

# High intercontinental migration rates and population admixture in the sapstain fungus *Ophiostoma ips*

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## Abstract

*Ophiostoma ips* is a common fungal associate of various conifer-infesting bark beetles in their native ranges and has been introduced into non-native pine plantations in the Southern Hemisphere. In this study, we used 10 microsatellite markers to investigate the population biology of *O. ips* in native (Cuba, France, Morocco and USA) and non-native (Australia, Chile and South Africa) areas to characterize host specificity, reproductive behaviour, and the potential origin as well as patterns of spread of the fungus and its insect vectors. The markers resolved a total of 41 alleles and 75 haplotypes. Higher genetic diversity was found in the native populations than in the introduced populations. Based on the origin of the insect vectors, the populations of *O. ips* in Australia would be expected to reflect a North American origin, and those in Chile and South Africa to reflect a European origin. However, most alleles observed in the native European population were also found in the native North American population; only the allele frequencies among the populations varied. This admixture made it impossible to confirm the origin of the introduced Southern Hemisphere (SH) populations of *O. ips*. There was also no evidence for specificity of the fungus to particular bark beetle vectors or hosts. Although *O. ips* is thought to be mainly self-fertilizing, evidence for recombination was found in the four native populations surveyed. The higher genetic diversity in the North American than in the European population suggests that North America could be the possible source region of *O. ips*.

**Keywords:** anthropogenic introduction, microsatellite marker, origin, population genetics, symbiosis

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## Introduction

Bark beetles (Coleoptera: Curculionidae, Scolytinae) consist of approximately 4100 species that typically colonize the phloem and cambial layers of trees, primarily in conifers (Wood & Bright 1992; Farrell *et al.* 2001). Many species are economically important forest pests, especially in conifers

in the Northern Hemisphere (NH). Although a large number of bark beetle species can infest and kill only stressed or dying trees and are considered secondary pests, some species aggressively attack and kill healthy trees and cause significant economic losses (Paine *et al.* 1997; Grégoire & Evans 2004).

*Pinus* and their bark beetle herbivores are native to the NH. Several European bark beetles have been introduced into North America and have become established there (Niemelä & Mattson 1996; Haack 2001). In contrast, far

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fewer forest insects have been introduced into Europe from North America (Niemelä & Mattson 1996). In the SH, various *Pinus* species have been introduced and are widely planted to sustain economically important forestry industries. During the course of the last 120 years, several bark beetle species have been accidentally introduced into these pine plantations (Wingfield *et al.* 2000).

Many bark beetle species are known to live in a close association with fungi, especially species of *Ophiostoma*, *Grosmannia* and *Ceratocystiopsis*, and their asexual states (Harrington & Cobb 1988; Wingfield *et al.* 1993; Jacobs & Wingfield 2001; Zipfel *et al.* 2006). These fungi are uniquely adapted to dissemination by insects. They possess sticky spores produced at the apices of stalked or long-necked fruiting structures that line the galleries and pupal chambers of the beetles. These spores are acquired by the insects in mycangia or on the exoskeleton before they emerge to infest new trees (Wingfield *et al.* 1993; Jacobs & Wingfield 2001; Six & Klepzig 2004). In some cases, mites phoretic on the beetles are also involved in vectoring these fungi (Klepzig *et al.* 2001).

*Ophiostoma ips* (Rumb.) Nannf. is a haploid ascomycete that has been reported to be pathogenic to conifers (Lieutier *et al.* 1989). More commonly, it causes sapstain on logs and freshly cut wood, imparting significant losses to the forestry industries (Rumbold 1931; Seifert 1993). The fungus is commonly associated with various bark beetles, including species of *Ips* and *Orthotomicus* (Rumbold 1931; Upadhyay 1981; Lieutier *et al.* 1989; Stone & Simpson 1990). *Ophiostoma ips* has been introduced into SH countries through the accidental introduction of various conifer-infesting bark beetles (Wingfield & Marasas 1980; Zhou *et al.* 2001, 2004a, 2005). These include the stem-infesting *Ips grandicollis*, which is native to North and Central America, and which has been introduced into Australia (Stone & Simpson 1990). Likewise, *Orthotomicus erosus*, which infests stems of *Pinus* spp., is native to Central and Southern Europe and countries around the Mediterranean Sea (Mendel & Halperin 1982), and has been introduced into South Africa (Tribe 1990). In addition, *O. ips* is commonly found in association with introduced populations of the root collar-infesting bark beetle, *Hylurgus ligniperda* in Chile (Zhou *et al.* 2004a)

and South Africa (Zhou *et al.* 2001). *Hylurgus ligniperda* is native to Europe (Swan 1942) and thus provides a common link for the origins of non-native conifer-infesting bark beetle in Chile and South Africa.

Very little is known regarding the biology of *O. ips*. Zhou *et al.* (2004b) showed that the fungus is homothallic (self-fertilizing) and that perithecia with viable ascospores are produced from its single haploid spore cultures. An intriguing question is whether *O. ips* might be used to study the history of forestry-related human mediated movement of its insect vectors. Because the fungus is exclusively spread by its bark beetle vectors, its history of introduction (as reflected in its population biology) in the exotic environment where the fungus has been introduced should also reflect that of its vectors. A small or single introduction of the insect vector together with the selection pressure of the novel environment would be indicated by low fungal population diversity (McDonald & Linde 2002).

In this study, we have analysed collections of *O. ips* isolated from various bark beetles (or their galleries) from trees located in both native pine ecosystems in the NH and from plantations of non-native pines in the SH. Genetic diversity and structure of these fungal populations are analysed using microsatellite markers specifically designed for *O. ips* (Zhou *et al.* 2002) in order to better understand the ecology, biology and origin of this fungus.

Specifically, we addressed the following questions: (i) how the genetic diversity between the geographical populations is structured; (ii) what the source population of *O. ips* is; (iii) how the global movement of the bark beetles affects the world-wide genetic diversity of the fungal associate; (iv) whether there is a relationship between insect vectors and fungal population structure; and (v) whether *O. ips* is self-fertilizing in nature or out-crossing.

## Materials and methods

### Fungal populations

A total of 248 isolates representing seven populations of *O. ips* collected from pine-infesting bark beetles and their galleries were analysed in this study (Table 1). Beetles were

**Table 1** Sampled populations of *Ophiostoma ips* included in this study

Country of origin	No. of isolates	Host	Insect vector
NSW, Australia	27	<i>Pinus radiata</i>	<i>Ips grandicollis</i>
Havana, Cuba	31	<i>P. caribaea</i>	<i>I. grandicollis</i>
Louisiana, USA	52	<i>P. taeda</i>	<i>I. grandicollis</i>
Middle Atlas Mountains, Morocco	31	<i>P. pinaster</i>	<i>Orthotomicus erosus</i>
Provence, France	30	<i>P. pinaster</i> , <i>P. halepensis</i>	<i>O. erosus</i>
Valdivia, Chile	20	<i>P. radiata</i>	<i>Hylurgus ligniperda</i>
Mpumalanga and KwaZulu-Natal, South Africa	57	<i>P. patula</i> , <i>P. elliottii</i>	<i>H. ligniperda</i> , <i>O. erosus</i>

squashed directly onto a selective medium (20 g Biolab malt extract, 20 g Biolab agar and 1000 mL deionized water, amended with 0.05% cycloheximide and 0.04% streptomycin). Galleries were carefully examined using a dissection microscope and spore masses were transferred to the selective medium. Cultures were incubated at 25 °C and purified by transferring mycelium from the edges of single colonies to fresh 2% MEA (20 g Biolab malt extract, 20 g Biolab agar and 1000 mL deionized water).

Sampling areas were defined based on accessibility of material and they were thus larger in South Africa than those from other countries. *Ophiostoma ips* was recovered from over 50% of beetles or galleries in all regions sampled. The ITS region of the rRNA of two to six randomly chosen isolates of *O. ips* from each population was sequenced as described by Zhou *et al.* (2004b) to confirm the identity of the isolates.

Populations of isolates from Australia (AUS) (27), Cuba (CUB) (31), and the United States of America (USA) (52) were collected from *Ips grandicollis*. This insect infests *Pinus radiata* in New South Wales where both the insect and the fungus have been introduced. In contrast, the insect infests native *Pinus taeda* in Louisiana and native *Pinus caribaea* in Havana (Table 1).

Isolates of *O. ips* from Middle Atlas Mountains of Morocco (MOR) (27) and the Provence region of France (FRA) (30) were from *Orthotomicus erosus* where the insect naturally infests *Pinus pinaster* and *Pinus halepensis*. The Chilean isolates (CHI) (20) were from *Hylurgus ligniperda* infesting *Pinus radiata*, both of which are exotic in that country (Table 1).

The South African (RSA) isolates originated from Mpumalanga (27) and KwaZulu-Natal (30). At both sites in RSA, the fungus was collected from the exotic pine-infesting bark beetle species, *Hylurgus ligniperda* and *Orthotomicus erosus*, during a two-year survey of *Ophiostoma* spp. associated with these beetles (Zhou *et al.* 2001). In Mpumalanga, the beetles had infested *Pinus patula*, whereas they were found infesting *Pinus elliottii* in KwaZulu-Natal (Table 1).

All cultures used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

#### DNA extraction, PCR, and genescan analysis

A haploid hyphal tip culture of each isolate was grown in 2% ME (20 g Biolab malt extract, and 1000 mL deionized water). DNA was extracted from the resulting cultures using PrepMan Ultra Sample reagent (Applied Biosystems) following the manufacturer's protocol. PCR was applied to all isolates using 10 of the 12 pairs of polymorphic fluorescent-labelled primers designed for *O. ips* (Zhou *et al.* 2002). Primer pairs of OI17/18 and OI27/28 used in Zhou *et al.* (2002) were not used since we failed to amplify

a number of isolates with them. The PCR reaction mixture (25 µL final volume) consisted of 1–2 ng DNA, 1X PCR reaction buffer, 2.5 mM MgCl<sub>2</sub>, 0.25 mM of each dNTP, 0.2 µM primer, and 1.25 U Super-Therm DNA Polymerase mixture (Hoffmann-La-Roche, US). PCR reactions were performed using an Eppendorf Mastercycler® Personal (Perkin-Elmer, Germany) with conditions similar to those described previously (Zhou *et al.* 2002) except that the annealing temperature was adjusted for some isolates. PCR products were visualized under UV illumination with 1% agarose gel.

Fluorescent-labelled PCR products were separated, and allele size was determined by comparing the mobility of the PCR products to that of TAMRA internal size standard, using GENESCAN® 2.1 and GENOTYPER® 3.0 analysis software (Perkin Elmer Corp.), as previously described (Zhou *et al.* 2002). For each isolate, a data matrix of multistate characters was compiled by assigning a different letter to each allele at each of the 10 loci (e.g. AABDCGDAFB), thus providing each isolate with a multilocus haplotype. Where two isolates had the same haplotype, they were considered to be clones. The Bonferroni correction was applied to significance levels of all  $\chi^2$  tests (Weir 1997).

#### Gene and genotypic diversity

Gene diversity was determined by allele frequencies at each locus, and gene diversity of each population was calculated using the formula,  $H = 1 - \sum_k x_k^2$ , where  $x_k$  is the frequency of the  $k^{\text{th}}$  genotype (Nei 1973), in POPGENE version 1.31 (<http://www.ualberta.ca/~fyeh/fyeh>). Gene diversity was calculated for both the whole and clone-corrected populations. Differences in allele frequencies for the 10 polymorphic loci across clone-corrected populations were calculated using contingency  $\chi^2$  tests (Workman & Niswander 1970). In the clone-corrected dataset each haplotype in a population was represented only once. This was done to ensure that estimates of allele frequencies are not biased by over-representation of a few clones. Genotypic diversity was determined by the number and frequency of combinations of alleles at multiple loci, and genotypic diversity of the populations (including all isolates) was calculated using the formula,  $G = 1 / \sum [f_x(x/n)^2]$ , 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  $\hat{G} = G/N \times 100$ , where  $N$  is the population size (McDonald *et al.* 1994).

#### Genetic distance and assignment tests

The genetic distance,  $\delta\mu^2$ , between haplotypes from each population based upon number of repeats at each allele for

each of the 10 loci was calculated using the program MICROSAT (Minch *et al.* 1995) and neighbour-joining trees were constructed using Molecular Evolutionary Genetic Analyses (MEGA) Version 3.1 (Kumar *et al.* 2004).  $\delta\mu^2$  is the square of the mean distance between two populations and was developed specifically for microsatellite data, assuming single-step mutations (Goldstein *et al.* 1995).

Assignment tests were conducted in Doh (Brzustowski, J. 'Doh assignment test calculator'. Available online at <http://www2.biology.ualberta.ca/jbrzusto/Doh.php>). This calculator takes genotypes (or haplotypes) of individuals from several populations and determines the population from which each individual is most likely to have come. This is achieved using the assignment index, which is the highest probability that the haplotype of an individual would occur in any of the populations (Paetkau *et al.* 1995). The NH populations were treated as native and theoretically assigned to a group based on their area of origin, while the SH populations were considered introduced and were not assigned. Thus, if there had been no mixture, each of the NH haplotypes would remain assigned to the same group and each of the introduced haplotypes would be assigned to the NH population from which it most probably originated. If admixture has occurred then haplotypes from all populations would be assigned to different groups.

### Population differentiation

In order to evaluate the level of differentiation between clone-corrected populations,  $\theta$ -values were calculated in MULTILOCUS version 1.2 (<http://www.bio.ic.au.uk/evolve/software/multilocus>).  $\theta$  is 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  $\theta$  equal to 0 indicates no population differentiation, while a value of  $\theta$  equal to 1 indicates no shared alleles between two populations. The significance of  $\theta$  was determined by comparing the observed value to that of 1000 randomized datasets in which individuals were randomized across populations. The null hypothesis that no population differentiation had occurred, can be rejected where the  $P$ -value is significant.

### Mode of reproduction

The Index of Association ( $I_A$ ) (Smith *et al.* 1993) was used to determine the mode of reproduction for each of the NH clone-corrected populations. The tests were performed on the 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 1000 calculated randomly recombining datasets.

Recombination within the population was then determined, comparing the observed data to the distribution range of the recombined data (Taylor *et al.* 1999). If the observed data fall within the distribution range, the population could be considered to be undergoing recombination, and *vice versa*.

## Results

### Isolate identities

Morphological characteristics and comparisons of ITS sequences obtained from 22 selected isolates with that of the ex-type culture of *O. ips* (CMW7075, AY546704) and those of other species in the *O. ips* complex (Zhou *et al.* 2004b) indicated that the obtained isolates represent *O. ips* (tree not shown).

### Genetic diversity

Loci of the 248 isolates of *O. ips* included in this study were amplified using 10 pairs of microsatellite markers. This gave a total of 41 alleles across the 10 loci examined (Table 2). Twenty-nine alleles were detected from the isolates from USA, 24 from those from Cuba, 22 from those from France, 21 from those from Morocco, 16 from those from Australia, 12 from those from Chile and 13 from those from South Africa. Monomorphic loci were observed in all populations, but made up a particularly large proportion of loci in the introduced populations from South Africa and Chile (Table 2). Unique alleles were observed in all of the native NH populations but not in any of the three introduced SH populations (Table 2).

The estimated gene diversity of the whole populations from the microsatellite profiles of isolates varied greatly (Table 2). The highest levels of gene diversity were found in three native populations from Cuba, USA, and France ( $H = 0.270, 0.243, 0.204$ , respectively). Moderately high gene diversity was observed for the native population from Morocco ( $H = 0.173$ ) and the introduced population from Australia ( $H = 0.161$ ), while the lowest diversity was observed for the introduced populations from Chile and South Africa ( $H = 0.087, 0.088$ , respectively).

The genotypic diversity estimated from the microsatellite profiles of isolates varied greatly among populations (Table 2). The highest maximum genotypic diversity ( $\hat{G} = 46.5\%$ ) was found in the native Cuban population, while the lowest ( $\hat{G} = 2.4\%$ ) was observed for the introduced South African population (Table 2).

### Genetic distance and assignment tests

A total of 93 haplotypes were identified among the 248 isolates of *O. ips* studied. These were distributed across

**Table 2** Allele size (bp) and frequency at 10 loci for *Ophiostoma ips* populations collected from France (FRA), Morocco (MOR), Cuba (CUB), United States of America (USA), Australia (AUS), Chile (CHI) and South Africa (RSA)

Locus	Allele size	FRA	MOR	CUB	USA	AUS	CHI	RSA
OI1	333	—	—	0.07	—	—	—	—
	336	—	—	0.03	0.02	—	—	—
	338	0.03	—	—	—	—	—	—
	339	0.97	1.00	0.90	0.98	1.00	1.00	1.00
OI3	204	—	—	—	0.04	0.04	—	—
	205	0.97	0.97	0.61	0.84	0.96	1.00	1.00
	206	—	0.03	0.10	0.04	—	—	—
	207	0.03	—	0.19	0.02	—	—	—
	211	—	—	0.10	—	—	—	—
	212	—	—	—	0.06	—	—	—
OI5	440	0.03	0.03	0.10	—	—	—	0.09
	444	0.23	—	0.19	0.02	—	—	—
	445	0.67	0.65	0.71	0.85	1.00	0.55	0.91
	446	—	—	—	0.13	—	—	—
	453	0.07	0.32	—	—	—	0.45	—
OI7	317	1.00	1.00	0.97	0.94	1.00	1.00	1.00
	318	—	—	0.03	—	—	—	—
	320	—	—	—	0.06	—	—	—
OI9	215	—	0.13	—	—	—	—	—
	216	0.03	—	—	—	0.33	—	—
	221	—	0.52	1.00	0.08	—	—	—
	222	0.97	0.35	—	0.90	0.67	1.00	1.00
	223	—	—	—	0.02	—	—	—
OI13	339	—	—	0.06	—	—	—	—
	341	0.30	0.10	—	0.06	1.00	—	—
	342	0.70	0.90	0.94	0.94	—	1.00	1.00
OI19	222	0.03	—	—	—	—	—	—
	229	0.17	0.03	—	0.11	—	—	0.14
	230	0.77	0.94	1.00	0.78	0.22	—	—
	231	0.03	0.03	—	0.11	0.78	1.00	0.86
OI23	177	—	—	0.10	—	—	—	—
	180	—	—	—	0.02	—	—	—
	181	0.20	0.97	0.67	0.67	0.89	—	—
	182	0.80	0.03	0.23	0.31	0.11	1.00	1.00
OI25	353	0.87	0.90	0.42	0.60	0.59	0.75	0.39
	362	0.13	0.10	0.58	0.40	0.41	0.25	0.61
OI31	314	—	0.03	0.07	0.02	—	—	—
	316	—	—	—	0.02	—	—	—
	317	1.00	0.97	0.90	0.92	0.96	1.00	1.00
	318	—	—	—	0.04	0.04	—	—
	321	—	—	0.03	—	—	—	—
N		30	31	31	52	27	20	57
No. of alleles		22	21	24	29	16	12	13
No. of unique alleles		2	1	6	6	0	0	0
H		0.204	0.173	0.270	0.243	0.161	0.087	0.088
No. of polymorphic loci		8	8	8	10	6	2	3
N(h)		15	14	20	25	10	4	5
G		3.13	8.74	14.43	15.07	5.53	3.08	1.39
$\hat{G}$		10.1	28.2	46.5	29.0	20.5	15.4	2.4

N = Number of isolates.

H = Gene diversity of the population (Nei 1973).

N(h) = Number of multilocus haplotypes.

G = Genotypic diversity (Stoddart &amp; Taylor 1988).

 $\hat{G}$  = G/N% = percent maximum diversity.

	Haplotypes	Group 1	Group 2	Group 3	Group 4
France	15	10 (67%)	1	3	1
Morocco	14	1	10 (71%)	2	1
Cuba	20	0	1	16 (80%)	3
USA	25	4	2	5	14 (56%)
Australia	10	6	2	0	2
Chile	4	2	0	0	2
South Africa	5	0	1	2	2
Total	93	23	17	28	25

**Table 3** Assignment of haplotypes from the four native and three introduced populations into four groups based upon the location of the native populations. The relationship between haplotypes assigned to each group can be seen in Fig. 1

populations as follows: USA (25), Cuba (20), France (15), Morocco (14), Australia (10), South Africa (5), and Chile (4) (Table 2). In a distance tree, the haplotypes of *O. ips* from the geographically adjacent and distant populations were distributed throughout the tree (Fig. 1). Nine haplotypes were also shared among populations (Fig. 1). For example haplotypes CHI3, RSA5, FRA15, CUB17 and USA 21 were identical even though they represent collections from four continents. Thus, the final number of unique haplotypes was 75 (Fig. 1).

Assignment tests based upon the four NH populations also emphasized the admixture of haplotypes from different countries and continents. The assignment tests grouped haplotypes with similar allele frequencies regardless of their origin. Thus, for the haplotypes originating in France, 67% were assigned to Group 1 together with one haplotype from Morocco, three from Cuba and one from USA (Table 3, Fig. 1). Similarly, for haplotypes originating in the USA, four were assigned to Group 1, two to Group 2, five to Group 3, leaving 14 haplotypes that were assigned to Group 4 (Table 3). Haplotypes from the introduced populations in the SH also had no clear origin. Six haplotypes from Australia and two from Chile were assigned to Group 1, two haplotypes from Australia and one from South Africa were assigned to the Group 2, two haplotypes from South Africa were assigned to the Group 3, while two haplotypes from Australia, two haplotypes from Chile and two haplotypes from South Africa were assigned to the Group 4 (Table 3). Because the native populations were an admixture of haplotypes, the assignment of haplotypes to a particular country (Group) does not imply that they originated from that country.

### Population differentiation

Only five haplotypes were found among the 57 isolates from South Africa. All of these were present in the 30 isolates from KwaZulu-Natal (*Pinus elliottii*), while four haplotypes were represented in the 27 isolates from Mpumalanga (*Pinus patula*). There was no pattern of association between the haplotype of *O. ips* and their insect

**Table 4** Contingency  $\chi^2$ -tests for differences in allele frequencies for 10 microsatellite loci across clone-corrected populations of *Ophiostoma ips* collected from (A) all bark beetle species in France (FRA), Morocco (MOR), Cuba (CUB), United States of America (USA), Australia (AUS), Chile (CHI) and South Africa (RSA); (B) *Ips grandicollis* (USA, CUB and AUS); (C) *Orthotomicus erosus* (FRA, MOR and RSA) and (D) combined populations from *Ips grandicollis* (USA, CUB and AUS) compared with combined populations from *Orthotomicus erosus* (FRA, MOR and RSA)

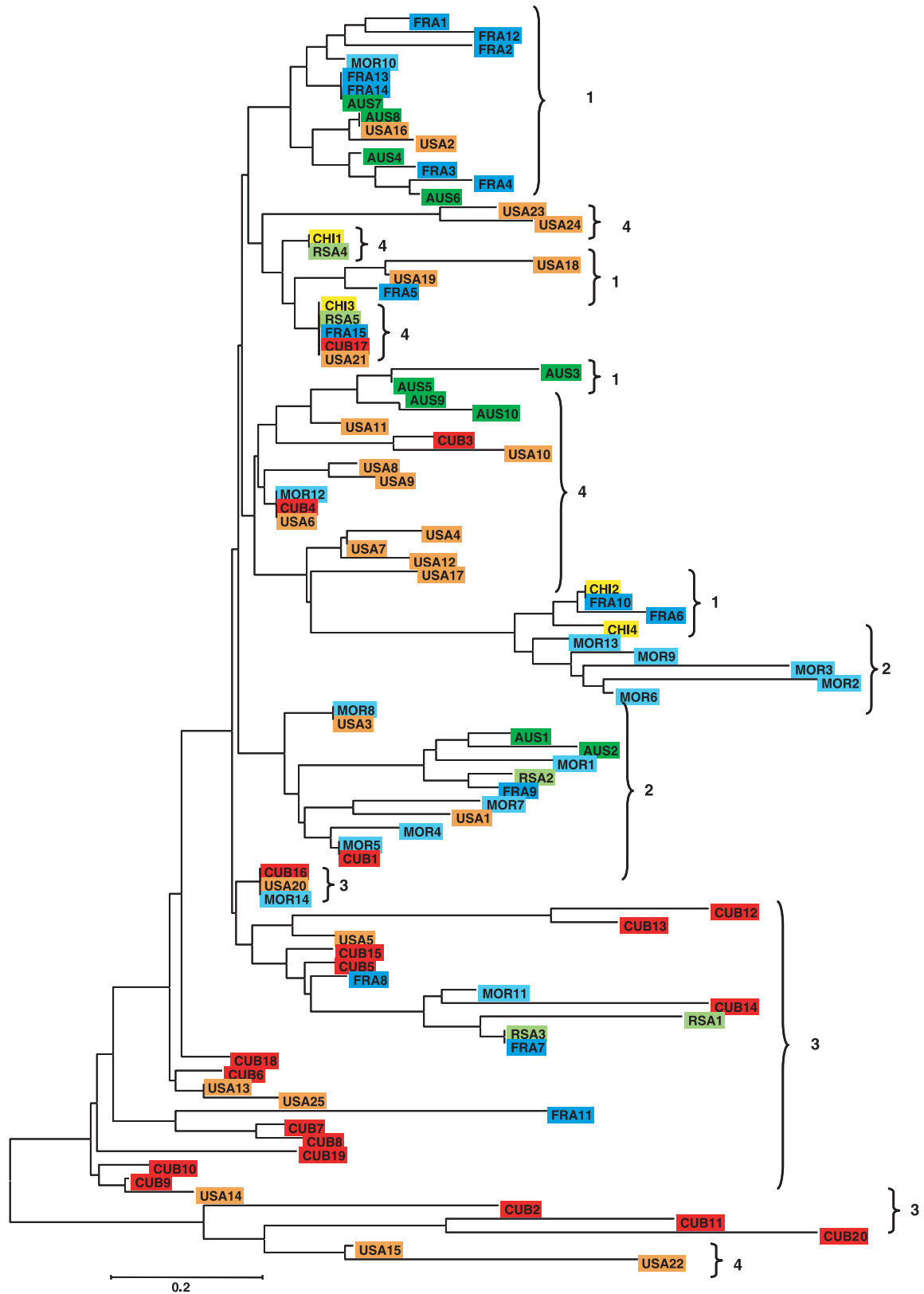
Locus†	(A)		(B)		(C)		(D)	
	$\chi^2$	Df	$\chi^2$	d.f.	$\chi^2$	d.f.	$\chi^2$	d.f.
OI1	15.0	18	3.2	4	1.3	2	4.1	3
OI3	45.0	36	14.2	10	2.7	4	8.4	6
OI5	68.5*	24	9.8	6	15.1	6	20.0*	4
OI7	12.1	14	5.3	4	—	—	2.6	2
OI9	55.0*	24	9.6	6	23.4*	6	7.7	3
OI13	59.2*	14	43.1*	4	7.8	2	1.8	2
OI19	41.1*	18	18.1*	4	5.4	9	6.0	3
OI23	39.0*	18	4.7	6	15.6*	2	4.0	3
OI25	3.7	7	0.4	2	0.7	2	2.1	1
OI31	15.1	24	4.6	8	1.5	2	3.7	

†significant ( $P < 0.05$ )  $\chi^2$  values are indicated by asterisks.

vectors, *Hylurgus ligniperda* and *Orthotomicus erosus*, hosts or geographical origin.

Chi-square tests indicated significant differences in allele frequencies at five loci when comparing across all populations (Table 4). Allele frequencies differed significantly at two loci when comparing populations from *Ips grandicollis* (CUB, USA and AUS) and two loci when comparing populations (RSA, FRA and MOR) from *Orthotomicus erosus* (Table 4). Interestingly when populations collected from the same bark beetle species were combined and compared, they were only significantly different at one locus (Table 4).

Most of the populations differed significantly from each other (Table 5). However, the  $\theta$ -values showed no population differentiation between USA and Cuban populations ( $\theta = 0.025$ ), and populations from Chile and South Africa



**Fig. 1** Neighbour-joining dendrogram of *Ophiostoma ips* showing the relationship between haplotypes from France (FRA-blue), Morocco (MOR-light blue), Cuba (CUB-red), United States of America (USA-orange), Chile (CHI-yellow), Australia (AUS-green) and South Africa (RSA-light green). The populations (1–4) to which the different haplotypes were assigned in the assignment test are indicated.

	FRA	MOR	USA	CUB	AUS	RSA	CHI
FRA	—	0.201***†	0.101***	0.145***	0.184***	0.144*	0.133*
MOR		—	0.087**	0.135***	0.351***	0.292***	0.314***
USA			—	0.025	0.234***	0.123*	0.142*
CUB				—	0.307***	0.138*	0.165***
AUS					—	0.449***	0.502***
RSA						—	0.085
CHI							—

†For  $\theta$ , asterisks represent level of significance (\*\* $P < 0.001$ , \*\* $P < 0.01$ , \* $P < 0.05$ ), no stars indicate no significant differentiation between populations.

**Table 5** Pairwise comparisons of population differentiation ( $\theta$ , above the diagonal) among clone-corrected populations of *Ophiostoma ips* collected from France (FRA), Morocco (MOR), Cuba (CUB), United States of America (USA), Australia (AUS), Chile (CHI) and South Africa (RSA)

were almost identical ( $\theta = 0.085$ ) (Table 5). The low levels of population differentiation indicate that gene flow is high. Alternatively, the time since the two populations both belonged to a common gene pool is relatively short and differentiation (through genetic drift or mutation) has not yet occurred.

### Mode of reproduction

The presence of clones (isolates with the same multilocus haplotype) in all populations, both native and introduced, is evidence of either asexual reproduction or a homothallic sexual cycle in *O. ips*. The Index of Association ( $I_A$ ) was thus calculated on clone-corrected populations to determine whether all loci are linked or if there is underlying recombination. The  $I_A$  was not calculated for the introduced populations. This was because founder effects and the introduction of limited haplotypes (and thus alleles) will often give such populations the appearance of being clonal even when recombination is occurring. This is especially apparent in the South African population that possessed only five haplotypes among 57 isolates. For native populations of *O. ips* from Cuba, France, Morocco, and USA, the  $I_A$  of the observed data for the clone-corrected populations fell within that for the recombined dataset (Fig. 2). This indicates that recombination has occurred within each of the populations.

### Discussion

In this study, we have analysed the population structure of *O. ips*, a self-fertilizing fungus that is associated with various pine-infesting bark beetle species. These beetles all originate from the NH and have been introduced into pine plantations in the SH. However, relatedness of isolates of the fungus was not consistent with the known origin of its insect vectors, and there was no phylogeographical structure for any of the *O. ips* populations sampled from five continents. This could be due to intercontinental transfer of wood material by humans, resulting in extensive gene flow between populations of *O. ips*.

### Population structure and migration

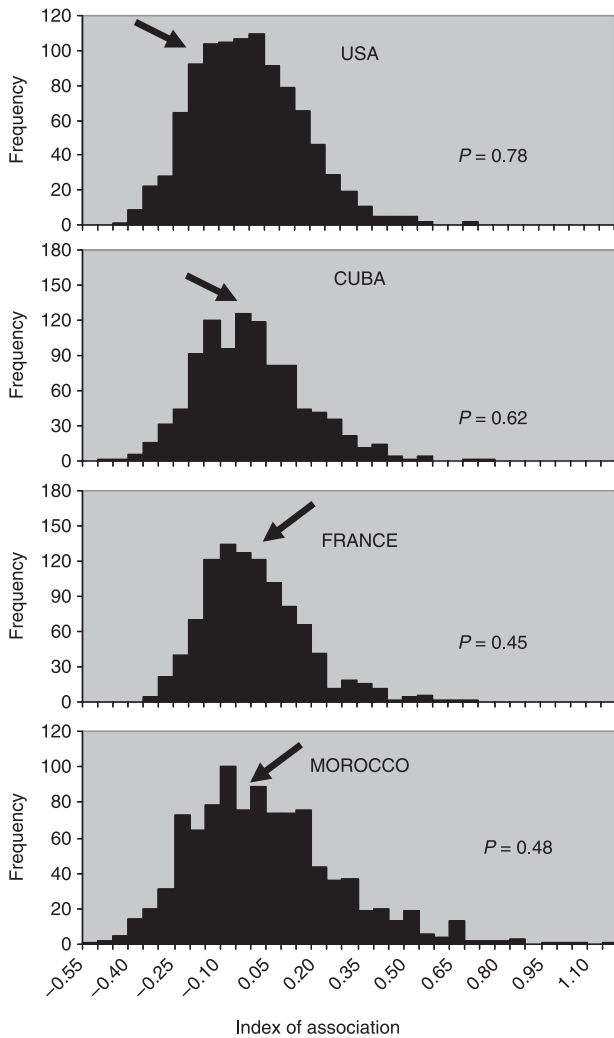
Populations of *O. ips* from North America had higher diversity than the European and North African populations. The first description of the fungus originated from the USA (Rumbold 1931). We collected populations from native vegetation in Cuba, France, Morocco, and USA in this study. Although the variability of the populations is also dependent on the effective population size over evolutionary time, higher diversity from Cuba and USA suggests that North America could possibly represent the origin of the fungus.

Genetic distance indicated that haplotypes from different geographical regions were intermixed. There was a tendency for some haplotypes from one region to group together (for example those from Cuba) but an equal proportion of the haplotypes were scattered throughout the dendrogram. This is indicative of admixture, a phenomenon generally attributed to anthropogenic disturbance of natural barriers to gene flow (Hughes *et al.* 2003; Wright *et al.* 2004). It appears most likely that European insect vectors introduced and established in North America (Niemelä & Mattson 1996; Haack 2001) have also introduced haplotypes, resulting in the admixture of the fungal populations. Assignment tests also support admixture and consequently the origin of introduced populations/haplotypes in the SH could not be determined. The significant population differentiation observed between most populations is particularly interesting and appears to be contrary to the concept of admixture. This emphasizes the fact that while the same alleles are present in all populations and the same multilocus haplotypes are observed on different continents, either selection in each of these populations or random genetic drift and genetic bottlenecks have a significant effect on these population structures. Thus, the allele frequencies (as measured by population differentiation) differ significantly.

### Relationship between host, beetles and fungal population structure

A comparison cannot be made between tree species in different countries as this is confounded by geographical





**Fig. 2** Histograms representing the distribution range of randomly recombining native populations of *O. ips* from USA, Cuba, France and Morocco using the Index of Association ( $I_A$ ). For all populations the  $I_A$  of the observed data falls within that of the randomized dataset indicating that recombination has occurred.

variation. With the exception of France and South Africa, the isolates of *O. ips* were collected from beetles on a different host tree species in each country. In South Africa there are two host trees and the same haplotypes were found on both of these. In France, isolates were not collected from the same host tree species at the same location. A comparison of haplotypes collected from the bark beetle *Ips grandicollis* with those from *Orthotomicus erosus* showed that the allele frequencies differed significantly at only one locus. Nine of the 75 haplotypes of *O. ips* were found on different beetle species on different continents. In South Africa the same haplotypes were found in isolates of *O. ips* from different bark beetle vectors and hosts. These results indicate no clear evidence of host

tree or insect specialization influencing the evolution and genetic change of *O. ips*. This is in contrast to the results of other studies where for example, the individual haplotypes of the fungus *Grosmannia clavigera* are distributed only through their specific association with a bark beetle vector (Six & Paine 1999). Thus, it seems as *O. ips* is a generalist fungus with low level of specificity to its vectors and that can move from one insect species to another when multiple beetles infest the same trees.

Very few haplotypes were observed among isolates of *O. ips* from South Africa. This finding is consistent with a founder effect indicating introductions with a very limited genetic base. The fungus was recorded in South Africa in 1980 (Wingfield & Marasas 1980) after its insect vector *Orthotomicus erosus* was discovered in the country in 1968 (Tribe 1990). The insect spread rapidly from the southwestern Cape Province throughout the country, and its associated fungus appears to have undergone very little change in this time period. Environmental selection pressure may also have had a role in perpetuating genetically-fit haplotypes, as shown by Burgess *et al.* (2004) for another exotic pine-infesting pathogen, *Diplodia pinea*.

Central to this study was the question as to whether there is an association between the origin of the bark beetle species and the origin of *O. ips* isolates for the introduced SH populations of the fungus. *Ophiostoma ips* in Australia is vectored by *Ips grandicollis*, which is native in the south-eastern USA and Central America. In Chile, *O. ips* is vectored by *Hylurgus ligniperda* and in South Africa the fungus is predominantly associated with *Orthotomicus erosus*. Both the latter insects are native to Europe (Swan 1942; Mendel & Halperin 1982; Neumann 1987). Thus, one would expect the populations of *O. ips* in Australia to reflect a North American origin and those in Chile and South Africa to reflect a European origin. In this regard, results of this study are inconclusive. At two loci, rare alleles only found in the North American population were present in the Australian population suggesting that this must be the source. But at another locus, an allele found only in France has a relatively high frequency in the Australian population. Most alleles found in the RSA and Chile populations are common to all populations. However, at one locus, a common allele in the Chile population is found only in the European populations.

There was no differentiation between populations of *O. ips* isolates from Chile and South Africa, and they were virtually identical. One explanation for this result would be that the relatively recent introduction of insect vector, *Hylurgus ligniperda* into Chile (Ciesla 1988) was probably from South Africa, where it has been present for more than 38 years. Alternatively, the European source of origin of this insect in South Africa and Chile might have been the same, resulting in genetically similar populations of the fungus in the two countries.

### Reproductive biology

Index of association tests for clone-corrected populations showed that recombination has occurred in *O. ips* in all four native populations surveyed. This result is interesting because the fungus is known to be homothallic (Zhou *et al.* 2004b) and thus inbreeding depression would be expected (Taylor *et al.* 1999). However, out-crossing can occur in homothallic fungi although this might not be the overall trend (Marra & Milgroom 2001). The survey by Taylor *et al.* (1999) showed that even mitosporic taxa (taxa with no known sexual cycle) exhibited the footprint of recombination in their population structure. Our results support their view that it is advantageous for the fungi to maintain sexual recombination as a life-history strategy (Taylor *et al.* 1999).

The microsatellite markers used in this study represent a powerful tool for studying population structure of the bark beetle-vectored fungus, *O. ips*. The remarkable lack of correlation between genetic and geographical distance indicates that there is no evidence of host or insect specialization influencing the genetic change in *O. ips*, but rather that there has been extensive intercontinental gene flow in this fungus. Most alleles observed in the European population were also found in the North American population, and this could be due to multiple introductions of European vectors to North America (Haack 2001), or *vice versa*. More detailed comparisons would be needed to confirm this hypothesis. Higher genetic diversity in the North American population than in the European population also suggests that North America would be the possible original source of *O. ips*.

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### References

Burgess TI, Wingfield MJ, Wingfield BD (2004) Global distribution of *Diplodia pinea* genotypes revealed using simple sequence repeat (SSR) markers. *Australian Plant Pathology*, **33**, 513–519.  
 Ciesla WM (1988) Pine bark beetles: a new pest management challenge for Chilean foresters. *Journal of Forestry*, **86**, 27–31.  
 Farrell BD, Sequeira AS, O'Meara BC, Normark BB, Chung JH, Jordal BH (2001) The evolution of agriculture in beetles (Curculionidae: Scolytinae and Platypodinae). *Evolution*, **55**, 2011–2027.  
 Goldstein DB, Ruiz-Linares A, Cavalli-Sforza LL, Feldman MW

(1995) An evaluation of genetic distances for use with microsatellite loci. *Proceedings of the National Academy of Science USA*, **92**, 6723–6727.  
 Grégoire JC, Evans HF (2004) Damage and control of BAWBILT organisms; an overview. In: *Bark and Wood Boring Insects in Living Trees in Europe, a Synthesis* (eds Lieutier F, Day KR, Battisti A, Grégoire JC, Evans HF), pp. 19–37. Kluwer Academic Publishers, Dordrecht.  
 Haack RA (2001) Intercepted Scolytidae (Coleoptera) at U.S. ports of entry: 1985–2000. *Integrated Pest Management Reviews*, **6**, 253–282.  
 Harrington TC, Cobb FW (1988) *Leptographium* root diseases on conifers. American Phytopathological Society Press, St. Paul, Minnesota.  
 Hughes J, Goudkamp K, Hurwood D, Hancock M, Bunn S (2003) Translocation causes extinction of a local population of the freshwater shrimp *Paratya australiensis*. *Conservation Biology*, **17**, 1007–1012.  
 Jacobs K, Wingfield MJ (2001) *Leptographium* species – tree pathogens, insect associates and agents of blue-stain. American Phytopathological Society Press, St. Paul, Minnesota.  
 Klepzig KD, Moser JC, Lombardero FJ, Hofstetter RW, Ayres MP (2001) Symbiosis and competition: complex interactions among beetles, fungi and mites. *Symbiosis*, **30**, 83–96.  
 Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. *Briefings in Bioinformatics*, **5**, 150–163.  
 Lieutier F, Yart A, Garcia J *et al.* (1989) Champignons phytopathogènes associés à deux coléoptères Scolytidae du pin sylvestre (*Pinus sylvestris* L.) et étude préliminaire de leur agresseivité envers l'hôte. *Annales Des Sciences Forestieres*, **46**, 201–216.  
 Marra RE, Milgroom MG (2001) The mating system of the fungus *Cryphonectria parasitica*: selfing and self-incompatibility. *Heredity*, **86**, 134–143.  
 McDonald BA, Linde C (2002) Pathogen population genetics, evolutionary potential, and durable resistance. *Annual Review of Phytopathology*, **40**, 349–379.  
 McDonald BA, Miles J, Nelson LR, Pettway RE (1994) Genetic variability in nuclear DNA in field populations of *Stagonospora nodorum*. *Phytopathology*, **84**, 250–255.  
 Mendel Z, Halperin J (1982) The biology and behaviour of *Orthotomicus erosus* in Israel. *Phytoparasitica*, **10**, 169–181.  
 Minch E, Ruiz-Linares A, Goldstein D, Feldman M, Cavalli-Sforza LL (1995) MICROSAT: a computer program for calculating various statistics on microsatellite data version 1.4 d. Stanford University, Stanford, California.  
 Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences USA*, **70**, 3321–3323.  
 Neumann FG (1987) Introduced bark beetles on exotic trees in Australia with special reference to infestations of *Ips grandicollis* in pine plantations. *Australian Forestry*, **50**, 166–178.  
 Niemelä P, Mattson WJ (1996) Invasion of North American forests by European phytophagous insects. Legacy of European crucibles? *Bioscience*, **46**, 742–753.  
 Paetkau D, Calvert W, Sterling I, Strobeck C (1995) Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology*, **4**, 347–354.  
 Paine TD, Raffa KF, Harrington TC (1997) Interactions among Scolytid bark beetles, their associated fungi, and live host conifers. *Annual Review of Entomology*, **42**, 179–206.

- Rumbold CT (1931) Two blue-staining fungi associated with bark-beetle infestation of pines. *Journal of Agricultural Research*, **43**, 847–873.
- Seifert KA (1993) Sapstain of commercial lumber by species of *Ophiostoma* and *Ceratocystis*. In: *Ceratocystis and Ophiostoma Taxonomy, Ecology and Pathogenicity* (eds Wingfield MJ, Seifert KA, Webber JF), pp. 141–151. American Phytopathological Press, St. Paul, Minnesota.
- Six DL, Klepzig K (2004) Bark beetle-fungal symbiosis: context dependency in complex associations. *Symbiosis*, **37**, 189–205.
- Six DL, Paine TD (1999) Allozyme diversity and gene flow in *Ophiostoma clavigerum* (Ophiostomatales: Ophiostomataceae), the mycangial fungus of the Jeffrey pine beetle, *Dendroctonus jeffreyi* (Coleoptera: Scolytidae). *Canadian Journal of Forest Research*, **29**, 324–331.
- Smith JM, Smith NH, O'Rourke M, Spratt BG (1993) How clonal are bacteria? *Proceedings of the National Academy of Sciences USA*, **90**, 4384–4388.
- Stoddart JA, Taylor JF (1988) Genotypic diversity: estimation and prediction in samples. *Genetics*, **118**, 705–711.
- Stone C, Simpson JA (1990) Species associations in *Ips grandicollis* galleries in *Pinus taeda*. *New Zealand Journal of Forestry Science*, **20**, 75–96.
- Swan DC (1942) The bark beetle — *Hylastes ater* Payk. (Coleoptera: Scolytidae) attacking pines in South Australia. *Journal of Agriculture*, **12**, 86–90.
- Taylor JW, Jacobson DJ, Fisher MC (1999) The evolution of asexual fungi: reproduction, speciation and classification. *Annual Review of Phytopathology*, **37**, 197–246.
- Tribe GD (1990) Phenology of *Pinus radiata* log colonization and reproduction by the European bark beetle *Orthotomicus erosus* (Wollaston) (Coleoptera: Scolytidae) in the south-western Cape Province. *Journal of the Entomological Society of Southern Africa*, **2**, 117–126.
- Upadhyay HP (1981) A monograph of *Ceratocystis* and *Ceratocystiopsis*. University of Georgia Press, Athens.
- Weir BS (1997). *Genetic Data Analysis II*. Sinauer Associates, Sunderland, MA.
- Wingfield MJ, Marasas WFO (1980) *Ceratocystis ips* associated with *Orthotomicus erosus* (Coleoptera: Scolytidae) on *Pinus* spp. in the Cape Province of South Africa. *Phytophylactica*, **12**, 65–69.
- Wingfield MJ, Seifert KA, Webber JF (1993) *Ceratocystis and Ophiostoma: Taxonomy, Ecology and Pathogenicity*. American Phytopathological Society Press, St. Paul, Minnesota.
- Wingfield MJ, Slippers B, Zhou XD *et al.* (2000) Global spread of insect-associated fungi on exotic plantation pines. XXI IUFRO World Congress, Kuala Lumpur, Malaysia, 7–12 August 2000. *IUFRO World Series*, **11**, 107–114.
- Wood SL, Bright DE (1992) A catalog of Scolytidae and Platypodidae (Coleoptera), Part 2: Taxonomic index. *Great Basin Naturalist Memoirs*, **A 13**, 1–6.
- Workman PL, Niswander JD (1970) Population studies in Southwestern Indian tribes. II. Local genetic variation in the Papago. *American Journal of Human Genetics*, **22**, 24–49.
- Wright SI, Lauga B, Charlesworth D (2004) Subdivision and haplotype structure in natural populations of *Arabidopsis lyrata*. *Molecular Ecology*, **12**, 1247–1264.
- Zhou XD, Burgess T, De Beer ZW, Wingfield BD, Wingfield MJ (2002) Development of polymorphic microsatellite markers for the tree pathogen and sapstain agent, *Ophiostoma ips*. *Molecular Ecology Notes*, **2**, 309–312.
- Zhou XD, De Beer ZW, Ahumada R, Wingfield BD, Wingfield MJ (2004a) *Ophiostoma* and *Ceratocystiopsis* spp. associated with two pine-infesting bark beetles in Chile. *Fungal Diversity*, **15**, 253–266.
- Zhou XD, De Beer ZW, Carnegie A *et al.* (2005) *Ophiostoma* spp. associated with the bark beetle, *Ips grandicollis*, on native and exotic *Pinus* spp. Proceedings of XXII IUFRO Congress 2005, Brisbane, Australia. *International Forestry Review*, **7**, 394.
- Zhou XD, De Beer ZW, Cibrián D, Wingfield BD, Wingfield MJ (2004b) Characterisation of *Ophiostoma* spp. associated with pine bark beetles from Mexico, including *O. pulvinisporum* sp. nov. *Mycological Research*, **108**, 690–698.
- Zhou XD, De Beer ZW, Wingfield BD, Wingfield MJ (2001) Ophiostomatoid fungi associated with three pine-infesting bark beetles in South Africa. *Sydowia*, **53**, 290–300.
- Zipfel RD, De Beer ZW, Jacobs K, Wingfield BD, Wingfield MJ (2006) Multigene phylogenies define *Ceratocystiopsis* and *Grosmannia* distinct from *Ophiostoma*. *Studies in Mycology*, **55**, 75–97.

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