele frequencies. Loci encoding for functionally similar isozymes were designated numerically by the mobilities of the encoded proteins and numbered starting from the cathodal end of the gel. Allozymes were designated by their mobilities relative to the most common allozyme, which was designated 100.

Allele frequencies were calculated according to each different taxon, namely *S. sapinea*, *S. sapinea* f. sp. *cupressi*, *L. theobromae*, *D. mutila* and *B. dothidea*. Nei's (1978) unbiased genetic distances were calculated from allele frequencies, between individual taxa. The matrix of genetic distance was analysed with the unweighted pair group method (UPGMA) of cluster analysis (Sneath & Sokal, 1973) to produce a dendrogram.

## RESULTS

***Comparison of conidia and conidial ontogeny.*** The seven isolates of *S. sapinea* lacked conidiophores and the conidia developed through holoblastic ontogeny (Minter, Kirk & Sutton, 1982). Conidiogenous cells that had undergone a single percurrent proliferation were occasionally observed. Mature conidia of all isolates of *S. sapinea* were oblong to

**Table 4.** Growth of *Sphaeropsis sapinea* and *S. sapinea* f. sp. *cupressi* on six agar media at 25°

| Isolate number* | Colony diameter after 3 d (mm)† MEA | PDA | CMA | WA | C-DOX | NA |
|---|---|---|---|---|---|---|
| CWS 1 | 67·0 cd | 75·7 ab | 64·7 cde | 44·8 klmno | 60·3 def | 41·7 mnopq |
| CWS 5 | 56·0 fgh | 67·3 cd | 66·3 cd | 37·2 pqrs | 60·7 def | 38·7 nopqr |
| CWS 8 | 54·0 fghi | 65·2 cde | 64·5 cde | 35·7 qrst | 47·0 ijklm | 34·0 rstu |
| CWS 10 | 63·7 cde | 74·8 ab | 69·2 bc | 44·3 lmnop | 70·5 abc | 45·8 jklmn |
| CWS 33 | 54·0 fghi | 76·9 a | 49·3 hijkl | 37·7 opqrs | 55·2 fgh | 35·7 qrst |
| CWS 59 | 52·7 ghij | 58·7 efg | 44·5 lmnop | 31·5 rstuv | 38·7 nopqr | 33·8 rstu |
| CWS 60 | 55·3 fgh | 65·2 cde | 52·0 ghijk | 39·3 nopqr | 51·5 ghijkl | 31·7 rstuv |
| CPP 1 | 27·2 uv | 34·5 qrstu | 28·7 tuv | 9·2 w | 7·2 w | 31·2 stuv |
| CPP 10 | 24·5 v | 36·0 qrst | 34·8 qrst | 11·0 w | 6·5 w | 27·3 uv |
| CPP 12 | 25·3 v | 33·8 rstu | 33·5 rstu | 11·2 w | 7·5 w | 24·6 v |

* Numbers having a CWS and CPP prefix, respectively, represent isolates of *S. sapinea* and *S. sapinea* f. sp. *cupressi* that are maintained at the Department of Plant Pathology, University of the Orange Free State, Bloemfontein, South Africa.

† Values are means of three replications. Interaction values followed by the same letter are not significantly different ($P = 0·01$) according to Tukey's HSD procedure.

**Table 6.** Number of loci and allelic frequencies for isolates of *Sphaeropsis sapinea* and related taxa

| Locus | Allele* | Taxon | | | | |
|---|---|---|---|---|---|---|
| | | *Sphaeropsis sapinea* | *S. sapinea* f. sp. *cupressi* | *Lasiodiplodia theobromae* | *Diplodia mutila* | *Botryosphaeria dothidea* |
| *Acp-1* | 106 | — | — | 0·500 | — | — |
| | 100 | 0·903 | 1·000 | 0·500 | — | 1·000 |
| | 95 | 0·097 | — | — | 1·000 | — |
| *Acp-2* | 109 | — | — | — | 1·000 | — |
| | 104 | — | — | 1·000 | — | — |
| | 100 | 1·000 | 1·000 | — | — | 1·000 |
| *Amy* | 200 | 0·109 | — | — | — | — |
| | 160 | 0·098 | 0·500 | — | — | — |
| | 130 | 0·159 | — | — | — | — |
| | 100 | 0·573 | 0·500 | 0·500 | 1·000 | — |
| | 50 | 0·061 | — | 0·500 | — | 1·000 |
| β-*Glu* | 120 | 0·195 | — | — | — | 1·000 |
| | 110 | 0·391 | — | — | — | — |
| | 100 | 0·402 | 1·000 | 1·000 | 1·000 | — |
| | 70 | 0·012 | — | — | — | — |
| *Est-1* | 115 | — | — | — | 1·000 | — |
| | 110 | — | 1·000 | — | — | — |
| | 108 | — | — | 1·000 | — | — |
| | 102 | 0·085 | — | — | — | — |
| | 100 | 0·915 | — | — | — | — |
| | 80 | — | — | — | — | 1·000 |
| *Est-2* | 110 | 0·012 | — | — | — | — |
| | 100 | 0·830 | 1·000 | — | 1·000 | 1·000 |
| | 96 | 0·146 | — | — | — | — |
| | 90 | 0·012 | — | — | — | — |
| | 85 | — | — | 1·000 | — | — |
| *Est-3* | 100 | 0·976 | 1·000 | — | 1·000 | 1·000 |
| | 90 | 0·024 | — | — | — | — |
| | 10 | — | — | 1·000 | — | — |
| *Est-4* | 100 | 1·000 | — | — | 1·000 | 1·000 |
| | 98 | — | 1·000 | — | — | — |
| | 95 | — | — | 1·000 | — | — |
| *Est-5* | 100 | 1·000 | 1·000 | — | 1·000 | — |
| | 95 | — | — | 1·000 | — | — |
| | 85 | — | — | — | — | 1·000 |
| *Gda* | 120 | — | — | 1·000 | — | — |
| | 115 | — | — | — | — | 1·000 |
| | 105 | 0·012 | — | — | — | — |
| | 100 | 0·951 | 1·000 | — | 1·000 | — |
| | 95 | 0·037 | — | — | — | — |
| *Gpi* | 107 | 0·036 | 0·500 | — | — | — |
| | 104 | 0·049 | 0·500 | — | — | 1·000 |
| | 100 | 0·915 | — | 1·000 | 1·000 | — |
| *Mdh* | 110 | 0·012 | — | — | — | — |
| | 100 | 0·976 | 1·000 | — | 1·000 | — |
| | 90 | 0·012 | — | — | — | — |
| | 85 | — | — | 1·000 | — | — |
| | 80 | — | — | — | — | 1·000 |
| *Pep-Gl* | 110 | 0·012 | — | — | — | — |
| | 100 | 0·927 | — | 1·000 | 0·500 | — |
| | 92 | — | — | — | 0·500 | — |
| | 88 | 0·037 | — | — | — | — |
| | 85 | 0·024 | — | — | — | — |
| | 80 | — | 1·000 | — | — | — |
| | 76 | — | — | — | — | 1·000 |
| *Pep-Ph* | 115 | 0·281 | — | 0·500 | — | — |
| | 100 | 0·707 | 1·000 | 0·500 | 1·000 | 1·000 |
| | 95 | 0·012 | — | — | — | — |

* Numbers indicate relative electrophoretic mobility, with 100 designating the most common allele.

clavate, rounded at the apex with a blunt base and predominantly aseptate (Fig. 1). Conidia varied in colour from hyaline, when immature, to yellowish or dark brown when mature with the outer surface of the conidial walls predominantly smooth, or sometimes appearing pitted. Conidial lengths varied between 34·6 and 44·9 µm and widths between 12·3 and 17·8 µm (Table 3). Conidia had a length/width ratio of 2·6. Spermatia (Wingfield & Knox-Davies, 1980) were observed in cultures of isolates CWS 8 and CWS 59.

The three cultures of *S. sapinea* f. sp. *cupressi* also lacked conidiophores and conidiogenous cells developed directly from the periphery of the pycnidia. Conidium ontogeny was holoblastic and percurrent proliferation of the conidiogenous cells was commonly observed (Figs 2, 3). Immature conidia had a granular cytoplasm with no septa and hyaline conidial walls. Mature conidia were predominantly ovoid, light to dark brown and medianly 1-euseptate with smooth conidial walls (Fig. 4). Although conidial widths were not significantly different from those of *S. sapinea*, conidial lengths were significantly shorter ($P = 0·01$). Conidia of *S. sapinea* f. sp. *cupressi* had a length/width ratio of 2·0. Spermatia, similar in size to those observed in cultures of *S. sapinea*, were observed in a culture of isolate CPP 10 (Fig. 5).

***Comparison of growth on different media.*** There was a significant interaction ($P = 0·01$) between different isolates and media (Table 4). All seven isolates of *S. sapinea* grew significantly faster ($P = 0·01$) than any single isolate of *S. sapinea* f. sp. *cupressi* on 5 of the 6 media tested. Growth of *S. sapinea* f. sp. *cupressi* on WA and CD was minimal. On NA, only three isolates of *S. sapinea* grew significantly faster ($P = 0·01$) than the three isolates of *S. sapinea* f. sp. *cupressi*. In general the greatest radial growth in all 10 isolates occurred on PDA.

The mycelium of all *S. sapinea* isolates varied from fluffy white to black on MEA, PDA, CMA and CD. Mycelium of the three isolates of *S. sapinea* f. sp. *cupressi* was indistinguishable in appearance from that of *S. sapinea* on MEA, PDA and CMA. After 10 d growth on WA and NA, all 10 isolates produced a very sparse mycelial mat on the agar surface and the three isolates of *S. sapinea* f. sp. *cupressi* had begun to sporulate on the agar.

***Comparison of growth at different temperatures.*** There was a significant interaction ($P = 0·01$) between different isolates and temperature (Table 5). All isolates of *S. sapinea* grew significantly faster ($P = 0·01$) than any single isolate of *S. sapinea* f. sp. *cupressi* at 10, 20, 25 and 30°. At 15°, only isolate CWS 59 did not grow significantly faster than all three isolates of *S. sapinea* f. sp. *cupressi*. The optimum growth for the seven isolates of *S. sapinea* occurred at 25° and was significantly different ($P = 0·01$) from that at 20 and 30°. Although the three isolates of *S. sapinea* f. sp. *cupressi* displayed the same tendency, differences in growth between 15 and 25° were not statistically significant ($P = 0·01$).

***Electrophoresis.*** There were no monomorphic loci for all five taxa screened but distinct differences in allelic differentiation and frequencies were observed (Table 6). Of the 13 alleles that occurred in both *S. sapinea* f. sp. *cupressi* and *S. sapinea*, two were monomorphic [*Acp-2* (100) and *Est-5* (100)]; three [*Amy* (160), *Gpi* (107) and *Gpi* (104)] had frequencies in *S. sapinea* of less than 0·100 compared to 0·500 for *S. sapinea* f. sp. *cupressi*; and eight had frequencies ranging between 0·402 and 0·976 for *S. sapinea* compared to 1·000 for *S. sapinea* f. sp. *cupressi*. Two alleles [*Amy* (160) and *Gpi* (107)], occurred only in *S. sapinea* and *S. sapinea* f. sp. *cupressi*; three alleles [*Est-1* (110), *Est-4* (98) and *Pep-Gl* (80)] were unique to *S. sapinea* f. sp. *cupressi*. *Sphaeropsis sapinea*, *Lasiodiplodia theobromae*, *B. dothidea* and *D. multila* respectively, had 18, 8, 5 and 2 unique alleles. The number of alleles common to *S. sapinea* f. sp. *cupressi* and *L. theobromae*, *B. dothidea* and *D. mutila*, numbered 4, 6 and 8, respectively.

Table 7. Nei's unbiased genetic distances (Nei, 1978) between fungal species are presented below the diagonal and standard errors (Nei & Roychoudhury, 1978) are presented above the diagonal

| | | | | | |
|---|---|---|---|---|---|
| 1* | | *0·409* | *0·153* | *0·291* | *0·182* |
| 2 | 1·55 | | *0·429* | *0·603* | *0·545* |
| 3 | 0·306 | 1·229 | | *0·433* | *0·231* |
| 4 | 0·780 | 2·113 | 1·216 | | *0·329* |
| 5 | 0·356 | 1·630 | 0·510 | 0·857 | |
| | 1 | 2 | 3 | 4 | 5 |

* 1, *Sphaeropsis sapinea*; 2, *Lasiodiplodia theobromae*; 3, *Diplodia mutila*; 4, *Botryosphaeria dothidea*; 5, *Sphaeropsis sapinea* f. sp. *cupressi*.

Nei's unbiased genetic distances provide an estimate of the number of codon differences per 100 loci that occur between two taxa. Genetic distances between taxa were a minimum of 0·306 between *S. sapinea* and *D. mutila*, and a maximum of 2·113 between *L. theobromae* and *B. dothidea* (Table 7). Cluster analysis of the genetic distances between the five taxa (Fig. 6) revealed that *S. sapinea* was more closely related to *D. mutila* than to *S. sapinea* f. sp. *cupressi*. *D. mutila* and *S. sapinea* f. sp. *cupressi* were, however, more closely related to each other than to *B. dothidea* and *L. theobromae*.

## DISCUSSION

The designation of the name *S. sapinea* f. sp. *cupressi* to the fungus causing canker of *C. sempervirens* implies a close phylogenetic relationship with *S. sapinea* but physiological differences. The present study has shown that *S. sapinea* f. sp. *cupressi* is distinctly different from *S. sapinea*, both morphologically and phylogenetically. Even when viewed in the context of the considerable genetic variation known to exist among isolates of *S. sapinea* (Palmer *et al.*, 1987; Swart *et al.*, 1991) differences observed between *S. sapinea* f. sp. *cupressi* and *S. sapinea* are distinctive. The most conclusive proof of significant differentiation between isolates of *S. sapinea* and *S. sapinea* f. sp. *cupressi* is based on conidial characteristics and allozyme diversity.

Conidial dimensions of *S. sapinea* are given as 30–45 × 10–16 µm by Sutton (1980). Although mean conidial widths of *S. sapinea* f. sp. *cupressi* [13 µm (12–15) µm] as shown by Solel *et al.* (1987) and the present study [12·4 µm (11–14·5) µm] easily fall within the range for *S. sapinea*, conidial lengths do not. The maximum length for conidia of

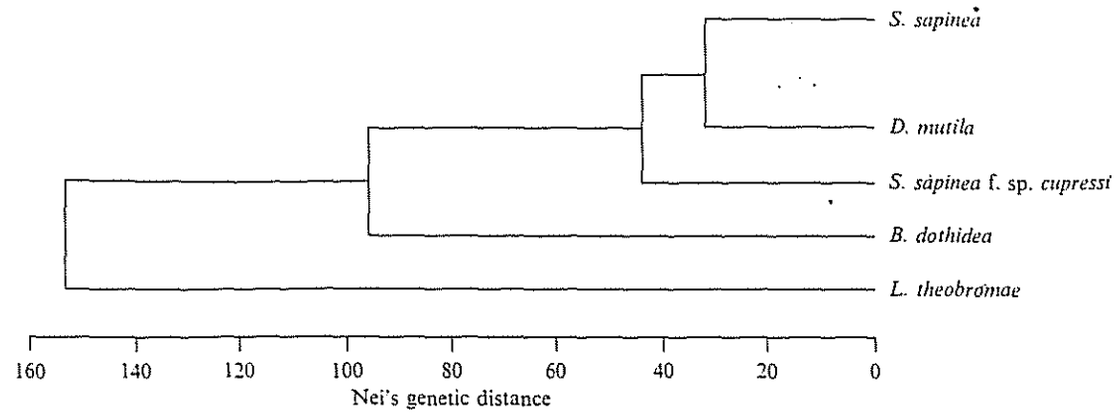


**Fig. 6.** Phenetic analysis of electrophoretic data of isolates of *Sphaeropsis sapinea*, *S. sapinea* f. sp. *cupressi*, *Lasiodiplodia theobromae*, *Diplodia mutila* and *Botryosphaeria dothidea*, using the UPGMA algorithms and Nei's unbiased genetic distance (Nei, 1978).

*S. sapinea* f. sp. *cupressi* according to Solel *et al.* (1987) [26 µm (20–30) µm] barely encompasses the minimum lengths given for *S. sapinea* by Sutton (1980). The significant difference ($P = 0{\cdot}01$) in length between conidia of *S. sapinea* and *S. sapinea* f. sp. *cupressi* recorded in the current study substantiates the previous observation. Although conidial dimensions of *S. sapinea* f. sp. *cupressi* agree closely with those given for *D. mutila* (27–31 × 12–13·5 µm) (Sutton, 1980), small differences are apparent. The length:width ratio of *D. mutila* conidia is given as 2·3 (Shoemaker, 1964) which is greater than that of *S. sapinea* f. sp. *cupressi*. Furthermore, Shoemaker (1964) reported a marked scarcity of 1-septate conidia for *D. mutila* compared to the prevalence of mature 1-septate conidia observed for *S. sapinea* f. sp. *cupressi* in the present study and by Solel *et al.* (1987).

Isozymic profiles of all isolates of *S. sapinea* were distinctly different from those of *S. sapinea* f. sp. *cupressi*, *L. theobromae*, *B. dothidea* and *D. mutila*. The latter three taxa were included in this study because of their morphological and pathological similarity to *S. sapinea* (Waterman, 1943; Sutton, 1980; Sutton & Dyko, 1989) and therefore serve as a frame of reference within which the putative relationship between *S. sapinea* and *S. sapinea* f. sp. *cupressi* can be better evaluated. On the basis of cluster analysis it appears that a closer relationship exists between *S. sapinea* and *D. mutila* than between *S. sapinea* and *S. sapinea* f. sp. *cupressi*. This provides strong evidence for lack of a phylogenetic relationship between *S. sapinea* and *S. sapinea* f. sp. *cupressi*.

Despite the larger genetic distance between *D. mutila* and *S. sapinea* f. sp. *cupressi* (0·510) than between the latter fungus and *S. sapinea* (0·356), the similar conidial dimensions of *D. mutila* and *S. sapinea* f. sp. *cupressi* suggest that there is a greater affinity between the latter taxa. Tuset (1979) isolated *D. mutila* from cankers on *C. sempervirens* and the possibility that *S. sapinea* f. sp. *cupressi* is synonymous with *D. mutila* was, therefore, considered. Based on Sutton's (1980) classification, the genus *Diplodia* is characterized by the presence of conidiophores and by having conidiogenous cells which form a single monoblastic conidium. This is inconsistent, however, with the conidial ontogeny of *S. sapinea* and that of *S. sapinea* f. sp. *cupressi* observed in this study. *S. sapinea* and *S. sapinea* f. sp. *cupressi* both lack conidiophores and have conidiogenous cells that undergo percurrent proliferation. The conidial ontogeny of *S. sapinea* f. sp. *cupressi* therefore indicates that it belongs in the genus *Sphaeropsis* despite its distinct differences in conidial morphology from *S. sapinea* and its relatively large genetic distance from the latter fungus. The validity of the generic separation of *Diplodia* and *Sphaeropsis* might be questioned by these results although greater numbers of species should be considered before any conclusions are drawn in this regard.

Many canker pathogens have been reported on *Cupressus* spp. although references to *Diplodia* spp. and *Sphaeropsis* spp. are scant. *Sphaeropsis sapinea* has been reported as a pathogen of *C. lusitanica* Mill. (Punithalingam & Waterston, 1970; Sutton, 1980). Brown (1968) reported *Diplodia pinea* as the cause of dieback on *C. macrocarpa* Hartw. ex Gord. and *C. sempervirens*. Various species of *Diplodia* have also been associated with dieback of cypress trees in Portugal (De Azevedo, 1979). These reports, however, all lack substantial pathogenic evidence and are, therefore, questionable.

The results of the present study provide substantial evidence for the lack of a close relationship between *S. sapinea* and *S. sapinea* f. sp. *cupressi*. The latter name is therefore misleading and should be avoided. We have resisted the temptation of providing a name for this fungus and believe that this should await a comprehensive examination of *Sphaeropsis* and *Diplodia* spp. occurring on Cupressaceae. For the present, we therefore suggest that the fungus studied by Solel *et al.* (1987) be referred to as *Sphaeropsis* sp. and that the species epithet *sapinea* be omitted in order to avoid further confusion.

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