Chapter 6

Epitypification of *Ophiostoma galeiformis* and phylogeny of species in the *O. galeiformis* complex

Ophiostoma galeiformis was first described from *Larix kaempferi* in Scotland in 1951, where it was associated with *Hylurgops palliatus*, *Dryocoetes autographus*, and *Trypodendron lineatum*. The taxonomy of this fungus has, however, been uncertain due to a lack of sexual structures on the type specimen and contamination of a preserved ex-type culture. The aim of this study was to designate an epitype for *O. galeiformis*, and to consider phylogenetic relationships of the species. Eighteen *O. galeiformis*-like isolates from different parts of the world were included in the study, including collections from *Pinus sylvestris* infested with *Tomicus piniperda* in Scotland. Both light microscopic study and ITS rDNA sequencing were used to study these fungi. Morphological characteristics of isolates from Scotland, Sweden, South Africa and Chile corresponded well with those described for *O. galeiformis* and an isolate from Scotland was designated as an epitype. An extended description is provided for this species, which should facilitate identification in the future. ITS rDNA sequence data showed that the isolates representing *O. galeiformis*, were phylogenetically separated from three isolates from the USA and Mexico that had been identified as this species. The latter fungi probably represent an undescribed taxon.

Keywords: bark beetle, sapstain, ITS, *Tomicus piniperda*.

INTRODUCTION

Bark beetles (Coleoptera: Scolytidae) commonly occur in most forest ecosystems and several species are regarded as important forest pests (Wood & Bright, 1992). Most bark beetles species act as vectors of fungi, especially ophiostomatoid fungi (Whitney, 1982; Beaver, 1989; Wingfield, Seifert & Webber, 1993; Paine, Raffa & Harrington, 1997). The genus *Ophiostoma* includes some primary tree pathogens as well as sapstain agents (Lagerberg, Lundberg & Melin, 1927; Brasier, 1979, 1991; Harrington, 1993; Seifert, 1993; Brasier & Mehrotra, 1995).

Ophiostoma galeiformis (Bakshi) Mathiesen-Käärik, originally described as *Ceratocystis galeiformis* Bakshi, is a sapstain fungus that was first described from Scotland (Bakshi, 1951; Mathiesen-Käärik, 1953). This fungus was isolated from the bark of *Larix kaempferi* infested with *Hylurgops palliates* (Gyll.), *Dryocoetes autographus* (Ratzeburg), and *Trypodendron lineatum* (Olivier) (Bakshi, 1951). Later, the fungus was found on *Picea* infested with *Hylastes cunicularius* (Errichson) in Sweden (Mathiesen-Käärik, 1953, 1960), and from unknown pine-infesting bark beetles (Hunt, 1956). *Ophiostoma galeiformis* is also associated with pine-infesting bark beetles occurring in Chile and South Africa (Chapter 2, 3). A single, *O. galeiformis*-like isolate has also recently been isolated from *Dendroctonus mexicanus* (Hopkins) infesting *Pinus pseudostrobus* in Mexico (Chapter 4).

The taxonomy of *O. galeiformis* has been confused for many years. This is largely due to the fact that the type specimen lacks sexual structures (Hunt, 1956; Upadhyay, 1981). Although Hunt (1956) included the species in his study of the genus *Ceratocystis*, Upadhyay (1981) and Seifert, Wingfield & Kendrick (1993) considered it a species of uncertain status. The International Code of Botanical Nomenclature (Art. 9.7) (Greuter *et al.*, 2000), allows the designation of a specimen and/or a culture as an epitype where the holotype does not show the necessary distinguishing characters. The epitype would then serve as the holotype in determining characteristics that

cannot be obtained from the holotype. The aim of this study was to reconsider the taxonomic status of *O. galeiformis* and designate an epitype for the species. Light microscopy was employed, and the ITS (internal transcribed spacer) region of the ribosomal RNA operon was sequenced to confirm its phylogenetic relationships.

MATERIALS AND METHODS

Fungal isolates and morphological investigation

The holotype of *O. galeiformis* (IMI20168) was studied based on available morphological structures. We intended to include the ex-type culture (CBS137.51) in this study, but it was found to be contaminated and repeated attempts to purify it failed. Seventeen other isolates, identified as *O. galeiformis* based on descriptions of the fungus (Bakshi, 1951; Hunt, 1956) and ecology, were included in the study (Table 1). Single conidial cultures were prepared for all these isolates and were grown on 2 % MEA (20 g Biolab malt extract, 20 g Biolab agar and 1000 ml distilled water), and examined. For the isolate producing perithecia (CMW5290), 25 sexual and asexual structures were examined and measured using a light microscope, and the ranges and averages were computed. All cultures are maintained in the culture collection (CMW) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

Mating experiments

Five isolates (CMW241 and CMW567 from South Africa, CMW4442 and CMW4444 from Scotland, and CMW9988 from Sweden) (Table 1) resembling *O. galeiformis* were chosen and crossed in every possible combination. All crossed cultures, including control crosses of isolates against themselves, were incubated on MEA with pine twigs as described by Harrington *et al.* (2001).

DNA sequencing and phylogenetic analysis

DNA extraction, PCR amplification, sequencing reactions, and phylogenetic analysis were conducted in a similar way as those described in Chapter 3. Eighteen single hyphal tip cultures of selected isolates were prepared for sequencing (Table 1). DNA was extracted using a modified version of the extraction method developed by Raeder and Broda (1985). The ITS1 and ITS2 (internal transcribed spacer) regions, including the 5.8S gene of the ribosomal RNA operon, were amplified, using primers ITS1-F (Gardes & Bruns, 1993) and ITS4 (White *et al.*, 1990). PCR products were sequenced with the same primers used for PCR, as well as two additional internal primers, CS2 (Wingfield *et al.*, 1996), and ITS3 (White *et al*, 1990). The obtained sequences were aligned using Sequence Navigator version 1.01 (ABI PRISM, PerkinElmer). Aligned data were analysed using PAUP (Phylogenetic Analysis Using Parsimony) (Swofford, 1998). Bootstrap analysis (1000 replicates) was run to determine confidence intervals of the branching points.

RESULTS

Anamorph morphology

Morphological comparisons showed no differences between the anamorph structures of the isolates from Scotland, Sweden, Chile and South Africa and those present on the holotype (IMI20168). *Pesotum* was dominant, and measurements of fruiting structures corresponded well with those described previously (Table 2). The three *O. galeiformis*-like isolates from Mexico (CMW9490), Georgia, USA (C527), and California, USA (C1293), were similar but differed slightly from isolates of *O. galeiformis* from Scotland, Sweden, Chile, and South Africa. Colony color of the isolates from Mexico and the USA was lighter than that of other isolates.

Mating experiments

Perithecia were formed on crosses using the five isolates from South Africa, Scotland and Sweden. The crosses of two South African isolates (CMW241 and CMW567), and two isolates from South Africa and Scotland (CMW567 and CMW4442), produced the most perithecia.

DNA Sequencing analysis

DNA fragments approximately 510 bp in size were amplified for all the isolates (Table 1), except the ex-type culture (CBS137.51), which had a fragment length of 463 bp. This sequence was subjected to a BLAST search, which revealed that it was a species of *Phialophora* and thus a contaminant. Manual alignment of the remaining sequences resulted in a total of 546 characters. Of these, 20 were parsimony-informative, 92 parsimony-uninformative, and 434 constant. Heuristic searches using *O. cucullatum* as the outgroup taxon, resulted in three most parsimonious trees (CI = 0.967, RI = 0.913, HI = 0.033) of which one (Fig. 1) was chosen for presentation here. Two main clades (Fig. 1) were evident in the three phylogenetic trees. The first clade, including two sub-clades, represented the *O. galeiformis* group. Isolates from Chile, South Africa and Sweden formed one subclade with a bootstrap support of 67 %, while the five isolates from Scotland formed the other subclade with a bootstrap support of 64 %. The second clade, with a bootstrap support of 100 %, included the three isolates from Mexico and the USA.

TAXONOMY

The five *O. galeiformis* isolates from Scotland are morphologically almost identical. The anamorph characteristics are also indistinguishable from those on the holotype specimen. These isolates share the same ITS sequences, and differed only by two base pairs from the isolates from

Chile, South Africa and Sweden. All these isolates are, therefore, considered to represent a single species whose morphological characteristics agree well with the original description of *O. galeiformis* (Bakshi, 1951; Mathiesen-Käärik, 1953). One of the Scottish isolates (CMW5290), is designated as the epitype strain of *O. galeiformis* since it originated from the same geographical region as the holotype specimen, and has been deposited in the culture collection (CMW5290) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa and in the Centraalbureau voor Schimmelcultures (CBS***), Utrecht, Holland. The epitype specimen, a dried culture grown on 1.5 % MEA with pine twigs, and bearing both perithecia and the asexual *Pesotum* state has been deposited in the National Collection of Fungal Specimens of South Africa (PREM***). The species description provided below is based on the epitype specimen (PREM***) and culture of *O. galeiformis* (CMW5290).

Ophiostoma galeiformis (Bakshi) Mathiesen-Käärik, Meddn. St. Skogsfor. Sk. Inst. 43: 47. 1953. = *Ceratocystis galeiformis* Bakshi, Mycol. Pap. 35: 13. 1951.

Colonies reaching 30 mm in diameter in 10 days on 2 % MEA at 25 °C. Colonies light grey (19''d) to dark brown (13'''''k) (Rayner, 1970) with age, appressed with yeasty appearance. *Perithecia* rarely produced in culture. *Perithecial bases* globose, dark brown to black, (105-) 340 (-545) µm in diameter (Fig. 2A), with few ornamental hyphae. *Perithecial necks* dark brown to black, (260-) 560 (-840) µm long, (20-) 50 (-93) µm wide at base, (8-) 27 (-54) µm wide at the apex. *Ostiolar hyphae* absent (Fig. 2B). *Asci* not observed. *Ascospore masses* in tendrils at the apex of perithecial necks (Fig. 2C). *Ascospores* hyaline, aseptate, with brim, bean shaped in side and face view, (2-) 3.5 (-6) x (1-) 1.7 (-2.0) µm (Fig. 2D).

Leptographium anamorph: rarely produced in culture. *Conidiophores* up to seven septate, 60 – 92 (- 130) µm long (Fig. 2E). *Conidia* hyaline, cylindrical to ellipsoid, with a truncate base, (1.9-) 3.6 (-4.7) x (1.0-) 1.4 (-2.2) µm (Fig. 2F).

Pesotum anamorph: predominant in culture. *Conidiophores* with apex hyaline to light grey, stalk brown, 50 – 140 (- 300) µm long, (7-) 19 (-29) µm wide at base, (10-) 47 (-190) µm wide at head (Fig. 2G). *Conidia* hyaline, cylindrical, (2.6-) 4.2 (-6.4) x (1.1-) 2.0 (-3.0) µm (Fig. 2H).

Additional strains examined: CMW4426, Scotland, Elgin, isolated from *P. sylvestris* infested with *T. piniperda*, 29 August 1997, M. J. Wingfield, CBS*** = PREM***. CMW4447, Scotland, Elgin, isolated from *P. sylvestris* infested with *T. piniperda*, 29 August 1997, M. J. Wingfield, CBS*** = PREM***.

Holotype: IMI20168, isolated from bark of *Larix kaempferi* (Japanese larch), associated with bark beetles *Hylurgops palliatus* and *Dryocoetes autographus*, Blaire Atholl, Perthshire, Scotland, 1951, B.K. Bakshi.

Epitype: PREM*** [CBS***: CMW5290; epitype designated herewith], Scotland, Elgin, isolated from *P. sylvestris* infested with *T. piniperda*, 29 August 1997, T. Kirisits.

DISCUSSION

In this study, we have confirmed previous reports that the holotype of *O. galeiformis* only contains the anamorph state of the fungus. We have, furthermore, shown that the ex-type culture deposited in the CBS is contaminated and cannot be used in taxonomic studies. An epitype based on a

collection from the same geographical area where *O. galeiformis* was first collected has thus been designated. This should ensure that future studies on the fungus are based on material known to represent the species. Results of this study have also confirmed that *O. galeiformis* occurs in Chile, South Africa, and Sweden. It, furthermore, represents the first report of the association between this fungus and *Tomicus piniperda*. Three isolates from the USA and Mexico thought to represent this species are different and probably represent an undescribed taxon.

In the descriptions of *O. galeiformis* by Bakshi (1951) and Hunt (1956), the conidial states were assigned to three genera: *Graphium, Leptographium*, and *Cephalosporium*. Both Mathiesen-Käärik (1953) and Hunt (1956) mentioned that the fungus formed a continuum of conidiophore structures varying from single, simple conidiophores to true synnemata, typical of the genus *Graphium*. Wingfield (1993) stated that it was difficult to assign a generic name to the anamorph of *O. galeiformis* since the species has both synnematous and mononematous states. Scanning electron microscopy studies further showed that there is a continuum in patterns of conidium development including those typical of *Sporothrix*, *Hyalorhinocladiella*, and *Graphium* (now *Pesotum*) (Benade, Wingfield & Van Wyk, 1997). In this study, both the *Pesotum* and *Leptographium* forms of the anamorph of *O. galeiformis* were observed, but the *Pesotum* form was predominant. We do not believe that it is necessary to provide a formal name for the anamorph but if these were selected, we would preferentially refer to it as *Pesotum*, because this state is dominant in cultures.

Analysis of sequence data of *O. galeiformis* isolates from Chile and South Africa presented in this study has shown that these isolates are closely related to those from Scotland and Sweden. Occurrence of mating between the isolates from South Africa, Scotland, and Sweden indicated that these isolates could represent a single species. Ophiostomatoid fungi in countries such as South Africa and Chile are carried by bark beetles, which were accidentally introduced into these countries from Europe (Ciesla, 1988; Tribe, 1992). *Ophiostoma galeiformis* is apparently

common in Europe associated with a wide range of bark beetles and it would have been introduced into South Africa and Chile with one or more of these insect species. In South Africa, *O. galeiformis* is associated with *Hylurgus ligniperda* (Fabricius) (Zhou *et al.*, 2001) and in Chile we have commonly isolated it from *Hylastes ater* (Chapter 3). None of these insects have been connected with *O. galeiformis* in their natural European habitat but this is probably due only to the lack of studies of fungi associated with these insects in Europe.

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Table 1. Fungal isolates included in this study.

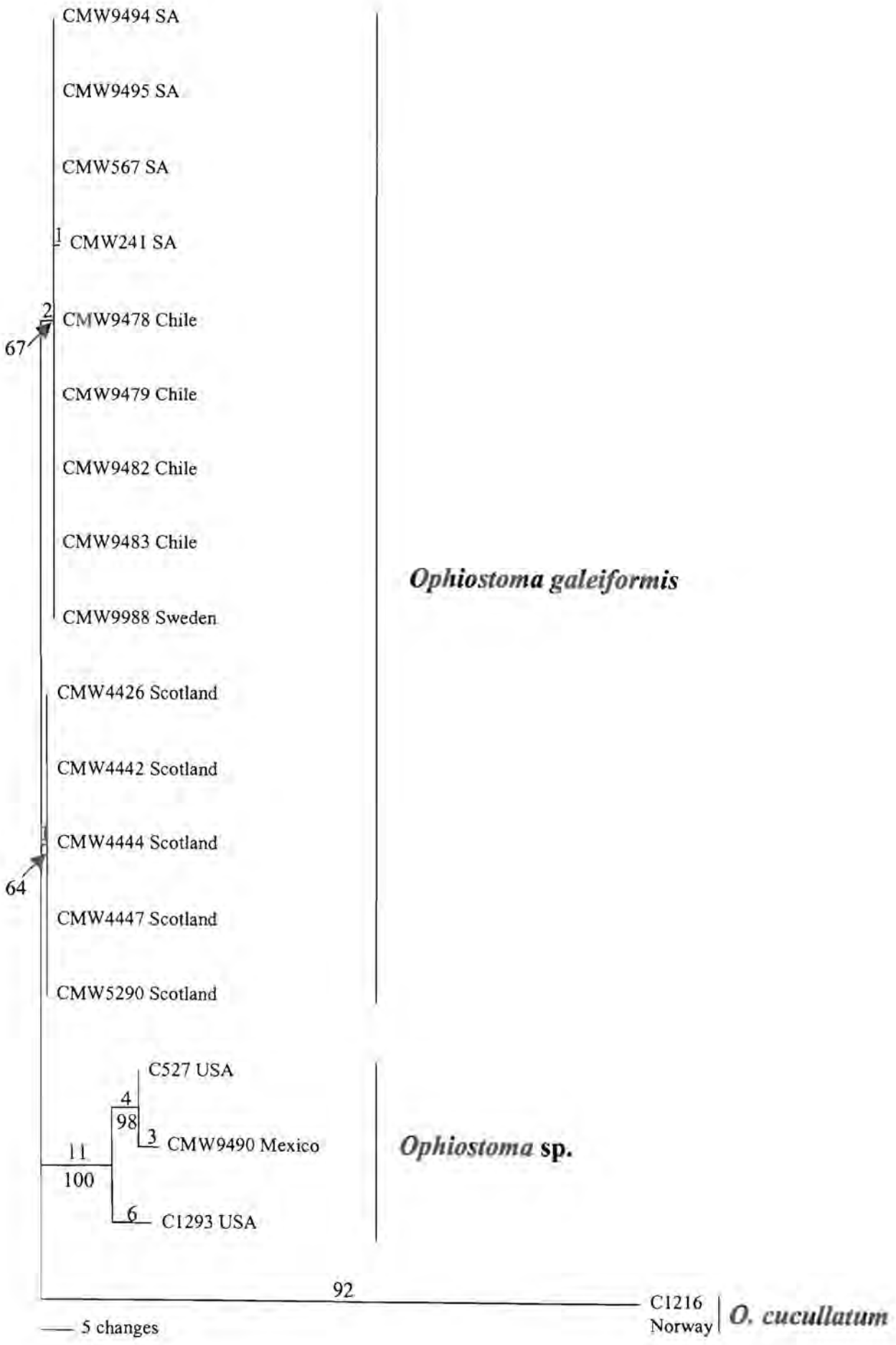
Species	Isolate No.	Other No.	Herbarium	Collector or supplier	Host	Insect	Origin	Sexual state	rDNA sequence
Ophiostoma	[1]CMW4426			MJ Wingfield & T Kirisits	*Pinus sylvestris*	*Tomicus piniperda* (Linnaeus)	Elgin, Scotland		
galeiformis	CMW4442			MJ Wingfield & T Kirisits	*P. sylvestris*	*T. piniperda*	Elgin, Scotland		
	CMW4444			MJ Wingfield & T Kirisits	*P. sylvestris*	*T. piniperda*	Elgin, Scotland		
	CMW4447			MJ Wingfield & T Kirisits	*P. sylvestris*	*T. piniperda*	Elgin, Scotland		
	CMW5290		[2]PREM???	MJ Wingfield & T Kirisits	*P. sylvestris*	*T. piniperda*	Elgin, Scotland	present	
	CMW9478			MJ Wingfield & XD Zhou	*P. radiata*	*Hylastes ater* (Paykull)	Valdivia, Chile		
	CMW9479			MJ Wingfield & XD Zhou	*P. radiata*	*H. ater*	Valdivia, Chile		
	CMW9482			MJ Wingfield & XD Zhou	*P. radiata*	*Hylurgus ligniperda* (Fabricius	Valdivia, Chile		
	CMW9483			MJ Wingfield & XD Zhou	*P. radiata*	*H. ligniperda*	Valdivia, Chile		
	CMW9494			XD Zhou	*P. elliiottii*	*H. ligniperda*	KwaZulu-Natal, South Africa		
	CMW9495			XD Zhou	*P. elliiottii*	*H. ligniperda*	KwaZulu-Natal, South Africa		
	CMW241			MJ Wingfield	*P. pinaster*		Grabouw, South Africa		
	CMW567			MJ Wingfield	*P. pinaster*		Grabouw, South Africa		
	CMW9988	[3]CBS150.54		A Mathiesen-Käärik	*Picea abies*	*Hylastes cunicularius*	Västerbotten, Sweden		
Ophiostoma		[4]C527		M Baldwin	*Pinus taeda*		Georgia, USA		
galeiformis-like		C1293		D Hofstra	*P. radiata*		California, USA		
	CMW9490			MJ Wingfield & XD Zhou	*P. pseudostrobus*	*Dendroctonus mexicanus*	Chiapas, Mexico		
O. cucullatum		C1216		H Solheim	*Picea abies*	*Ips typographus*	Norway		

Notes: [1]CMW is the Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa. [2]PREM – The National Collection of Fungi, South Africa. [3]CBS – Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; [4]C – Culture Collection of T.C Harrington, Department of Plant Pathology, Iowa State University, Iowa, USA.

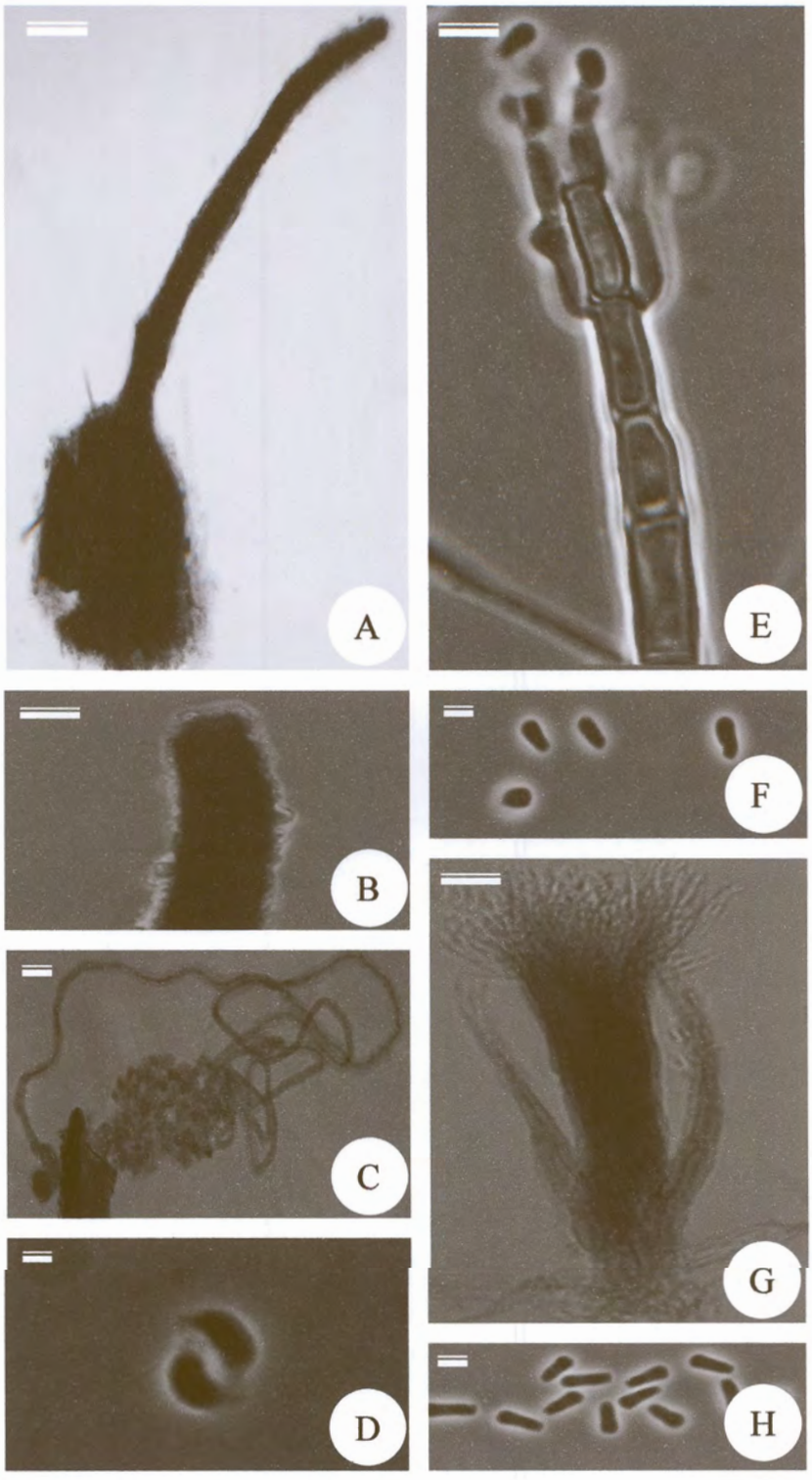
Table 2. Comparison of the epitype specimen and previous descriptions of *O. galeiformis* (all measurements in µm).

			Epitype	Bakshi, 1951	Mathiesen-Käärik, 1953	Hunt, 1956
Teleomorph	Perithecial base	Colour	Dark brown to black	Brown to black	Black	Same as those of Bakshi (1951)
		Diameter	(105-) 340 (-545)	(182-) 218 (- 273)	(184-) 221 (-255)	
		Ornamentation	Very few hairs	Present	Hairless or with few single hairs	
	Perithecial neck	Length	(260–) 560 (–840)	(539-) 640 (-700)	(620-) 760 (-930)	
		Base width	(20–) 50 (-93)	(39-) 49 (–60)	(45–) 51 (–65)	
		Apex width	(8-) 27 (-54)	(15–) 25 (-28)	(22–) 25 (-28)	
	Ostiolar hyphae		Absent	Absent	Absent	
	Ascospores	Colour	Hyaline	Hyaline	Hyaline	
		Septation	Absent	Absent	Absent	
		Shape	Bean shaped, with brim	Bean shaped, with brim	Bean shaped, with appendages	
		Size	(2.0-) 3.5 (– 6.0) x (1.0-) 1.7 (– 3.0)	(4.0–) 4.6 (-5.3) x (2.1–) 2.5 (-3)	3.8 – 2.0 um, without sheath	
Anamorph	*Pesotum* anamorph		Conidia (2.0–) 4.0 (- 6.0) x (1.0–) 2.0 (-3.0)	Conidia one-celled, elongate, (4.0-) 4.6 (-5.2) x (1.9-) 2.0 (-2.2)	Real synnemata: stipe 200 – 400 long, head up to 100 - 500, hyaline or light greenish.	Stalks brown to black at base, hyaline in the upper part, up to 300 x 60, conidia same as those of *Leptographium*.
	Leptographium anamorph		Conidia cylindrical to ellipsoid (2.0–) 3.6 (-4.7) x (1.0-) 1.5 (–2.0)	Conidia one-celled, elongate, (4.0-) 4.6 (-5.2) x (1.9-) 2.0 (-2.2)	Simple conidiosphore: stipe 150 – 250 long, 4 - 8 septate, head up to 50 - 60, conidia egg-shaped, (4.0-) 4.8 (-5.5) x (2.0-) 2.2 (-2.4)	Stalks brown, thick-walled, up to 8 septate, up to 300 x 3-5, head up to 50, conidia hyaline, cylindrical to ellipsoid, 5 – 6 x 2.5 - 3
	'Cephalosporium' anamorph			Conidia one-celled, oval, hyaline, (2.2-) 2.7 (-3.1) x (1.5-) 1.7 (-2.0)	*Cephalosporium*-like head: one celled, hyaline, ova, (2.2-) 2.8 (-3.6) x (1.2-) 1.9 (- 2.3)	Hyaline (conidiphores and conidia), spores ellipsoid to oval, 3 - 3.5 x 2 - 2.5

Fig. 1. Phylogram of the *Ophiostoma galeiformis* complex based on analyses of ITS sequences (ITS1 and ITS2 regions, as well as 5.8S rRNA gene). *Ophiostoma cucullatum* was used as outgroup. Base substitution numbers are indicated above the branches and the bootstrap values (1000 bootstrap repeats) below the branches.



Figs. 2A – 2H. *Ophiostoma galeiformis* (CMW 5290) on 1.5 % MEA with pine twigs. A. Dark perithecia with long neck (Bar = 85 µm). B. Apex of the neck without ostiolar hyphae (Bar = 15 µm). C. Tendril of ascospore masses (Bar = 50 µm). D. Bean shaped ascospores (Bar = 2.5 µm). E. *Leptographium* anamorph (Bar = 7 µm). E. Conidia of *Leptographium* anamorph (Bar = 5 µm). F. *Pesotum* anamorph (Bar = 23 µm). G. Conidia of *Pesotum* anamorph (Bar = 4 µm).



Chapter 7

Pathogenicity of *Ophiostoma ips*, *Leptographium serpens* and *L. lundbergii* to pines in South Africa*

Three exotic bark beetles (Coleoptera: Scolytidae), *Hylastes angustatus*, *Hylurgus ligniperda*, and *Orthotomicus erosus*, infest *Pinus* spp. in South Africa. These beetles are generally considered as secondary pests, but can also act as vectors of ophiostomatoid fungi. In South Africa, at least 12 ophiostomatoid fungi are associated with the three beetle species, of which *Ophiostoma ips*, *Leptographium serpens*, and *L. lundbergii*, occur most frequently. The aim of this study was to test the pathogenicity of the three fungi to pines in South Africa. Two isolates of each fungus were inoculated on various species of pines in different areas of South Africa. The inoculated fungi caused resin exudation and sapwood discoloration around inoculation points. There were significant differences in lesion length between species inoculated, times of inoculation and plantation areas. Although *O. ips* gave rise to longer lesions than *L. serpens* and *L. lundbergii*, our results suggest that none of these species should be considered as serious pathogens.

Keywords: *Pinus*, bark beetles, *Hylastes angustatus*, *Hylurgus ligniperda*, *Orthotomicus erosus*.

* Zhou, X. D., De Beer, Z. W., Wingfield, B. D & Wingfield, M. J. (2002) Infection sequence and pathogenicitiy of *Ophiostoma ips*, *Leptographium serpens* and *L. lundbergii* to pines in South Africa. In: Fungal Succession (eds. K. D. Hyde and E. B. G. Jones). *Fungal Diversity* **10**: 229-240.

INTRODUCTION

Three exotic bark beetle species, *Hylastes angustatus* (Herbst), *Hylurgus ligniperda* (Fabricius), and *Orthotomicus erosus* (Wollaston), native to Europe and the Mediterranean Basin, infest *Pinus* spp. in South Africa (Tribe, 1992). *Hylurgus ligniperda* and *O. erosus* are generally considered as secondary pests. *Hylastes angustatus*, however, is more aggressive than the other two bark beetle species, and is considered as a primary pest. This insect damages pine seedlings during maturation feeding and thus, causes significant losses in newly established pine plantations (Anonymous, 1946; Tribe, 1992).

Bark beetles are well-known vectors of fungi, and particularly *Ophiostoma* and *Ceratocystis* spp. (Münch, 1907; Whitney, 1982; Harrington, 1988; Beaver, 1989; Wingfield *et al.*, 1993; Paine *et al.*, 1997; Jacobs and Wingfield, 2001). These fungi generally sporulate in the galleries of their bark beetle vectors and are either carried in mycangia, on the exoskeletons, or in the guts of the beetles (Beaver, 1989; Paine *et al.*, 1997). The relationship between ophiostomatoid fungi and their bark beetle vectors, however, varies among different hosts, fungal species and their insect vectors (Harrington, 1993a; Wingfield *et al.*, 1995; Paine *et al.*, 1997).

Many ophiostomatoid species cause sapstain of freshly cut wood (Münch, 1907; Lagerberg *et al.*, 1927; Seifert, 1993). Several species are also pathogenic to plants. *Ophiostoma ulmi* (Buisman) Nannf. and *O. novo-ulmi* Brasier, which cause Dutch elm disease, have killed millions of elm trees in the Northern Hemisphere during the past century (Brasier, 1990; Brasier & Mehrotra, 1995). Three host-specific varieties of *Leptographium wageneri* (Kendrick) M. J. Wingfield, which cause black stain root disease of conifers, have led to severe losses to forestry in United States and Canada (Harrington and Cobb, 1988). Less pathogenic species such as *O. minus* (Hedgcock) H. & P. Sydow, *L. wingfieldii* M. Morelet and *L. terebrantis* Barras & Perry,

can cause significant lesions, or even kill the trees when mass inoculated (Wingfield, 1986; Harrington, 1993b; Solheim *et al.*, 1993).

In South Africa, at least 12 ophiostomatoid species are associated with the three pine-infesting bark beetles (Zhou *et al.*, 2001). Of the 12 species, *Ophiostoma ips* (Rumb.) Nannf., *L. lundbergii* Lagerb. & Melin and *L. serpens* (Goid.) M. J. Wingfield, are most frequently encountered (Zhou *et al.*, 2001). These three species have been reported to be pathogenic to conifers in many parts of the world (Mathre, 1964; Lorenzini and Gambogi, 1976; Lieutier *et al.*, 1989; Kaneko and Harrington, 1990; Otrosina *et al.*, 1997).

A number of preliminary pathogenicity trials have been conducted with these species on pines in South Africa (Wingfield and Knox-Davies, 1980; Wingfield and Marasas, 1980, 1983; Wingfield and Swart, 1989; Dunn *et al.*, 2002). Little is, however, known regarding their relative importance or pathogenicity to pines in the area. The aim of this study was, therefore, to test and compare the pathogenicity of the three most frequently encountered fungal associates of pine-infesting bark beetles in South Africa. These tests were conducted on two-year-old pines representing a number of key species and in two different geographic areas.

MATERIALS AND METHODS

Screening of fungal isolates

All isolates used in this study were obtained during a survey of ophiostomatoid fungi associated with the three pine infesting bark beetle species in South Africa (Zhou *et al.*, 2001). Fungal isolates were selected based on their relative growth rate in culture, because this was shown in preliminary trials (Wingfield, unpublished) to correlate strongly with pathogenicity. Initially, 139 isolates of *O. ips*, 116 of *L. serpens*, and 138 of *L. lundbergii* were screened on 2 % MEA (Malt Extract Agar: 20 g Biolab malt extract, 20 g Biolab agar and 1000 mL distilled water) at

25 C in the dark for two weeks. The two fastest-growing isolates of each species were chosen for the pathogenicity trials. All isolates used in this study are maintained in the Culture Collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, Republic of South Africa.

Inoculation experiments

Pinus spp., which are known hosts of the bark beetles and the three fungal species, were chosen for the pathogenicity trials. The availability of trees, as well as locations where the bark beetles occur, were also considered. Field inoculations were conducted in two-year-old plantations in the Western Cape province (Knysna, 23° 04' 00" E, 33° 56' 00" S) and Mpumalanga province (Sabie, 30° 39' 00" E, 25° 08' 00" S), South Africa. In Knysna, two pine species, *P. radiata* and *P. elliottii*, were selected for inoculations. In Sabie, *P. elliottii*, and a hybrid of *P. elliottii* and *P. caribaea*, were used.

Inoculum was prepared by growing fungal isolates on 2 % MEA at 25 C in the dark for two weeks. After this period, cultures had commenced sporulation and the agar surface was covered with dark mycelium.

Twenty trees of each pine species were inoculated with each of the six isolates. An equal number of trees of each species served as controls. One branch per tree, with an average of 20 mm in diameter, was inoculated. A plug of bark was removed, using a sterile 10 mm-diameter cork borer, to expose the cambium. An agar plug of equal size, bearing the test fungus, was placed mycelium side down, in each wound. Sterile agar plugs were used as controls. All inoculation points were sealed with masking tape to reduce desiccation. Six weeks after first inoculation, all trials were repeated by inoculating a second branch of the same trees inoculated during the first trial.

Branches were examined six weeks after inoculation by removing the bark and exposing the cambium. Lesion lengths and branch diameters were measured. Reisolations were done by transferring pieces of freshly cut, discoloured cambium to 2 % MEA. Cultures were incubated at 25 C for two weeks, after which they were microscopically examined to confirm that the lesions had been caused by the inoculated fungi.

Data analysis

All data sets were analysed separately. Isolates belonged strictly to a specific fungal species and measurements were done on two different inoculated branches of the same tree. Therefore, a hierarchical ANOVA was employed for the analysis. The treatment variances differed somewhat, and to improve the accuracy of the ANOVA by eliminating the effect of branch diameter, branch diameter was used as a covariate in an ANCOVA. However, branch diameter was non-significant, and had no influence over lesion measurements. Differences between times of trials, species and isolates were evaluated by using a multiple comparison method adjusted to maintain the accuracy of the comparisons (Tukey – Kramer) (Anonymous, 1989).

RESULTS

Screening of fungal isolates

The fastest growing isolates of *L. lundbergii* (CMW6185 and CMW6186) and *L. serpens* (CMW6187 and CMW6188), originated from *H. angustatus* infesting *P. patula* in Mpumalanga province. Isolates of *O. ips* selected, however, came from *O. erosus* infesting *P. patula* in Mpumalanga province (CMW6189), and *P. elliottii* in Kwazulu-Natal (CMW6190), respectively.

Inoculation experiments

Six weeks after inoculation, resin exudation was visible, and the inoculated fungi caused discoloration of sapwood on inoculated branches. However, no signs of dieback were seen. The branches inoculated with test fungi had more resin around inoculation points than controls. Reisolations from inoculated branches consistently yielded the inoculated fungi.

Ophiostoma ips was more pathogenic than *L. serpens* and *L. lundbergii*, and generally gave rise to longer lesions. The lesion length average of *O. ips* from the four sites was 33.3 mm, varying between 28.7 mm and 48.5 mm. For *L. serpens*, lesion length average was 27.8 mm (between 15.2 mm and 44.9 mm), and for *L. lundbergii*, it was 29.3 mm (between 15.4 mm and 37.2 mm) (Tables 1, 2). In the Sabie area, however, *O. ips* (with lesion length average 31.1 mm) caused slightly shorter lesions than *L. serpens* (32.7 mm) and *L. lundbergii* (31.4 mm) (Table 2).

The hybrid of *P. elliottii* and *P. caribaea* was generally more susceptible to the test fungi than *P. elliottii* and *P. radiata*. This hybrid had a lesion length average of 32.6 mm, while the average lesion lengths for *P. radiata*, and *P. elliottii* in Sabie, and *P. elliottii* in Knysna, were 28.9 mm, 30.1 mm, 28.9 mm, respectively. Interestingly, *P. elliottii* in Sabie (with lesion length average 29.6 mm) was more resistant to *O. ips* than it was in Knysna (38.9 mm). However, *P. elliottii* was more susceptible to *L. serpens* in Sabie (31.9 mm), than it was in Knysna (21.4 mm) (Table 2).

In general, longer lesion lengths were recorded in the Sabie area than in the Knysna area. In Sabie, the lesion length average was 31.4 mm, while it was 28.9 mm in the Knysna area. *Ophiostoma ips*, however, gave rise to a different trend. In the Knysna area, the fungus had a lesion length average of 36.5 mm, while it was 31.1 mm in the Sabie area (Table 2).

Multiple comparisons of lesion length showed that there were no significant differences between the lesion lengths for the two trials with *L. lundbergii* and *L. serpens*, or for *O. ips* in the

Sabie area (Table 3). However, for *O. ips*, there were significant differences on the two pine species in the Knysna area (P = 0.0012, P = 0.0001) (Table 3).

Combined analysis of variance (Table 4) for lesion length of the two trials at each of the four sites, showed that there are significant differences between experiment site (p = 0.0003), species (p = 0.0001), site x species (p = 0.0001), times of trials (p = 0.0001), species x times of trials (p = 0.0009), site x times of trials (p = 0.0002), and site x species x times of trials (p = 0.0364). No significant differences were found between trees at each site (Table 4).

DISCUSSION

Results of this study showed that *O. ips*, *L. serpens* and *L. lundbergii* can cause lesions in the cambium of *Pinus* spp. in South Africa. However, none of the three species inoculated caused outward symptoms such as die-back on trees. This suggests that they are weak pathogens and confirms the results of previous studies where these fungi have been tested separately on a limited number of tree species (Wingfield and Knox-Davies, 1980; Wingfield and Marasas, 1980, 1983; Wingfield and Swart, 1989). Of the three species tested, *O. ips* caused the longest lesions. *Leptographium serpens* and *L. lundbergii* gave rise to similar lesion lengths, which were generally shorter than those associated with *O. ips*.

Our results have shown that *O. ips* can cause lesions, but is not particularly pathogenic to pines in South Africa. This is in agreement with the studies of Wingfield and Marasas (1980), Rane and Tattar (1987), Parmeter *et al.* (1989), and Dunn *et al.* (2002). There are other studies, however, showing that the fungus was pathogenic to pines. In western Japan, *O. ips*, the associate of an *Ips* sp. infesting *P. densiflora* and *P. thunbergii*, infests the roots and has been reported to cause death of living pine trees in forests (Nisikado and Yamauti, 1933). The fungus

has also been shown to significantly inhibit sapflow of infected *Pinus ponderosa* (Mathre, 1964). In France, it is pathogenic to Scots pines and possibly plays a role in the establishment of *Ips sexdentatus* (Boerner) on trees (Lieutier *et al.*, 1989). In the United States, *O. ips*, together with *L. terebrantis* and *L. procerum* (Kendrick) M. J. Wingfield, is important in the dynamics of susceptibility of southern pines to the attack by the southern pine beetle, *Dendroctonus frontalis* (Zimmermann) (Otrosina *et al.*, 1997).

Neither *L. lundbergii* nor *L. serpens* was pathogenic to living healthy pines in South Africa. This is interesting, since *L. serpens* has been recorded to be associated with a root disease of *P. pinea* in Italy (Lorenzini and Gambogi, 1976), and *P. pinaster* and *P. radiata* in South Africa (Wingfield and Knox-Davies, 1980; Wingfield *et al.*, 1988). *Leptographium lundbergii* has been found to be weakly pathogenic to severely stressed red and black pines in Japan (Kaneko and Harrington, 1990).

Ophiostoma ips, which was more pathogenic than *L. serpens* and *L. lundbergii*, is primarily vectored by the non-aggressive *O. erosus* (Zhou *et al.*, 2001). The two *Leptographium* spp. are mainly isolated from *H. angustatus*, which is considerably more aggressive than *O. erosus* (Zhou *et al.*, 2001). This situation, where the less aggressive bark beetle carries the more virulent fungus, has also been observed in other studies (Owen, 1987; Harrington, 1993a, b). Owen (1987) found that the more virulent fungus, *L. terebrantis*, was vectored by a less aggressive bark beetle, *Dentroctonus valens* (LeConte). There are, however, also studies indicating that more aggressive conifer–infesting bark beetle species vector more virulent fungi (Krokene and Solheim, 1998; Solheim *et al.*, 2001). For example, *Ophiostoma canum* (Münch) H. & P Sydow, the major associate of *Tomicus minor* (Hartig), was found to be less virulent than *L. wingfieldii* and *O. minus*, the main associates of *T. piniperda* (Linnaeus) (Solheim *et al.*, 2001). Långström and Hellqvist (1993) showed that *T. minor* is less aggressive than *T. piniperda*.

In our study, the hybrid of *P. elliottii* and *P. caribaea* was more susceptible to the test fungi than *P. elliottii* and *P. radiata*. *Pinus elliottii* was more resistant to *O. ips*, while more susceptible to *L. serpens* in the Sabie area than in the Knysna area. These results suggest that different hosts differ in their response to fungal penetration. This is in agreement with the study of Raffa and Smalley (1995), where *P. resinosa* and *P. banksiana* showed different response patterns to *O. ips* and *O. nigrocarpum* (R. W. Davidson) De Hoog.

In the Sabie area, the tested fungi caused longer lesions than in the Knysna area, with the exception of *O. ips*. This might be explained by interactions between hosts, fungal species, climatic and other conditions in the two areas. This would be consistent with the fact that forest stand density has an influence on the infection by blue-stain fungi (Christiansen, 1985), that high water tables can increase the rate of black-stain root disease (Kulhavy *et al.*, 1978), and that stand conditions affect the expression of host resistance (Peter and Lorio, 1993).

Our results have shown that between the first and second trials, there were no significant differences in lesion length for *L. serpens* and *L. lundbergii* in the two areas, and of *O. ips* in the Sabie area. However, lesion lengths for the two trials using *O. ips* differed significantly in the Knysna area. The differences could be due to the interactions of hosts, fungi, and stand conditions, rather than seasonal difference. This is in agreement with the study of Parmeter *et al.* (1989), though other reports suggest seasonal difference affects the host response (Paine, 1984; Lorio, 1986).

Analysis of the combined ANOVA confirmed that interactions were significant, not only between sites, times of trials, fungal species inoculated, but also between site x times of trials, site x species, species x times of trials, and site x times of trials x species. Similar results have been found by Dunn *et al* (2002). They showed that pathogenicity of *O. piliferum* (Fr.) H. & P. Sydow, *O. ips*, and *Sphaeropsis sapinea* (Fr.: Fr.) Dyko & Sutton interacted strongly with host species, location, and season.

Overall, our results have confirmed that *O. ips*, *L. serpens* and *L. lundbergii* should not be considered as serious pathogens of above ground parts of *P. elliottii*, *P. radiata*, or the *P. elliottii* / *P. caribaea* hybrid in South Africa. But both *O. ips* and *L. lundbergii* are well-known sapstain agents on pines (Lagerberg *et al.*, 1927; Davidson, 1935; Gibbs, 1993; Seifert, 1993; Farrell *et al.*, 1997). Therefore, the ophiostomatoid fungi, together with their bark beetle vectors, should be taken into account when disease resistant clones, or control strategies against sapstain, are developed.

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Table 1. Lesion length means (mm) for different isolates of *Ophiostoma ips*, *Leptographium lundbergii*, and *L. serpens* at the various inoculation sites.

Fungal Species	Isolate No.	Trial [a] No.	No. of trees	Lesion Length Mean			
				Sabie (*P. elliottii* x *P. caribaea*)	Sabie (*P. elliottii*)	Knysna (*P. radiata*)	Knysna (*P. elliottii*)
L.Lundbergii	CMW6185	1	20	28.4	24.6	22.3	15.4
L. lundbergii	CMW6186	1	20	36.6	37.2	34.0	33.8
L. lundbergii	CMW6185	2	20	36.2	26.2	28.6	27.8
L. lundbergii	CMW6186	2	20	34.4	27.9	26.9	28.5
L. serpens	CMW6187	1	20	31.0	28.1	21.3	15.2
L. serpens	CMW6188	1	20	27.8	33.7	21.4	20.2
L. serpens	CMW6187	2	20	30.1	30.0	25.6	25.2
L. serpens	CMW6188	2	20	44.9	35.8	29.9	25.1
O. ips	CMW6189	1	20	31.6	29.7	28.8	32.4
O. ips	CMW6190	1	20	29.7	29.2	28.8	33.6
O. ips	CMW6189	2	20	31.8	30.6	44.5	48.5
O. ips	CMW6190	2	20	29.1	28.7	34.3	41.2

[a] The first set of trials at each site is referred to as 1, and the repetition of the complete trial on the second branch of each tree, is referred to as 2.

Table 2. Lesion lengths (mm) associated with inoculation of *Ophiostoma ips*, *Leptographium lundbergii*, and *L. serpens* onto various pine species in two geographic areas of South Africa.

	Area			Area			
Species	Sabie (*P. elliottii* x *P. caribaea*)	Sabie (*P. elliottii*)	Mean (Sabie)	Knysna (*P. radiata*)	Knysna (*P. elliottii*)	Mean (Knysna)	Mean
L.Lundbergii	33.9	29.0	31.4	28.0	26.4	27.2	29.3
L. serpens	33.5	31.9	32.7	24.6	21.4	23.0	27.8
O. ips	30.6	29.6	31.1	34.1	38.9	36.5	33.3
Mean	32.6	30.1	31.4	28.9	28.9	28.9	

Table 3. Comparison of the differences between lesion lengths (mm) after inoculations with *Ophiostoma ips*, *Leptographium lundbergii*, and *L. serpens* at two different times.

Species	Index	Sabie (*P. elliottii* x *P. caribaea*)	Sabie (*P. elliottii*)	Knysna (*P. radiata*)	Knysna (*P. elliottii*)
L. lundbergii	[a] D	-2.7 ([c] 32.5, [d] 35.2)	3.9 (30.9, 27.0)	0.2 (28.1, 27.9)	-3.1 (25.0, 28.1)
	[b] P	1.0000	0.9931	1.0000	0.9997
L. serpens	D	-7.9 (29.3, 37.2)	-2.0 (30.9, 32.9)	-6.4 (21.3, 27.7)	-7.4 (17.7, 25.1)
	P	0.1123	1.0000	0.4476	0.1650
O. ips	D	0.2 (30.6, 30.4)	-0.3 (29.4, 29.7)	-10.6 (28.8, 39.4)	-11.8 (33.0, 44.8)
	P	1.0000	1.0000	0.0012	0.0001

[a] D - Difference of the lesion length means between the first and second trials.
[b] P - Probability value.
[c] Lesion length mean of the first trial.
[d] Lesion length mean of the second trial.

Table 4. Combined ANOVA for lesion length measurements of the two trials at each of the four sites.

	[b] DF	[c] SS	[d] MS	[e] F	[f] P
Site	3	2001.37	667.12	6.33	0.0003
Trees at each site	76	8597.14	113.12	1.07	0.3193
Species	2	5085.55	2542.77	24.13	0.0001
Site x Species	6	12382.22	2063.70	19.58	0.0001
[a] Times	1	3771.78	3771.78	35.79	0.0001
Species x Times	2	1487.75	743.88	7.06	0.0009
Site x Times	3	2115.42	705.14	6.69	0.0002
Site x Species x Times	6	1425.77	237.63	2.26	0.0364

[a] Times - Initial and repeated inoculations
[b] DF - Degree of Freedom
[c] SS - Sum of Squares
[d] MS - Mean Square
[e] F - F value
[f] P - Probability Value

Chapter 8

Development of polymorphic microsatellite markers for the tree pathogen and sapstain agent, *Ophiostoma ips*[*]

Twelve pairs of simple sequence repeat markers (SSR) were developed using a single ascospore isolate of *Opiostoma ips*, isolated from the bark beetle, *Orthotomicus erosus*, infesting *Pinus elliottii* in South Africa. All markers were found to be polymorphic when tested on 7 isolates of *O. ips* collected from Austria, Chile, Israel, Mexico, South Africa, Sweden, and USA.

Keywords: SSR, *Orthotomicus*, *Pinus*, polymorphic.

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Opiostoma ips, is a fungus commonly associated with pine-infesting bark beetles in the Northern Hemisphere. These insects are important forest pests and their associated fungi degrade wood through sapstain (Seifert, 1992) and might also contribute to tree death (Zhou *et al.*, 2001). *Ophiostoma ips* has been introduced into Southern Hemisphere pine-growing countries such as South Africa, together with bark beetles (Wingfield & Marasas, 1980; Tribe, 1990). The close association between the beetles and the fungus, provides a unique system to examine the frequency and number of introductions into a new area. In order to understand patterns of introduction and spread, the diversity of the fungal population must be studied. The most effective means to achieve this goal is to develop co-dominant molecular markers. Thus, the aim of this study was to develop polymorphic microsatellite markers for *O. ips*.

DNA from the single ascospore isolate (CMW6418) was randomly amplified using ISSR primers 5' DHB$(CGA)_5$, 5' DDB$(CCA)_5$, 5' DBD$(CAC)_5$, 5' NDB$(CA)_7$C, 5' NDV$(CT)_8$, 5' HBDB$(GACA)_4$, and M13 (Meyer & Mitchell, 1995; Buscot *et al.*, 1996; Hantula, Dusabenyagasani & Hamelin, 1996). PCR volume of 50 uL consisted of 5 ng DNA, 0.2 mM of each dNTP, 0.6 uM primer, 3.5 U Expand High Fidelity PCR System enzyme mix, and 5 uL of Expand HF buffer, 10 X conc., with 15 mM $MgCl_2$ (Roche Molecular Biochemicals, Alameda, CA). PCR reactions were performed on an Eppendorf Mastercycler® Personal (Perkin-Elmer, Germany), and conditions were as follows: 95°C for 2 min followed by 40 cycles of 30 s at 95°C, 45 s at 48°C, and 2 min at 95°C, and a final step at 72°C for 10 min. PCR products were visualised under UV illumination on 1 % agrose gel (Promega, Madison, Wisconsin), purified using High Pure PCR Product Purification Kit (Boehringer, Mannheim, Germany), and different sizes of products were cloned using the pGEM®-T Easy Vector System (Promega Corporation, Madison, Wisconsin, USA).

Bacterial colonies containing recombinant plasmids were selected using the technique described by Burgess *et al.* (2001), and plasmid DNA was recovered using alkaline lysis

(Sambrook & Russell, 2001). Plasmid DNA was then digested with *Eco* RI (Roche Molecular Biochemicals, Alameda, CA) to release the inserts. Different sized inserts were sequenced using an ABI PRISM™ 377 Autosequencer (Applied Biosystems, Inc., Forster City, Calif.) with the BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) using the T7 and Sp6 primers.

Sequence electropherograms were analysed using Sequence Navigator version 1. 0. 1 (Applied Biosystems) and screened for microsatellite regions. SSR primer pairs were then designed to flank the microsatellite regions. For some sequences, the microsatellite region of interest was at the beginning or end of the insert. In these cases, genome walking was used to obtain the full repeat sequence (Burgess *et al.*, 2001). In total, twelve SSR primer pairs were designed, based on these sequences, to amplify a DNA fragment in the range of between 180-450 bp (Tab. 1). The primer pairs were designed to amplify a variety of tandem repeats including GA, CT, GT and GTT (Tab. 1).

SSR-PCR was conducted with DNA from 7 isolates of *O. ips*, 5 isolates believed to be native in Austria, Israel, Mexico, Sweden, and USA, and 2 isolates from introduced populations in Chile and South Africa. PCR volume of 25 uL consisted of 2 ng DNA, 0.1 mM of each dNTP, 0.3 uM primer, 0.7 U Expand High Fidelity PCR System enzyme mix, and 2.5 uL of Expand HF buffer, 10 X conc., with 15 mM $MgCl_2$ (Roche Molecular Biochemicals, Alameda, CA). PCR conditions were the same as those described by Barnes *et al.* (2001), except that an annealing temperature of 60°C was used for all primers. Polymorphisms of primer pairs were identified on PAGE gel (6 % acrylamide in 50 mM Tris-borate-EDTA buffer, 7 h at 140 v) followed by silver staining (Blum, Beier & Gross, 1987). All twelve primer pairs proved to be polymorphic (Tab 1) and one of each primer pair was labelled with a phosphoramidite fluorescent dye, HEX or FAM (MWG, Ebersberg, Germany).

Fluorescent-labelled PCR products of those 7 isolates with 12 primer pairs were separated on an ABI PRISM™ 377 sequencer as described by Burgess *et al.* (2001). Sizes of alleles were determined by using a combination of GeneScan® 2.1 analysis software (Perkin Elmer Corp.) and Genotyper® 3.0 (Perkin Elmer Corp.), comparing to the TAMRA internal size standard.
At each of the 12 loci amplified by the markers, 2-4 alleles were amplified to give a total of 35 alleles across all loci (Tab. 2). For each isolate, a data matrix of characters was compiled by scoring the presence or absence of each allele at each locus. Parsimony analysis was performed on the data set using PAUP* (Swofford, 1998). The most parsimonious trees were obtained using heuristic searches with random addition in 1000 replicates, with the tree bisection-reconnection branch-swapping option "on" and the steepest-descent option "off". Bootstrap consensus trees were constructed using the same conditions.

The data matrix comprised of 35 characters, each character representing an individual allele at one of the 12 polymorphic SSR loci. Of the 35 characters, 13 were parsimony-informative. Heuristic searches using parsimony resulted in 9 trees of 39 steps (Fig 1). Bootstrap analysis supported strong branches separating the American from the European isolates. Isolates from introduced populations in South Africa and Chile clustered with the European isolates, in particular the isolate from Austria.

The primary aim of this study was to produce microsatellite markers for future *O. ips* population analyses. However, patters emerging from this preliminary study suggest that isolates from different geographic regions have different profiles. If proves to be the case, it should be possible to determine the origin of introduced populations in the Southern Hemisphere. The primers developed can now be used in population studies of *O. ips* from many parts of the world.

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Table 1. Characteristics of polymorphic microsatellite markers designed for plant pathogen and sapstain agent, *Ophiostoma ips*.

SSR Primer Pair	PO	Sequence	Flourescent Label	Expected Size(bp)	Calculated T_m (°C)	Annealing Temp (°C)	Core Sequence	Band Pattern
OI-1	F	5' CAA GGT GAA GTG GTG GGG AC	FAM	340	64	60	(GGAAGGAGGA)$_2$(AG)$_2$*(AG)$_3$	Single; Polymorphic
OI-2	R	5' CGC CCC TGA TTT CCC GAT TC			64	60	(GAGGA)$_2$*(GA)$_4$, and rich in G, A	Single; Polymorphic
OI-3	F	5' CAC CTT GCG CAG CCA GTT AC	FAM	210	64	60	GA$_3$GA$_8$G$_3$A$_9$GA$_5$GAG$_4$A	Single; Polymorphic
OI-4	R	5' CGT AGC GGT GGA GTC AAG CG			66	60		Single; Polymorphic
OI-5	F	5' CCA CTC ACC TCT CTT TAC GAC	FAM	441	64	60	CT$_4$CT$_7$CT$_5$CT$_6$*(CTT)$_3$*(TC)$_3$*	Single; Polymorphic
OI-6	R	5' CTC CTC TGC AAA CTC GTC CC			64	60	(TTTG)$_3$*(CT)$_3$, and rich in T	Single; Polymorphic
OI-7	F	5' GCT GTG GCG AGA CGA TGT CG	HEX	318	66	60	(GA)$_3$(GT)$_3$*(GGA)$_6$*(AGG)$_3$*	Single; Polymorphic
OI-8	R	5' CAT GCC AGC CGT TTC ATG TGC			66	60	(AGC)$_3$*(AGG)$_4$	Single; Polymorphic
OI-9	F	5' GAT GTC GCG GAG AAT GAC GG	HEX	221	64	60	(GTT)$_2$*T$_3$G$_2$T$_3$GTGT$_2$G$_2$T$_6$G$_2$T$_2$*	Single; Polymorphic
OI-10	R	5' GAT ATT AAA TCG CCC CCT CCC			62	60	(GT)$_3$*(TAGG)$_2$	Single; Polymorphic
OI-13	F	5' GCC TGG ACC GCT TCA TTG TCG	FAM	346	68	60	(CG)$_4$(CCG)$_2$*(TGC)$_6$*	Single; Polymorphic
OI-14	R	5' GAC GGT TTC GCC AGC GAG TAG			68	60		Single; Polymorphic
OI-17	F	5' CAT CCT GGC CAA CCG ACT GG	FAM	253	66	60	(GGC)$_2$*(TTC)$_2$*AGA$_9$GAT$_2$A$_9$*	Single; Polymorphic
OI-18	R	5' CTC CGA ATC TGG AGA GCC AG			64	60	(AC)$_3$, and rich in A	Single; Polymorphic
OI-19	F	5' GAG GAG AGA GAT GCG CCA GC	HEX	235	66	60	T$_8$CAT$_5$CAT$_7$AT$_4$*(CACTTTTT)$_2$*	Single; Polymorphic
OI-20	R	5' GTC TGC GTC GAA ATT GCC CC			64	60	(CTT)$_3$*(TTA)$_2$ *,and rich in T	Single; Polymorphic
OI-23	F	5' CAC GCG CAA GTT TGC CGA GG	FAM	184	66	60	(GGC)$_6$*(CGG)$_2$ *, and rich in G	Single; Polymorphic
OI-24	R	5' GCA CGT TGT TGT AGT ACC GCG			66	60		Single; Polymorphic
OI-25	F	5' GCT CCA TCC ACC ACT TAC AAC	HEX	365	64	60	(CCACCACAT)$_3$*(ACTTCCACC)$_2$*	Single; Polymorphic
OI-26	R	5' GCC GGT CAA GGA GAC AGT AAG			66	60	(CCACCACAT)$_2$(CCA)$_2$	Single; Polymorphic
OI-27	F	5' GGG CAT CGC CAT TGC CCT G	FAM	242	64	60	(GTT)$_7$*(TGG)$_5$*(GC)$_3$*	Single; Polymorphic
OI-28	R	5' GAG GTA CTC GAC CTG GAA CG			64	60		Single; Polymorphic
OI-31	F	5' CAG GTA CAG CGA GGG CGT G	HEX	320	64	60	(GT)$_3$*(GTT)$_3$*(GGT)$_6$	Single; Polymorphic
OI-32	R	5' GAC ACC TCC CCT AGC TCT AG			64	60		Single; Polymorphic

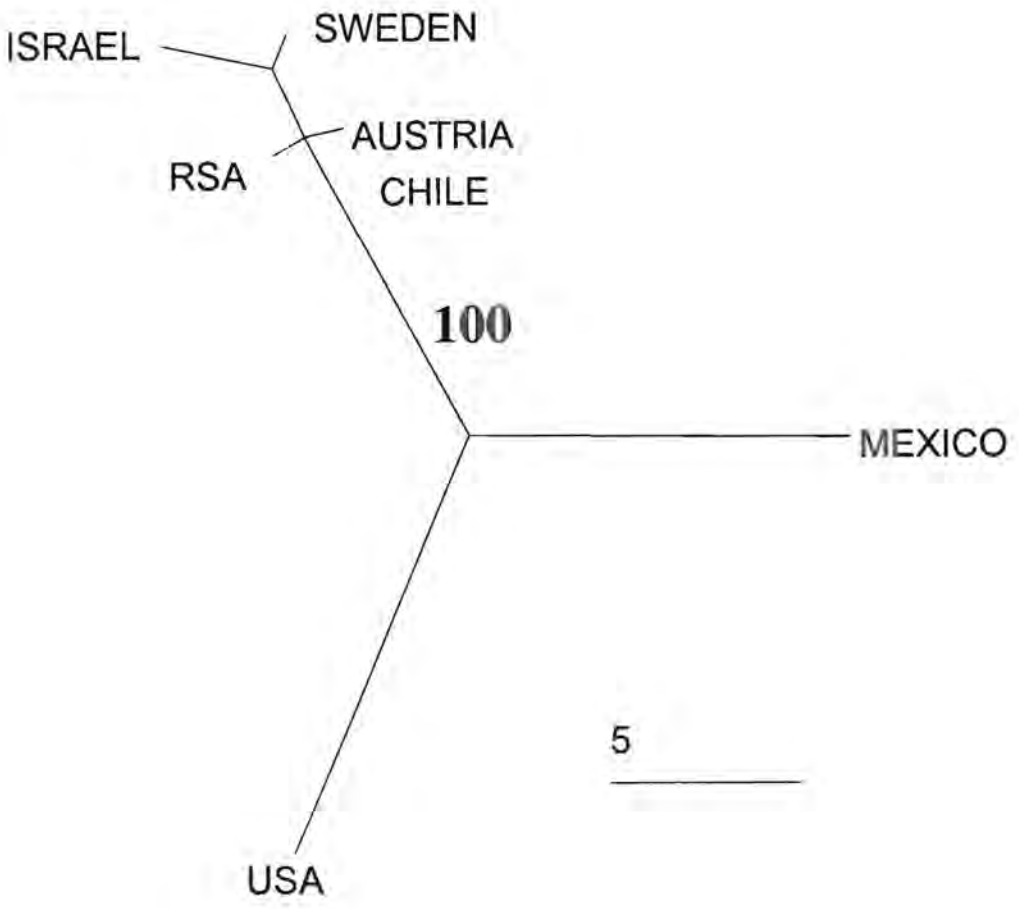
Notes: PO, primer orientation; F, forward; R, reverse primer.

Table 2. Alletic properties of designed polymorphic primers on 7 different isolates of *Ophiostoma ips*.

Isolate No.	Other No.	Origin	OI-1/2	OI-3/4	OI-5/6	OI-7/8	OI-9/10	OI-13/14	OI-17/18	OI-19/20	OI-23/24	OI-25/26	OI-27/28	OI-31/32
CMW7076	CBS 151.54	Sweden	329	209	447	317	223	346	253	235	185	363	239	318
CMW1173	SFP 215	Israel	329	209	455	317	223	346	253	235	185	354	239	318
CMW7079	CBS 438.94	Austria	337	209	447	317	223	346	253	235	185	354	239	318
CMW6416		Chile	337	209	447	317	223	346	253	235	185	354	239	318
CMW6418		South Africa	337	209	447	317	223	346	253	235	185	363	239	318
CMW7075	CBS 137.36	USA	337	210	391	318	214	318	259	249	191	332	213	295
CMW9020		Mexico	340	203	438	314	223	327	259	235	189	346	233	319

Notes: CMW – the Culture Collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002, Republic of South Africa.

Figure 1. Unrooted phylogram of one of the 9 most parsimonous trees generated from SSR polymorphic data showing strong bootstrap support separating the European from American isolates.



Chapter 9

Microsatellite (SSR) markers reveal genetic diversity among isolates of *Ophiostoma ips* from South Africa, Chile, Europe, and the USA

In the Northern Hemisphere, many conifer-infesting bark beetles (Coleoptera: Scolytidae) are vectors of the tree pathogen and sapstain agent, *Ophiostoma ips* (Rumb.) Nannf. In the Southern Hemisphere, *O. ips* has been introduced into exotic pine-growing countries together with bark beetles. Very little is known regarding the population biology of *O. ips*. The aim of this study was to evaluate the genetic diversity of populations of the fungus using SSR markers in order to gain an understanding of its introduction patterns. Twelve pairs of polymorphic markers were used to examine the population structure for five populations of *O. ips*, one each from Chile, Europe, and the USA, and two from South Africa. The SSR markers produced a total of 74 alleles across the 12 loci examined. The highest gene diversity was found in the USA population, while the lowest was observed for the European population, together with the introduced Mpumalanga population. Forty-four genotypes were found across all populations. The highest genotypic diversity was found in the USA population while the lowest was observed for the European population. A high gene flow was observed between Mpumalanga and KwaZulu-Natal populations, while moderate gene flow was present between the other populations. A neighbour-joining dendrogram showed that the three introduced populations from Chile and South Africa clustered more closely with the European population than with the USA population. The USA population had the highest genetic diversity, and is most likely to be the centre of origin of *O. ips*.

Keywords: Polymorphic marker, population genetics.

INTRODUCTION

Ophiostoma spp. represent an important fungal group that includes many primary tree pathogens (Harrington & Cobb, 1988; Brasier, 1990). *Ophiostoma ips* (Rumbold) Nannfeldt has been reported as pathogenic to conifers (Lieutier *et al.*, 1989; Otrosina *et al.*, 1997), killing living pine trees in extreme cases (Nisikado & Yamauti, 1933). Other studies, however, showed that *O. ips* is not severely pathogenic, causing lesions, but not killing trees, when artificially inoculated (Parmeter *et al.*, 1989; Zhou *et al.*, 2002a). Like many other *Ophiostoma* spp., *O. ips* causes sapstain on logs and freshly cut wood that leads to significant losses to the forestry industry globally (Rumbold, 1931; Hutchison & Reid, 1988; Stone & Simpson, 1990; Seifert, 1993; Marmolejo & García-Ocañas, 1993; Farrell *et al.*, 1997).

Ophiostoma ips has been found casually associated with different conifer-infesting bark beetles such as *Ips* spp. (Rumbold, 1931; Upadhyay, 1981; Lieutier *et al.*, 1991), *Dendroctonus* spp. (Rumbold, 1931; Hunt, 1956; Upadhyay, 1981; Perry, 1991), *Orthotomicus* spp. (Mathiesen-Käärik, 1960; Wingfield & Marasas, 1980), and *Tomicus piniperda* (Linnaeus) (Mathiesen, 1950; Mathiesen-Käärik, 1953; Masuya *et al.*, 1999). The fungus has typically been reported from Northern Hemisphere countries such as the USA, France, Sweden, and Japan (Mathiesen-Käärik, 1960; Lieutier *et al.*, 1991; Perry, 1991; Masuya *et al.*, 1999). It was also introduced into exotic pine-growing countries in the Southern Hemisphere such as Chile and South Africa (Chapter2, 3), together with bark beetles of European origin.

The bark beetle vectors of *O. ips* in Chile and South Africa include *Hylurgus ligniperda* (Fabricius), *Hylastes ater* (Paykull), *H. angustatus* (Herbst), and *Orthotomicus erosus* (Wollaston) (Chapter 1). Although some research has been conducted on the taxonomy and biology of the fungi associated with these beetles (Chapters 1, 2, 3), nothing is known regarding the population structure of *O. ips* or indeed any of these fungi.

Population structure generally refers to the amount and distribution of genetic variation within and between populations. Fungal populations with higher levels of genetic variation are likely to adapt more rapidly to fungicides or resistant hosts (McDonald & McDermott, 1993; Milgroom, 1996; McDonald, 1997). Understanding population structure of pathogens and pests is, therefore, an important component of effective disease and pest management. One of the most effective tools for studying population structure is using co-dominant markers, such as simple short repeats (SSR) markers (Burgess, Wingfield & Wingfield, 2001). In a previous study, twelve pairs of polymorphic SSR markers were developed for *O. ips* (Zhou *et al.*, 2002b). The objective of the present study was to use these markers to compare genetic diversity in different populations of *O. ips*. The genetic variation, genetic distance, and mode of reproduction within and between the different populations were considered.

MATERIALS AND METHODS

Fungal Isolates

Five populations of *O. ips* were investigated in this study (Table 1). The two South African populations (30 isolates each) were isolated from three exotic pine-infesting bark beetle species, *H. ligniperda*, *Hylastes angustatus* and *O. erosus*, during a two-year survey of *Ophiostoma* spp. associated with these beetles in two geographic regions (Chapter 2). In Mpumalanga, the beetles infested *P. patula*, while they were found on *P. elliottii* in KwaZulu-Natal. The Chilean population consisted of 21 isolates from *H. ligniperda* infesting *Pinus radiata* in the Valdivia area. The European population was comprised of 15 isolates from *Ips acuminatus* (Gyllenhal) infesting *P. sylvestris* in Sweden, and 15 from *I. sexdentatus* (Boerner) also infesting *P. sylvestris* in France. Two additional isolates (CBS151.54 and CBS 438.94) from Sweden and Austria respectively, and one (CMW1173) from Israel, were also included in the European

population. A further six isolates originated from the USA, isolated from *P. resinosa* and *P. ponderosa*, and the bark beetle *I. integer* (Eichhogg), were also included. All cultures used in this study are maintained in the culture collection (CMW) of Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

DNA Extractions

A single hyphal tip culture of each isolate was grown in 2 % ME (20 g Biolab malt extract, and 1000 ml distilled water). DNA was extracted using a modified version of the extraction method developed by Raeder and Broda (1985) as described in detail in Chapter 3.

SSR-PCR

SSR-PCR was conducted with all isolates using twelve pairs of polymorphic fluorescent-labelled microsatellite primers designed for *O. ips* (Zhou *et al.*, 2002b). PCR reaction mixtures and conditions were the same as those described previously (Zhou *et al.*, 2002b). PCR products were visualized, but not purified.

Genescan analysis

Fluorescent-labelled PCR products were arranged according to the expected size of the PCR product and fluorescent label type (HEX or FARM) attached to the primers. SSR PCR products were then separated and analysed as described previously (Zhou *et al.*, 2002b).

Gene and Genotypic Diversity

For each isolate, a data matrix of multistate characters was compiled by assigning each allele at each of the 12 loci a different letter (e.g. AABDCGDAFBGB). Two functions of gene and genotypic diversities were used to evaluate the genetic variation within the populations studied.

Gene diversity was determined by allele frequencies at each locus, while genotypic diversity was determined by the number and frequency of combinations of alleles at multiple loci. Gene diversity of each population was calculated according to the formula, $H = 1 - \Sigma_k x_k^2$, where x_k is the frequency of the k^{th} genotype (Nei, 1973), using POPGENE version 1.31 (http://www.ualberta.ca/~fyeh/fyeh). Genotypic diversity of the populations was calculated using the formula, $G = 1 / \Sigma [f_x (x/n)^2]$, where n is the sample size, and f_x is the number of genotypes occurring x times in the sample (Stoddart & Taylor, 1988). To compare the genotypic diversities between populations, the maximum percentage of genotypic diversity was obtained using the formula $\hat{G} = G / N*100$, where N is the population size (McDonald *et al.*, 1994).

Genetic distance

$\delta\mu^2$ and D_{AD} were used to calculate the genetic distance between populations based on microsatellite data. $\delta\mu^2$ is the square of the mean distance between two populations, and D_{AD} is based on absolute distance, which is not squared (Goldstein *et al.*, 1995). Genetic distances between populations were calculated using the programme MICROSAT (http://human.stanford.edu/microsat), and neighbour-joining trees were constructed in MEGA version 2.1 (http://www.megasoftware.net).

Population differentiation and gene flow

θ and G_{ST} were calculated to evaluate the level of population differentiation. θ was calculated in Multilocus version 1.2 (http://www.bio.ic.ac.uk/evolve/software/multilocus), and an estimate of F_{ST}, using the equation $\theta = Q - q / 1 - q$, where Q is the probability that two alleles from the same population are the same, and q is the probability that two alleles from different populations are the same. A value of θ equal to 0 indicates no population differentiation, while a value of θ equal to 1 indicates totally different alleles between two populations. The significance of θ was

determined by comparing the observed value to that of 1000 randomized datasets in which individuals were randomized across populations. The null hypothesis of no population differentiation can be rejected where the P value is significant.

Another measurement, G_{ST}, was calculated in POPGENE version 1.31 (http://www.ualberta.ca/~fyeh/fyeh), using the equation $G_{ST} = (H_T - H_S) / H_T$, where H_T is the mean total gene diversity and H_S is the mean within population diversity. A value of G_{ST} closer to zero reflects less genetic variation between populations, while values of 0 to 0.05 reflect little, and values above 0.25 large genetic differentiation.

The level of gene flow (N_m) was calculated from the estimate of G_{ST}, where $N_m = 0.5 (1 - G_{ST}) / G_{ST}$. A N_m value of zero indicates that populations are completely isolated, and share no alleles.

Mode of reproduction

The Index of Association (I_A) (Maynard *et al.*, 1993) was used to determine the mode of reproduction for each population. I_A was calculated for populations including all isolates, while the clone corrected population contains only one representative of each genotype. The tests were performed on a data matrix of multistate characters for each allele at each locus, and calculated using Multilocus version 1.2. The observed data were compared with the expected data for a 1000 randomly recombining data sets calculated. Recombination within the population was then determined, comparing the observed data to the distribution range of the recombined data (Taylor, Jacobson & Fisher, 1999). If the observed data fall within the distribution range, the population is likely to be undergoing recombination. The population is, however, not undergoing recombination where the observed data fall outside the distribution range.

RESULTS

Allelic diversity

One hundred and twenty isolates of *O. ips* were amplified with 12 pairs of SSR markers, and the markers produced a total of 74 alleles across the 12 loci examined (Table 2). Individually, there were 22 alleles in the Chile population, 18 in Europe, 20 in Mpumalanga, 28 in KwaZulu-Natal, and 46 in the USA population.

Monomorphic alleles were present at locus OI1 (Mpumalanga and USA), OI3 (Europe and Mpumalanga), OI7 (Chile, Europe, and Mpumalanga), OI9 (Chile and Europe), OI13 (Chile, Europe, KwaZulu-Natal, and Mpumalanga), OI17 (Europe and Mpumalanga), OI19 (Europe), OI23 (Chile, KwaZulu-Natal, and Mpumalanga), OI27 (Chile), and OI31 (Mpumalanga) (Table 2).

Unique alleles were observed in the different populations. In total, the Chilean and Mpumalanga populations each had two unique alleles over two loci. The European population had three unique alleles over three loci, the KwaZulu-Natal population had four over four loci, and the USA population had 26 over 11 loci (Table 2).

The gene diversity estimated from the SSR profile of isolates varied greatly between populations (Table 2). The highest gene diversity (h = 0.63) was found in the USA population, while the lowest (h = 0.10) was observed for the Mpumalanga and European populations. Moderately high diversities were found in the populations from Chile (h = 0.16) and KwaZulu-Natal (h = 0.20).

Genotypic diversity

Forty-four genotypes, representing isolates of *O. ips* studied, were obtained: eight in the Chile population, six in Europe, 15 in KwaZulu-Natal, nine in Mpumalanga, and six in the USA population (Table 3).

A number of genotypes were shared across the populations (Table 3). For instance, genotype OI24 was shared by six isolates from Chile, 17 isolates from Europe, four isolates from KwaZulu-Natal, and seven isolates from Mpumalanga. None of genotypes obtained from the USA isolates was shared with other isolates (Table 3).

The genotypic diversity estimated from the SSR profile of isolates varied greatly among populations (Table 3). The highest maximum genotypic diversity ($\hat{G}$ = 100 %) was found in the USA population while the lowest ($\hat{G}$ = 8.5 %) was observed for the European population. Moderately high diversities were found in the three introduced populations from Chile ($\hat{G}$ = 21.2%), KwaZulu-Natal ($\hat{G}$ = 21.5 %), and Mpumalanga ($\hat{G}$ = 15.2 %).

A neighbour-joining dendrogram based on the genotypes of each population, showed that there were no specific groups based on hosts, insect vectors and geographic areas in the populations (Figure 1). Most genotypes presented clustered closely, except the three isolates from USA, which were very far from the rest (Figure 1).

Genetic distance

The neighbour-joining dendrogram based on the genetic distance between the populations showed that there were two main clades in the populations studied (Figure 2). In the first clade, the two introduced South African populations (Mpumalanga and KwaZulu-Natal) clustered more closely with the introduced Chilean population than to the European population. The USA population, however, was distant to all the other populations.

Little genetic differentiation (G_{ST} = 0.02, θ = -0.03) was found between KwaZulu-Natal and Mpumalanga populations, and moderate genetic differentiation was observed between the other populations (Table 4).

The amount of gene flow varied between populations. High gene flow (N_m = 22.71) was observed between Mpumalanga and KwaZulu-Natal populations, while moderate gene flow was

found between the other populations (Table 4). The lowest gene flow existed between the USA and Europe, and the USA and Mpumalanga populations.

Mode of reproduction

The European population was found to be clonal, 30 isolates represented only 3 genotypes, while each of the other three isolates represented another genotype. The latter three isolates were from *Orthotomicus proximus* (Gyllenhal), *Ips sexdentatus* (Boerner), and *Crypturgus mediteranous*, respectively, and collected many years ago. The lack of diversity rendered these data unsuitable for analysis of IOA. For the USA population, linked alleles indicated clonal reproduction, or in case of fungi, homothallism. The introduced populations from Chile and KwaZulu-Natal appear clonal, while the Mpumalanga population appears to be undergoing some recombination (Figure 3).

DISCUSSION

To the best of our knowledge, this study is the first to consider the population structure of the tree pathogen and sapstain agent, *Ophiostoma ips*. SSR markers were used to evaluate the genetic diversity, genetic distance, and mode of reproduction of different populations from Chile, Europe, South Africa, and the USA. The USA population had the highest genetic diversity. The highest gene flow was observed between the two South African populations. The three introduced populations from Chile and South Africa clustered most closely with the European population, and were clearly separated from the USA population.

According to McDonald (1997), a population from the centre of origin of a species would be expected to have higher gene diversity than other populations (McDonald, 1997). In the case of

O. ips, the USA population had the highest genetic diversity, indicating that North America is most likely the centre of origin for this fungus, *O. ips*.

The relatively lower gene diversity (h = 0.10) observed in the native European population suggested that *O. ips* has developed clonal lineages. *Ophiostoma ips* is homothallic (Chapter 4), and one individual of a homothallic fungus is capable of producing sexual spores genetically identical to the parent strain (Taylor *et al.*, 1999), which supports the hypothesis of clonal lineages.

Neighbour-joining distance analysis showed that the three introduced populations from Chile and South Africa clustered more closely with the European population than with the USA population. The three introduced populations of *O. ips* were isolated from exotic bark beetles which are native to Europe (Swan, 1942; Neumann 1987). Our results support the view that the fungus was introduced into exotic pine-growing countries in the Southern Hemisphere by bark beetles originating from Europe. In addition, a much lower gene flow (N_m = 1.78) was found between the native European and USA populations.

The neighbour-joining dendrogram based on genotypes of each population showed that there were no specific groups within the populations. This indicates that there is, at present, no evidence of host or insect specialization influencing the evolution of *O. ips*. In this study, populations from South Africa were isolated from three bark beetle species infesting *P. patula* and *P. elliottii*, the Chilean population from *H. ligniperda* infesting *P. radiata*, the European population mainly from *I. acuminatus* and *I. sexdentatus* infesting *P. sylvestris*, and the USA population from *P. resinosa*, *P. ponderosa*, and *I. integer*. Other studies have, however, shown that host specialization can play an important role influencing the evolution of fungi such as *Magnaporthe grisea* (Hebert) Barr (Zeigler, 1998), and *Ceratocystis fimbriata* Ellis & Halst (Barnes *et al.*, 2001).

The high gene flow (N_m = 22.71) and little genetic differentiation (G_{ST} = 0.02) observed between the KwaZulu-Natal and Mpumalanga populations from South Africa probably resulted from the spread of bark beetles within the country. This is not surprising considering that there is no geographical barrier between the two populations. McDonald (1997) stated that absence of gene flow among populations could be used to define the geographic boundaries of populations. In our study, however, it appeared that there was not much genetic differentiation and gene flow (Table 4), between all other populations and the USA population. This is because G_{ST} is underestimated while N_m is overestimated for the USA population as a result of the small number of isolates in the population.

I_A tests for populations including all isolates, as well as for clone corrected populations, showed that most recombination occurred in the introduced Mpumalanga population. This population, however, had lower genetic diversity. Our hypothesis for this is that the population has been in this area for a longer period than in KwaZulu-Natal, and that a few specific genotypes have been selected during spread. Populations from Chile and KwaZulu-Natal, which had a higher genetic diversity and were not undergoing recombination, could have originated from multiple introductions of insect vectors. In the case of KwaZulu-Natal, the population was collected within a 30 km radius of the Richard Bay harbour, from where large volumes of pine logs from all over South Africa are exported annually. This would inevitably lead to a higher genetic diversity in that area.

The SSR markers used in this study represent powerful molecular tools, making it possible to understand the structure of fungal populations and introduction patterns. Our results support the view that *O. ips* was introduced into exotic pine-growing countries such as Chile and South Africa, together with the bark beetles native to Europe. The USA population, however, with the highest genetic diversity, is most likely to be the centre of origin for *O. ips*. Further investigation

with higher numbers of isolates from the USA, and populations from other parts of the world will be necessary to better understand the global patterns of spread of *O. ips*.

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Table 1: Isolates of *Ophiostoma ips* used in this study.

Isolate numbers (number of isolates)	Country of origin	Host	Insect vector
CMW6418 – 6423 (6), CMW6432 – 6434 (3), CMW6436 – 6440 (5)	KwaZulu-Natal, South Africa	*Pinus elliottii*	*Orthotomicus erosus* (Wollaston)
CMW6446 – 6454 (9), CMW6456, CMW6458 – 6460 (3), CMW6463, CMW6465		*P. elliottii*	*Hylurgus ligniperda* (Fabricius)
CMW7215		*P. elliottii*	*O. erosus*
CMW6442 – 6444 (3), CMW6470 – 6471 (2)	Mpumalanga, South Africa	*P. patula*	*Hylastes angustatus* (Herbst)
CMW6472 – 6474 (3), CMW6476 – 6478 (3), CMW6480 – 6488 (9), CMW6490 – 6495 (6), CMW7211 – 7214 (4)		*P. patula*	*O. erosus*
CMW6401 – 6417 (17), CMW5089, CMW5179, CMW7209 – 7210 (2)	Valdivia, Chile	*P. radiata*	*H. ligniperda*
CMW9005 – 9019 (15)	Uppsala, Sweden	*P. sylvestris*	*Ips acuminatus* (Gyllenhal)
CBS151.54	Dalarna, Sweden		*O. proximus* (Eichhoff)
CMW9310 – 9324 (15)	East France	*P. sylvestris*	*I. sexdentatus* (Boerner)
CBS438.94	Flatz, Austria		*I. sexdentatus*
CMW1173	Israel		*Crypturgus mediteranous*
CMW311 – 313 (3)	Minnesota, USA	*P. resinosa*	
CMW1760 – 1761 (2)	Idaho, USA	*P. ponderosa*	
CBS137.36	Oregon, USA		*I. integer* (Eichhoff)

Table 2: Allele size (bp) at 12 loci and allelic diversity for *Ophiostoma ips* collected from Chile, Europe, KwaZulu-Natal, Mpumalanga, and USA.

Locus	Allele	Chile	Europe	KwaZulu-Natal	Mpumalanga	USA
OI-1	329	-	2	-	-	-
	333	-	-	1	-	-
	337	20	30	26	30	6
	341	1	-	3	-	-
OI-3	179	-	-	-	-	1
	202	-	-	-	-	1
	205	-	-	1	-	-
	209	20	32	29	30	3
	210	-	-	-	-	1
	213	1	-	-	-	-
OI-5	391	-	-	-	-	1
	442	-	-	4	1	-
	444	-	-	1	-	-
	447	11	31	25	29	-
	448	-	-	-	-	2
	451	1	-	-	-	-
	455	9	1	-	-	1
OI-7	311	-	-	-	-	1
	317	21	32	28	30	4
	318	-	-	2	-	1
OI-9	210	-	-	-	-	1
	214	-	-	-	-	1
	217	-	-	-	-	2
	218	-	-	5	3	-
	223	21	32	25	27	2
OI-13	313	-	-	-	-	1
	318	-	-	-	-	1
	346	21	32	30	30	3
	349	-	-			1
OI-17	235	-	-	-	-	1
	247	-	-	-	-	1
	249	-	-	1	-	-
	253	20	32	27	30	-
	254	-	-	-	-	2
	257	1	-	2	-	-
	259	-	-	-	-	1
	263	-	-	-	-	1
OI-19	207	-	-	-	-	1
	226	-	-	-	-	1
	228	-	-	-	-	1
	230	-	-	1	-	-
	234	10	-	-	1	-
	235	10	32	28	29	2
	238	1	-	-	-	
	239	-	-	1	-	-
	249	-		-	-	1
OI-23	170	-	-	-	-	1
	179	-	-	-	-	3
	184	-	9	-	-	-
	185	21	23	30	30	1
	191	-			-	1
OI-25	332	-	-	-	-	1
	337	-	-	-	1	-
	350	-	-	-	-	1
	354	15	31	10	11	2
	355	-	-	-	-	1
	358	1	-	1	-	1
	359	-	-	2	-	-
	363	5	1	17	17	-
	367	-	-	-	1	-
OI-27	213	-	-	-	-	2
	223	-	-	-	-	1
	239	21	18	21	24	2
	240	-	14	-	-	-
	242	-	-	9	4	-
	243	-	-	-	2	-
	274	-	-	-		1
OI-31	278	-	-	-	-	1
	295	-	-	-	-	1
	296	-	-	-	-	1
	314	-	-	1	-	-
	318	20	31	29	30	3
	322	1	-	-	-	-
	327	-	1	-	-	-
Population size		21	32	30	30	6
No. of alleles		22	18	28	20	46
No. of unique alleles		2	3	4	2	26
No. of polymorphic loci		7	6	10	5	11
Gene diversity		0.16	0.10	0.20	0.10	0.63

Table 3: Multilocus genotypes and genotypic diversity obtained for *Ophiostoma ips* isolates from Chile, Europe, KwaZulu-Natal, Mpumalanga, and USA.

	Chile	Europe	KwaZulu-Natal	Mpumalanga	USA
OI1	-	-	-	1	-
OI2	3	-	10	11	-
OI3	-	1	-	-	-
OI4	1	-	-	-	-
OI5	-	-	1	-	-
OI6	-	-	1	-	-
OI7	-	-	3	4	-
OI8	-	-	1	-	-
OI9	-	-	1	-	-
OI10	1	-	-	-	-
OI11	1	-	-	-	-
OI12	-	1	-	-	-
OI13	7	-	-	-	-
OI14	1	-	-	-	-
OI15	-	-	1	-	-
OI16	-	-	1	-	-
OI17	1	-	-	1	-
OI18	-	1	-	-	-
OI19	-	-	-	2	-
OI20	-	-	2	-	-
OI21	-	9	-	-	-
OI22	-	4	-	-	-
OI23	-	-	-	1	-
OI24	6	17	4	7	-
OI25	-	-	1	2	-
OI26	-	-	1	-	-
OI27	-	-	1	-	-
OI28	-	-	1	-	-
OI29	-	-	1	1	-
OI30	-	-	-	-	1
OI31	-	-	-	-	1
OI32	-	-	-	-	1
OI33	-	-	-	-	1
OI34	-	-	-	-	1
OI35	-	-	-	-	1
	-	-	-	-	-
Number of isolates	21	33	30	30	6
Number of genotypes	8	6	15	9	6
Genotypic diversity (G)	4.46	2.80	6.44	4.56	6
Max. genotypic diversity ($\hat{G}$)	21.2%	8.5%	21.5%	15.2%	100%

Table 4: Amount of gene flow between populations.

	KwaZulu-Natal	Chile	Europe	USA
Mpumalanga	[a] θ = -0.03	θ = 0.092	θ = 0.055	θ = 0.258
	(P = 0.423)	(P = 0.038)	(P = 0.096)	(P < 0.001)
	[b] G_{st} = 0.02	G_{st} = 0.16	G_{st} = 0.18	G_{st} = 0.23
	[c] N_m = 22.71	N_m = 2.65	N_m = 2.21	N_m = 1.70
KwaZulu-Natal		θ = 0.097	θ = 0.069	θ = 0.213
		(P = 0.017)	(P = 0.075)	(P < 0.001)
		G_{st} = 0.13	G_{st} = 0.15	G_{st} = 0.19
		N_m = 3.35	N_m = 2.77	N_m = 2.14
Chile			θ = 0.118	θ = 0.208
			(P = 0.02)	(P = 0.002)
			G_{st} = 0.19	G_{st} = 0.20
			N_m = 2.07	N_m = 2.02
Europe				θ = 0.204
				(P = 0.006)
				G_{st} = 0.22
				N_m = 1.78

[a] θ = Population differentiation (Agapow & Burt, 2000);
[b] G_{st} = Genetic differentiation coefficient (Nei, 1973);
[c] N_m = Estimate of gene flow from G_{st} (McDermott & McDonald, 1993).

Figure 1: Neighbor-joining dendrogram of *Ophiostoma ips* isolates from Chile, Europe, KwaZulu-Natal, Mpumalanga, and the USA based on genotypes (Duplicate genotypes within a population were removed in the analysis).

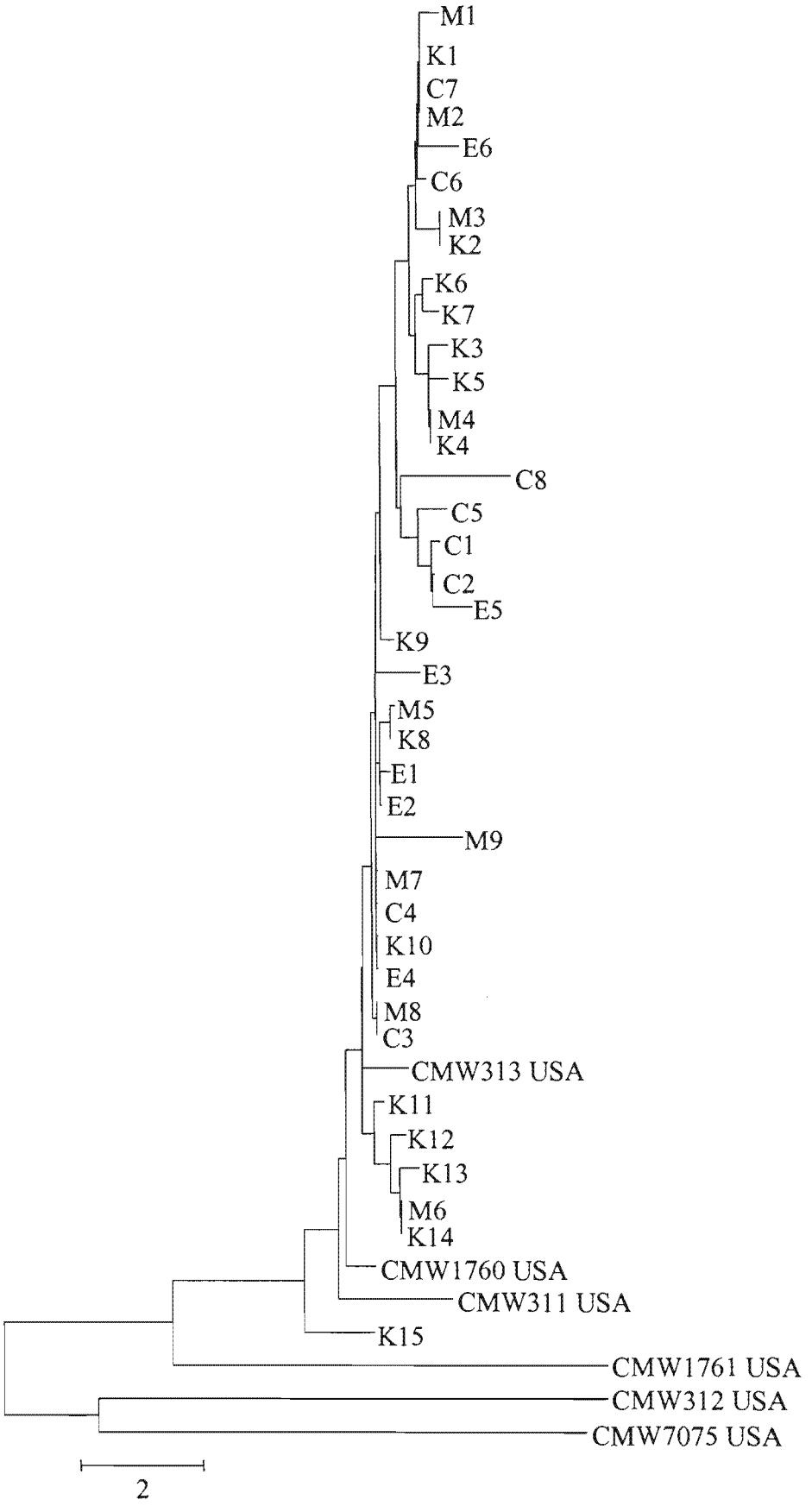


Figure 2: Neighbor-joining dendrogram of *Ophiostoma ips* populations from Chile, Europe, KwaZulu-Natal, Mpumalanga, and the USA.

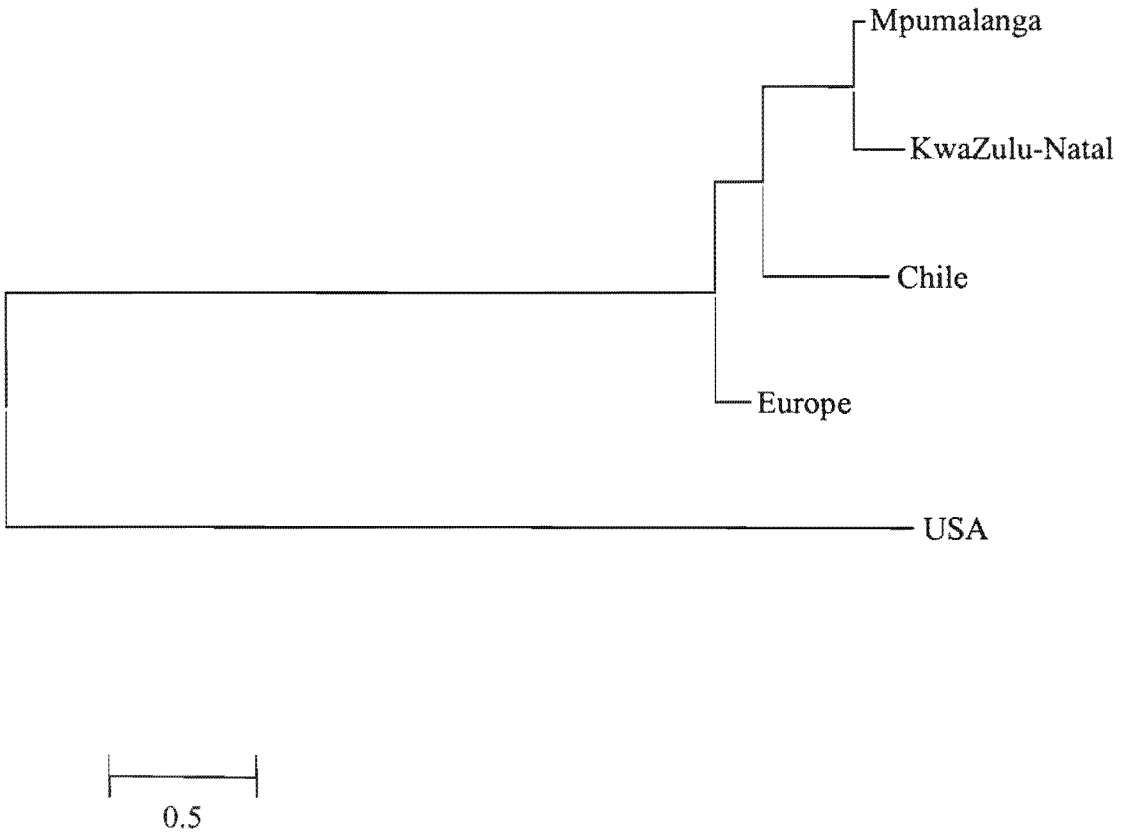
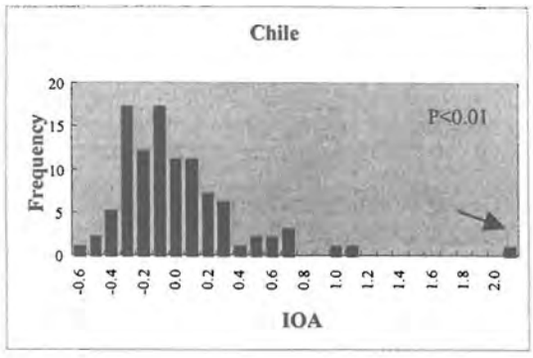
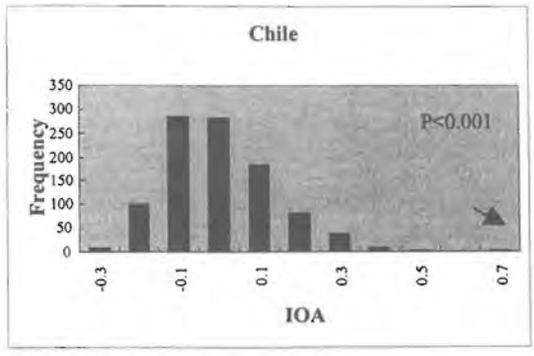
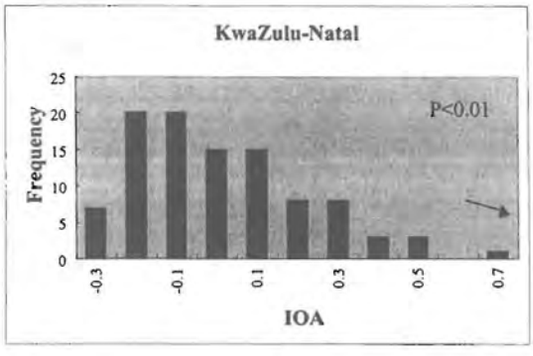
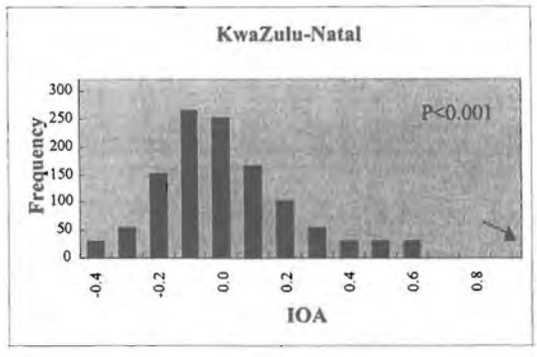


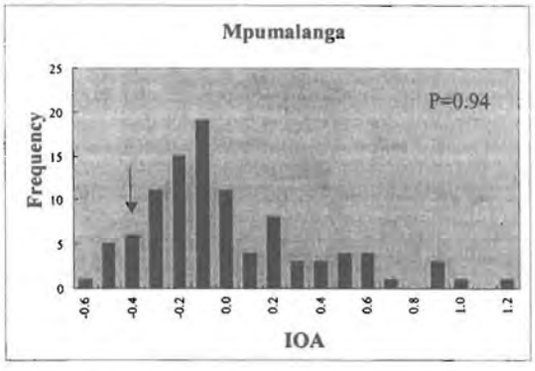
Figure 3: Histograms representing the distribution range of randomly recombining populations using the index of association (I_A).

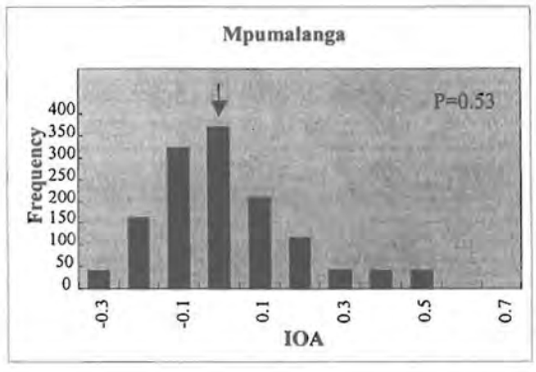


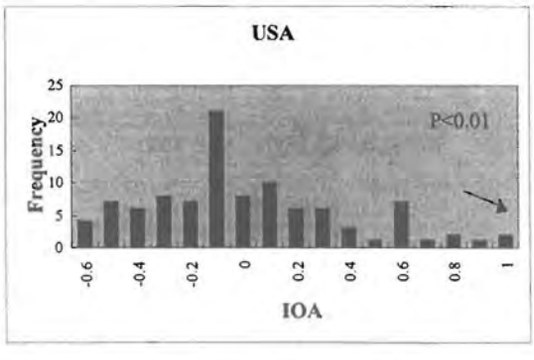


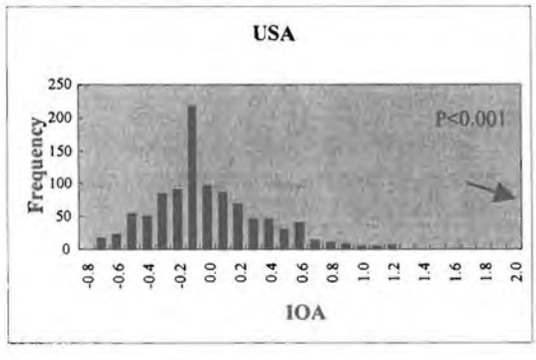












Clone corrected populations

Population including all isolates