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Single sequence repeat markers reflect diversity and geographic barriers in Eurasian populations of the conifer pathogen *Ceratocystis polonica*

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Summary

The blue-stain fungus and vascular stain pathogen *Ceratocystis polonica* and its associated bark beetle vectors, particularly *Ips typographus* and *I. typographus japonicus*, cause significant losses to several spruce species in Eurasia. Nothing is, however, known about the population genetics of this conifer pathogen. In this study, a set of single sequence repeat (SSR) markers were developed to determine the population structure and genetic diversity of *C. polonica* in Europe and Japan. ISSR-PCR primers were used to target SSR-rich regions and specific primers were designed flanking the SSR regions found in these amplicons. The SSR primers developed for *C. polonica* were found to be transferable to six other *Ceratocystis* species from conifers, residing in the *Ceratocystis coerulescens* complex. Ninety-eight isolates representing four populations of *C. polonica* (Austria, Norway, Poland and Japan) were tested using 10 selected polymorphic SSR markers. A high level of gene diversity was found in *C. polonica* as a whole (H = 0.53). Analysis of G statistics showed a low degree of population structure in Europe and a high level of gene flow between populations (Gst = 0.05, Nm = 8.5). In contrast, the Japanese and the European populations of *C. polonica* displayed strong genetic separation, which is likely caused by geographic isolation. The low level of population structure of *C. polonica* in Europe and the differentiation between the European and the Japanese fungal populations mirror previous findings for *I. typographus* and *I. typographus japonicus*, the main insect vectors of this fungus. These results support the view that the fungus and the insect have closely co-evolved together. This study also suggests that movement of *C. polonica* and its vectors between Europe and Asia pose a threat to forestry on both continents and this should clearly be avoided.

1 Introduction

Ceratocystis polonica (Siemaszko) C. Moreau is an economically important blue-stain fungus and vascular stain pathogen of various spruce species (*Picea* spp.) in Eurasia. This fungus is associated with the bark beetles *Ips typographus* L., *Ips amitinus* Eichh. and *Ips duplicatus* Sahlb. on Norway spruce (*Picea abies* [L.] Karst.) in Europe (Solheim 1986; Krokene and Solheim 1996; Kirisits 2004; Jankowiak 2005) and with *I. typographus* L. *japonicus* Niijima on Yezo spruce (*Picea jezoensis* [Sieb. & Zucc.] Carr.) in Japan (Yamaoka et al. 1997). The *I. typographus/C. polonica* complex represents a serious threat to Norway spruce in Europe, where it has caused immense losses to forestry (Christiansen and Bakke 1988). Large-scale outbreaks of *I. typographus,* where millions of spruce trees are killed, are well-known in Northern and Central Europe (Postner 1974;

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Christiansen and Bakke 1988; Führer 1996). Furthermore, *C. polonica* causes intensive and extensive blue-stain in the sapwood of bark beetle-infested spruce trees and logs, resulting in substantial economic losses, because of the downgraded blue-stained lumber (Christiansen and Bakke 1988).

Ceratocystis polonica was first isolated in Poland and described by Siemaszko (1939) as *Ophiostoma polonicum* Siemaszko. Considerable confusion has surrounded its generic placement (e.g. Upadhyay 1981; Solheim 1986). This has hinged largely on disputes relating to differences between the genera *Ophiostoma* and *Ceratocystis* (De Hoog and Scheffer 1984; Wingfield et al. 1993). It has subsequently been shown based on DNA sequence comparisons that despite their morphological similarities, these genera are phylogenetically unrelated (Hausner et al. 1993a,b; Spatafora and Blackwell 1994). They can also be easily separated based on morphology, where *Ophiostoma* spp. have anamorphs in genera such as *Leptographium*, *Pesotum* and *Sporothrix*, and *Ceratocystis* spp. have anamorphs in *Thielaviopsis* (De Hoog and Scheffer 1984; Wingfield et al. 1993; Jacobs and Wingfield 2001; Paulin-Mahady et al. 2002). The taxonomic confusion surrounding *C. polonica* emerged when this taxon was first described having a *Leptographium* state (Siemaszko 1939) and the species was thus treated in *Ophiostoma* for a long period of time (e.g. Solheim 1986). Visser et al. (1995), however, reported the presence of a *Chalara* (now *Thielaviopsis*, Paulin-Mahady et al. 2002) state in cultures of *O. polonicum* and this character, together with DNA sequence comparisons was used to show that it is a species of *Ceratocystis sensu stricto*.

Ceratocystis polonica forms part of the *Ceratocystis coerulescens* species complex. This group of fungi occurs predominantly on conifers and most species have ascospores surrounded by relatively uniform sheaths (Harrington and Wingfield 1998). *Ceratocystis polonica*, *Ceratocystis laricicola* Redfern & Minter and *C. rufipenni* M.J. Wingf., T.C. Harr. & H. Solheim are unusual species in that they are closely associated with specific bark beetle (Coleoptera, Scolytinae) vectors (Solheim 1986; Redfern et al. 1987; Wingfield et al. 1997; Kirisits 2004). This is unlike other *Ceratocystis* spp. that are vectored by non-specific insects such as flies (Diptera) and nitidulid beetles (Coleoptera, Nitidulidae) (Chang and Jensen 1974; Juzwik and French 1983). The bark beetle-associated species of *Ceratocystis* do not produce intensive fruity aromas and their relationship with insects is debated to be mutualistic (Paine et al. 1997; Harrington and Wingfield 1998; Kirisits 2004).

Ips typographus and its Asian relative, *I. typographus japonicus*, carry a wide array of fungi, most of which contribute to blue-stain (Solheim 1986; Yamaoka et al. 1997; Kirisits 2004; Jankowiak 2005). Amongst these, *C. polonica* is a common species in some areas of Europe and in Japan (Solheim 1986; Yamaoka et al. 1997; Kirisits 2004). It is also the most virulent fungus associated with *I. typographus* and *I. typographus japonicus* (Solheim 1988; Kirisits 1998; Yamaoka et al. 2000; Kirisits and Offenthaler 2002). Considerable debate has surrounded the importance of blue-stain fungi that are vectored by bark beetles (Harrington 1993; Paine et al. 1997; Kirisits 2004). In the case of *C. polonica*, there is substantial evidence that it is a pathogenic fungus contributing to the death of spruce trees infested by its insect vectors (Christiansen 1985; Solheim 1988; Kirisits 1998; Yamaoka et al. 2000; Kirisits and Offenthaler 2002).

Despite its importance and intriguing relationships with *I. typographus* and other *Ips* bark beetles, nothing is known regarding the population biology of *C. polonica*. In contrast, the phylogeography of *I. typographus* has been reasonably studied (Stauffer et al. 1999; Stauffer and Lakatos 2000; Sallé et al. 2007). Isolates of *C. polonica* associated with *I. typographus* in Europe and *I. typographus japonicus* in Japan are morphologically indistinguishable (Yamaoka et al. 1997), interfertile with each other (Harrington et al. 2002) and phylogenetically closely related (Harrington et al. 2002; Marin et al. 2005). This clearly indicates that European and Japanese isolates of the fungus

belong to the same species. It would, however, be intriguing to know how close different populations of *C. polonica* are genetically related and whether the population structure of the fungus parallels that of its principal insect vector, *I. typographus*.

Different DNA-based techniques have been used to study populations of various *Ceratocystis* species (Morin et al. 2004; Barnes et al. 2005; Engelbrecht et al. 2007). Single sequence repeat (SSR) markers have been developed for various fungi and applied in population studies (e.g. Burgess et al. 2001; Barnes et al. 2001b; Steimel et al. 2004). They would thus provide useful tools to consider questions relating to the population biology of *C. polonica*. In contrast to other techniques, they hold the advantage of being highly polymorphic, robust in PCR amplification, abundant and dispersed throughout most eukaryotic genomes (Hayden and Sharp 2001).

The aim of this study was to develop SSR markers to consider the population structure and genetic diversity of three populations of *C. polonica* from Europe and one from Japan. Microsatellite regions were sequenced to establish the source of polymorphisms between isolates. In addition, SSR primers developed for *C. polonica* were tested for amplification in 10 other species in the *C. coerulescens* complex.

2 Materials and methods

2.1 Isolates

Ninety-eight isolates representing four populations of *C. polonica* (Austria, Poland, Norway and Japan) and one isolate each from France and the Czech Republic were used in this study. These isolates originated from our own collections and those from various colleagues (Table 1). Most isolates of *C. polonica* from Europe were from Norway spruce infested by *I. typographus*; however, three isolates were obtained from Norway spruce infested by *I. amitinus* and a single isolate came from Scots pine (*Pinus sylvestris* L.) infested by the bark beetle *Tomicus minor* Hartig. The isolates from Japan were obtained from ascospores taken from perithecia occurring in the galleries of *I. typographus japonicus* on Yezo spruce in Hokkaido. Isolates from Austria, Poland and Norway originated from several localities at various parts of the respective countries (Table 1).

In addition to strains of *C. polonica*, 13 isolates representing species in the *C. coerulescens* complex (Table 2) were used to test the utility of SSR markers beyond *C. polonica*, for which these markers were developed. A single isolate of *Ceratocystis radicicola* (Bliss) C. Moreau was included as outgroup for testing primer amplification (Table 2). All isolates used in this study are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria.

2.2 Microsatellite screening

DNA extractions were performed as described by Barnes et al. (2001a). DNA from *C. polonica* isolate CMW7148 was amplified using the ISSR primers: NDV $(CT)_8$, DBD $(CCA)_5$, HV $(GT)_5G$, DBD $(CAC)_5$, HBDB $(GACA)_4$, HVH $(GTG)_5$, NDB $(CAC)_7C$ and DHB $(CGA)_5$. PCR amplification reactions were undertaken with single primers and with different primer pair combinations. PCR amplifications were conducted as described by Barnes et al. (2001b), but using an annealing temperature of 45°C.

The amplified products were purified with the High Pure PCR product purification kit (Roche Molecular Biochemicals, Mannheim, Germany) and cloned using the pGEM-T Easy Vector following the manufacturer's protocols (Promega Corp, Madison, WI, USA). Clones were grown overnight in Luria-Bertani broth medium containing 100 μg ml^{-1} of ampicillin (Sigma Chemicals Co, St Louis, MO, USA) for plasmid DNA extraction

Table 1. **Isolates of *Ceratocystis polonica* used in this study. If not otherwise indicated, isolates were obtained from *Picea abies* infested by *Ips typographus*.**

Isolate (CMW)[1,2]	Country	Province/locality	Collector/s
7149, 8813	Austria	Carinthia[3,4]	R. Grubelnik T. Kirisits
5002, 5075, 5102, 5112, 5119, 7133, 7134, 7135, 7144, 7145, 7146, 7148, 7754, 8812, 8792, 8818, 8829, 8844[13]	Austria	Lower Austria[3,5]	T. Kirisits R. Grubelnik
5026, 7143[14], 7147, 8821, 8825, 8827[14], 8828[14], 10838	Austria	Tyrol[3,6]	T. Kirisits
5011, 7140, 7141, 7749, 7753	Austria	Upper Austria[3,7]	R. Grubelnik T. Kirisits
7750	Austria	Styria[3]	R. Grubelnik T. Kirisits
8817	Austria	Burgenland[3]	R. Grubelnik T. Kirisits
7152	Austria	Salzburg[3]	R. Grubelnik T. Kirisits
10522	Poland	Unknown	W. Siemaszko
8291, 8293, 8295, 8296, 8297, 8298, 8299, 8300, 8302, 8303	Poland	Łopuszna[8]	R. Jankowiak
8287, 8288, 8289, 8290	Poland	Kopciowa[8]	R. Jankowiak
8304, 8306, 8307	Poland	Brenna[8]	R. Jankowiak
11092, 11094, 11105, 11112, 11114, 11120, 11125, 11127, 11129, 11136	Poland	Białowieza[9]	T. Kirisits
2210, 2272, 2273, 2284, 2285, 2286 8276, 8277, 8278[15]	Japan	Hokkaido	Y. Yamaoka
8085, 8088, 8089, 8091, 8092, 8830, 8874, 8875, 11074, 11075, 11078, 11081, 11082, 11083, 11084, 11085, 11086, 11087	Norway	Akershus[10]	H. Solheim
8873, 11079, 11080	Norway	Nord-Trøndelag[11]	H. Solheim
8872, 11076, 11077	Norway	Telemark[12]	H. Solheim
1165	Norway	Unknown	H. Solheim
7748	Czech Republic	Sumava	T. Kirisits R. Jakus
8831	France	Alsace	F. Lieutier

[1]CMW: Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.
[2]A portion of the isolates was investigated by Marin et al. (2005), where further collection information can be found.
[3]Austrian province.
[4]Isolates originated from two localities.
[5]Isolates originated from nine localities.
[6]Isolates originated from three localities.
[7]Isolates originated from one locality.
[8]Localities in the Beskids mountain range in Southern Poland (see Jankowiak 2005).
[9]Locality in North-Eastern Poland.
[10]Province in South-Eastern Norway.
[11]Province in North-Central Norway.
[12]Province in Southern Norway.
[13]Isolate obtained from *Pinus sylvestris* infested by *Tomicus minor*.
[14]Isolates obtained from *Picea abies* infested by *Ips amitinus*.
[15]Isolates obtained from galleries of *Ips typographus japonicus* on *Picea jezoensis* in Hokkaido (see Yamaoka et al. 1997).

Table 2. ***Ceratocystis*** **species tested with the** ***Ceratocystis polonica*** **SSR markers developed in this study.**

Isolate[1]	Species	Country	Host	Collector/s
CMW3185, CBS146.59	*Ceratocystis radicicola*	USA	Unknown	C. Moreau
CMW3230, CBS 140.37, C313	*C. coerulescens*	Germany	*Picea abies*	Unknown
CMW10523	*C. virescens*	USA	*Acer saccharum*	D. Houston
CMW1323, C490	*C. pinicola*	England	*Pinus sp.*	J. Gibbs
CMW3273, C708	*C. pinicola*	Norway	*Picea abies*	H. Solheim
CMW3255, C639	*C. eucalypti*	Australia	*Eucalyptus sieberi*	G. Kile
CMW3229, C278	*C. resinifera*	Norway	*Picea abies*	H. Solheim
CMW2332	*Thielaviopsis australis*	Australia	*Nothofagus cunninghamii*	M. Hall
CMW3270, C694	*T. neocaledoniae*	New Caledonia	*Coffea robusta*	E. Kiffer
CMW10524	*C. rufipenni*	Prince George	*Picea glauca*	H. Solheim
CMW10525 CBS556.97, C324	*C. douglasii*	USA	*Pseudtsuga menziesii*	R.W. Davidson
CMW4546	*C. laricicola*	Scotland	*Larix decidua*	T.Kirisits M.J. Wingfield D.B. Redfern
CMW7760 CBS109260 IF SA/II/2/1/5SHT	*C. laricicola*	Austria	*Larix decidua*	T. Kirisits
CMW8842 IF SA/I/3/3/7	*C. laricicola*	Austria	*Larix decidua*	T. Kirisits

[1]CMW, Culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; C, Culture collection of T.C. Harrington, Iowa State University, USA; IF, Culture collection of the Institute of Forest Entomology, Forest Pathology and Forest Protection (IFFF), Department of Forest and Soil Sciences, University of Natural Resources and Applied Life Sciences, Vienna (BOKU), Vienna, Austria.

(Sambrook et al. 1989). Purified plasmid DNA was digested with the restriction enzyme *Eco*RI (Roche Molecular Biochemicals) to screen for positive clones.

Plasmid insert sizes were determined using agarose gel electrophoresis and those larger than 500 bp were sequenced with the T7 and Sp6 universal primers using an ABI PRISM Big DYE Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). DNA sequences were determined with an ABI PRISM 377 DNA automated sequencer (Applied Biosystems).

2.3 SSR primer design and testing

Sequences were analysed for SSR regions using the program Sequence Navigator version 1.0.1 (Applied Biosystems). Specific primer pairs were designed to flank these regions and to amplify PCR fragments between 200 and 500 bp facilitating the GENESCAN analysis. When microsatellite regions were located at one of the ends of the insert, it was necessary to use the 'genome walking' technique described by Siebert et al. (1995) and Burgess et al. (2001) to obtain sequences from both sides of the microsatellite region.

All the primers designed were tested for the amplification of DNA from four *C. polonica* isolates (CMW2210, CMW7133, CMW7148 and CMW10522). PCR reactions were conducted as described by Barnes et al. (2001b) using annealing

Table 3. ***Ceratocystis polonica*** **SSR primer sequences and characteristics of microsatellite regions.**

SSR primers	Primer sequence	T_a (°C)[1]	SSR motif	Range of repeats[2]	No. of alleles	Genebank accession nos.[3]
P1-A	5′ CGC ATT CAC ATT GCC ACT TGC G 3′	60	TA	5–8	4	AY236093, AY236094, AY236090
P1-B	5′ CGT TAC TAG CGG GAG AGG CTG C 3′					AY236091, AY236095, AY236092
P3-A	5′ GGA TTT TCC TGC ACG AAG GTG G 3′	66	GAT	5–7	5	AY236107, AY236106, AY236102
P3-B	5′ CGG GCA TGG AAT TTG GTG TGG 3′					AY236103, AY236112, AY236104
P4-A	5′ CGA AGC GTC TCG ATA TAG CCT CG 3′	60	AAGC	3–6	8	AY236115, AY236120, AY236121
P4-B	5′ CCA CCA CCT TCA GTT ATC CTA CAC 3′		Region rich in T	6–9		AY236118, AY236126, - - - - - -
			Region rich in GA	47–63		
P5-A	5′ CTC CAT CCT CAA ACA TTG CCA G 3′	66	CAACAG	2–7	7	AY236133, AY236127, AY236130
P5-B	5′ CGC TAA GCT GTT CTG GGC GC 3′		Region rich in T	5–7		AY236131, AY236139, AY236132
P6-A	5′ CAT TCA CCG TCA GTG CCG CCG TAG G 3′	60	CAGAAAA	2–8	10	AY236145, AY236140, AY236143
P6-B	5′ GCA GAG CAA CGC TGA TGA AGG C 3′					AY236144, - - - - - -, AY236148[4]
P7-A	5′ GTC ACT TGT GCC CAT CGG TGC 3′	60	Region rich in A	47–73	9	AY237016, AY237017, AY237015
P7-B	5′ GAT ACG TTA CGG TCG CTG TGG C 3′					AY237014, AY237023, AY237011
P8-A	5′ CTG TCT GAG AGA ATG CAA CTG GG 3′	60	CTCTT	4–5	4	AY237030, AY237029, AY237027
P8-B	5′ CAG AAT GAG CGA GAG CAA TAG CG 3′		Region rich in T	8–12		AY237028, - - - - - -, AY237024
P9-A[5]	5′ CAC AGT TCA GAG TTG GAT TCC GG 3′	56	TA	2–4	?	AY237042, AY237045, AY237041
P9-B[5]	5′ GCT AAC TGA TGT AGA CAC ATC ATG CC 3′		Region rich in T	53–83		AY237037, - - - - - -, AY237038
P10-A	5′ CCA ATC TGG CGT CGT TTG ATT GC 3′	58	AC	5–12	5	AY237055, AY237054, AY237056
P10-B	5′ GGA AGT TAA GCG TCC ACC CAA C 3′		Region rich in T	7–12		AY237057, - - - - - -, AY237049
			Region rich in C	1–9		
P11-A	5′ GGG TGG ATG ATG GGA CTG TTA CGG 3′	58	GA	6–10	9	AY237069, AY237068, AY237064
P11-B	5′ CCA TCG CTT CCA CAG CAA GAC 3′		AC	5–11		AY237065, AY237073, AY237066
			Region rich in G	12–19		
P12-A	5′ CAT GAA ATC ATC AGA CGG AAG GG 3′	58	TC	6–8	4	AY237082, AY237081, AY237077
P12-B	5′ GGG AAG GAA AAT TGT ATT TGT CG 3′					AY237078, AY237087, AY237079
P13-A[5]	5′ TTG TTG CCA GAC GAT GAG AGT GC 3′	58	GA	7–8	?	AY237088, AY237096, AY237092
P13-B[5]	5′ GAC AAC CGC CGC TGC GAG C 3′					AY237089, AY237101, AY237094

[1]Optimal annealing temperature to PCR amplification of DNA from *C. polonica*.
[2]No. of SSR repeats for four *C. polonica* isolates (CMW7148, CMW7133, CMW2210 and CMW10522), one *C. pinicola* isolate (CMW3273) and two *C. laricicola* isolates (CMW4556 or CMW7760).
[3]Accession nos. are given in order for isolates: CMW7148, CMW7133, CMW2210, CMW10522, CMW3273 and CMW4546. Dashes indicate that there is no sequence deposited for that isolate.
[4]Accession no. for *C. laricicola* isolate CMW7760.
[5]Primer pairs not used in the population study, therefore the number of alleles for these loci were not determined.

temperatures between 56 and 66°C (Table 3). PCR products were purified and sequenced using both specific primers in separate reactions as described above. Sequences were aligned manually and analysed using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b (SWOFFORD 1998). Where polymorphisms between isolate sequences were found in the number of repeats of microsatellite motifs, one primer of each pair was labeled with either FAM or HEX fluorescent dye (MWG, Ebersberg, Germany) and these were used in analyses of the fungal populations.

2.4 Population biology

PCR amplifications using DNA from all the fungal isolates were conducted with each of 10 selected SSR primer pairs (Table 3). Fluorescent-labelled PCR products (approximately 2 ng of DNA) were mixed with 0.5 μl of the internal standard GENESCAN-TAMRA (Applied Biosystems), 1.5 μl of loading buffer and then separated by PAGE using an ABI Prism 377 DNA sequencer (Applied Biosystems). Allele size was determined using the software packages GENESCAN 2.1 and GENOTYPER 3.0 (Applied Biosystems).

Allele size was scored at each locus as present (1) or absent (0) for all isolates. Unweighted pair–group mean arithmetic (UPGMA) was used for cluster analysis using PAUP software (SWOFFORD 1998). The computer program POPGENE version 1.32 (YEH et al. 1999) was used to analyse the population data. Genetic diversity was quantified using measures of the number of polymorphic loci, allele frequencies and NEI's (1973) gene diversity. Population genetic differentiation was quantified by the Gst coefficient [Gst = (Ht − Hs)/Ht, where Hs is the average heterozygosity among organisms within random mating subpopulations and Ht is the average heterozygosity among organisms within the total area] (NEI 1987). Gene flow between populations was calculated by estimating the number of migrants (Nm) [Nm = 0.5(1 − Gst)/Gst] (MCDERMOTT and MCDONALD 1993). Distance and similarity measurements were calculated between population pairs according to NEI (1972). Mode of reproduction within each population was estimated through the gametic linkage disequilibrium (LD) between pairs of SSR loci (chi-squared test, $p < 0.05$) (WEIR 1979).

2.5 Utility of SSR primers on other species of *Ceratocystis*

The 12 SSR primer pairs developed for *C. polonica* were tested for PCR amplification in species belonging to the *C. coerulescens* complex and in *C. radicicola*, which was used as the outgroup species (Table 2). DNA extraction and PCR conditions were as described above. PCR products of both *C. laricicola* (CMW4546 or CMW7760) and *C. pinicola* (CMW3273) were also sequenced with SSR primers P4A-B, P5A-B, P6A-B, P10A-B and P11A-B and manually aligned with sequences obtained for *C. polonica*.

3 Results

3.1 Microsatellite screening

A total of 61 plasmid clones containing potential SSRs were sequenced. Twenty-two of these contained SSR regions, representing a success rate of 36%. Twelve of these regions were selected to design specific primers (Table 3). In six SSR regions, the microsatellite was found either at the beginning or end of the cloned PCR product. In these cases, it was necessary to amplify the adjacent DNA by genome walking.

3.2 SSR primer design and testing

Amplified loci contained SSR motifs with 2–7 nucleotides, and 4–12 repeats (Table 3). Some regions were rich in a specific nucleotide and were usually interrupted by short sequences of other nucleotides (Table 3). Such regions were selected because they showed high variability in the number of mononucleotides between isolates (Fig. 1). Six loci contained more than one SSR region, and these usually had higher numbers of alleles (Table 3).

Sixty-five alleles were obtained from the 10 selected SSR loci. The primer pair P6A-B amplified the most variable locus, for which 10 alleles were found. The primer pairs, P7A-B and P11A-B, amplified nine different alleles. The markers P1, P8 and P12 were the least variable, with four alleles present in all *C. polonica* isolates tested (Table 3).

3.3 Population biology

A high level of genetic diversity was found in *C. polonica* as a whole (H = 0.53), with the Austrian population being most genetically diverse (H = 0.51) and the Norwegian population least diverse (H = 0.39) (Table 4). All the loci studied were polymorphic across the four populations (Table 4). Allele frequency analyses showed that 14 alleles were amplified across all the populations (Austria, Norway, Poland and Japan), while 25 alleles were equally amplified in the three European populations. The Norwegian population did not contain unique alleles, while eight alleles were exclusively amplified in the Polish populations and six each in the Austrian and the Japanese populations.

G statistic analysis showed a low degree of population sub-structure in Europe (Gst = 0.05) and a high level of gene flow between populations (Nm = 8.5). The highest number of migrants per generation was found between the Austrian and the Polish populations (Nm = 13.6). In contrast, high population sub-division was found between the Japanese and the European populations, with Gst values equal to or higher than 0.2 and Nm lower than 2 in all cases (Table 4).

The UPGMA dendrogram (Fig. 2) illustrates the high level of genetic diversity for *C. polonica* and a low level of differentiation between the European populations, with most of the clusters occupied by isolates recovered from different countries. Most of the Japanese isolates grouped together in a separate clade. One of the Japanese isolates (CMW8278) was not present in this clade and grouped with isolates from Poland and Austria. Isolates CMW2286 from Japan and CMW7152 from Austria did not group in any of the two main clades.

Pairwise distance measures between populations indicated high similarity between the Austrian, Norwegian and the Polish populations (I ≥ 0.92) (Table 4). Distance data clearly illustrated the isolation of the Japanese population from the European populations, as the genetic identity index between these populations ranged between only 0.45 and 0.52 (Table 4). The largest genetic distance was found between the Norwegian and the Japanese populations (D = 0.79). The percentage of loci in disequilibrium ranged from 4.4% in the Polish population to 13% in the Japanese population (Table 4).

3.4 Utility of SSR primers on other species of *Ceratocystis*

Most of the SSR primer pairs developed for *C. polonica* amplified DNA from six other *Ceratocystis* spp. from conifers and belonging to *C. coerulescens* complex (Table 5). All 12 primer pairs amplified DNA from *C. laricicola*. Eleven primer pairs amplified DNA from *C. pinicola* T.C. Harr. & M.J. Wingf., *C. coerulescens* (Münch) Bakshi, *C. douglasii* (R.W. Davidson) M.J. Wingf. & T.C. Harr. and *C. rufipenni* and nine from *C. resinifera* T.C. Harr. & M.J. Wingf. Four primer pairs amplified DNA from *Ceratocystis eucalypti* Z.Q.

Primers P4A – P4B

```
C.polonica.Austria.CMW7133     GCGACGTGTAGAATTAGAGGCAAGCAAGC--------GGG
C.polonica.Japan.CMW2210       GCGACGTGTAGAATTAGAGGCAAGCAAGCAAGCAAGCGGG
C.polonica.Poland.CMW10522     GCGACGTGTAGAATTAGAGGCAAGCAAGC--------GGG
C.polonica.Austria.CMW7148     GCGACGTGTAGAATTAGAGGCAAGCAAGCAAGCAAGCGGG
C.pinicola.Norway.CMW3273      GCGACGTGTAGAATTAGAGGCAAGCAAGC--------GGG
```

Primers P5A – P5B

```
C.polonica.Austria.CMW7133      CAAGACATA-TTCAACAGCAACAGCAACAGCAACAGCAACAGCAACAGCAA------GCCGAAAAAAGCAATG
C.polonica.Japan.CMW2210        CAAGACATA-TTCAACAGCAACAGCAACAGCAACAGCAACAGCAA----------GCCGAAAAAAGCAATG
C.polonica.Poland.CMW10522      CAAGACATA-TTCAACAGCAACAGCAACAGCAACAGCAACAGCAACAGCAACAGCAAGCCGAAAAAAGCAATG
C.polonica.Austria.CMW7148      CAAGACATA-TTCAACAGCAACAGCAACAGCAACAGCAACAGCAACAGCAACAGCAAGCCGAAAAAAGCAATG
C.pinicola.Norway.CMW3273       CAAGACAT--T-CATCAGCAACAGCAA----------GCCGAAAAA-GCAATG
C.laricicola.Scotland.CMW4546   CAAGACATAAT-CATCAGCAACAACAACAGCAACAGCAACAGCAA----------GCCGAAAAA-GCAATG
```

Primers P6A – P6B

```
C.polonica.Austria.CMW7133     TCAAGACAGAAAACAGAAAACAGAAAACAGAAAACAGAAAACAGAAAACAGAAAACAGAAGAAAAAA---GTAAATATACCAGTGAATGTGTAGTAGAT
C.polonica.Japan.CMW2210       TCAAGACAGAAAACAGAAAACAGAAAACAGAA----------GAAAAAA---GTAAATATACCAGTGAATGTGTAGTAGAT
C.polonica.Poland.CMW10522     TCAAGACAGAAAACAGAAAACAGAAAACAGAAAACAGAAAACAGAA----------GAAAAAA---GTAAATATACCAGTGAATGTGTAGTAGAT
C.polonica.Austria.CMW7148     TCAAGACAGAAAACAGAAAACAGAAAACAGAAAACAGAA----------GAAAAAAAAAGTAAATATACCAGTGAATGTGTAGTAGAT
C.laricicola.Austria.CMW7760   TCAAGACAGAAAACAGAAAACAGAA----------GAAAAAA---GTAAATATACCAGTGAATGTGTAGTAGAT
```

Primers P10A – P10B

```
C.polonica.Austria. CMW7133          CAATG----------TAGGTAGACTAATGATTAGTTGAAAAAAATTGTAGGTAGACGTTGAATATACACCCACACACACACACACACACA----CT
C.polonica.Japan.CMW2210             CAATG----------TAGGTAGACTAATGATTAGTTGAAAAAAATTGTAGGTAGACGTTGAATATACA--CACACACACACACACACACACACT
C.polonica.Poland.CMW10522           CAATG----------TAGGTAGACTAATGATTAGTTGAAAAAAATTGTAGGTAGACGTTGAATATACACCCACACACACACACACACACA----CT
C.polonica.Austria.CMW7148           CAATG----------TAGGTAGACTAATGATTAGTTGAAAAAAATTGTAGGTAGACGTTGAATATACACCCACACACACACACACACACA----CT
C.laricicola.Scotland.CMW4546        CAATGATTAGTTGAGATAAAATGTAGGTAGAC----------GTTGAATATGCACCCACACACACACACACA--------CT
```

Primers P11A – P11B

```
C.polonica.Austria.CMW7133        TTATCTGTTCCTTAGCATGATGAGGGGAGGGAAGGGGGGGGGTATAGAGCAC(217b)-TGAACACACACACACACACACA------GAGAGAGAGAGA------GAAAA--TTAATG
C.polonica.Japan.CMW2210          TTATCTGTTCCTTAGCATGATGAGGGGAGGGAAGGGGGGGGGTATAGAGCAC(217b)-TGAACACACACACACACACACACACAGAGAGAGAGAGA------GAAAA--TTAATG
C.polonica.Poland.CMW10522        TTATCTGTTCCTTAGCATGATGAGGGGAGGGAAGGGGGGGGGTATAGAGCAC(217b)-TGAACACACACACACACACA------GAGAGAGAGAGA------GAAAA--TTAATG
C.polonica.Austria.CMW7148        TTATCTGTTCCTTAGCATAATGAGGGGGGGGGGGGGGGG---TATAGAGCAC(217b)-TGATACACACACACACACACACA---GAGAGAGAGAGAGAGAGAGAAAA--TTAATG
C.pinicola.Norway.CMW3273         TTATCTGTTCCTTGGCATAAAGAGGGGAGGGGGGG-------TATAGAGCAC(217b)-TGAACACACACACACACACA------GAGAGAGAGAGAGAGAGA------ATCAATG
C.laricicola.Scotland.CMW4546     TTATCTGTTCCTTAGCATAATGAGGGGGGGGGGGGGGGG---TATAGAGCAC(217b)-TGAACACACACACA------------GAGAGAGAGAGAGAGA--GAAAAAATTAATG
```

- indicates gaps between sequences.
(217b) indicates a sequence of 217 bases between two polymorphic regions which is not given.

Fig. 1. A comparison of DNA sequences from SSR loci amplified with the primer pairs P4A-B, P5A-B, P6A-B, P10A-B and P11A-B using DNA from *Ceratocystis polonica* (CMW2210, CMW7133, CMW7148, CMW10522), *Ceratocystis laricicola* (CMW4546, CMW7760) and *Ceratocystis pinicola* (CMW3273).

Table 4. **Population genetic analysis of four *Ceratocystis polonica* populations, three from Europe and one from Japan.**

Population	No. of isolates	H	Polymorphic loci	% pol. loci	Ht	Hs	Gst	Nm		Poland	Japan	Norway	Pairs in LD	% LD
Austria	36	0.51	10	100					D	0.07	0.67	0.08	64	6.7
									I	0.92	0.50	0.92		
									Nm	13.60	1.97	9.91		
									Gst	0.03	0.20	0.04		
Poland	28	0.48	10	100					D		0.65	0.07	44	4.4
									I		0.52	0.93		
									Nm		1.91	10.87		
									Gst		0.20	0.04		
Japan	9	0.47	10	100					D			0.79	53	13
									I			0.45		
									Nm			1.40		
									Gst			0.26		
Norway	25	0.39	10	100									44	7.8
Europe	89	0.49	10	100	0.49	0.46	0.05	8.5						
Total	98	0.53	10	100	0.58	0.48	0.16	2.53						

H, Nei's genetic diversity; % pol. loci, percentage of polymorphic loci; Ht, average of total heterozygosity; Hs, average of heterozygosity within populations; Gst, G statistics (referring to population genetic differentiation); Nm, no. migrants per generation; D and I, Nei's genetic distance and identity; LD, linkage disequilibrium.

Fig. 2. UPGMA dendrogram generated with 10 SSR loci for 100 isolates of *Ceratocytsis polonica*. Numbers refer to the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

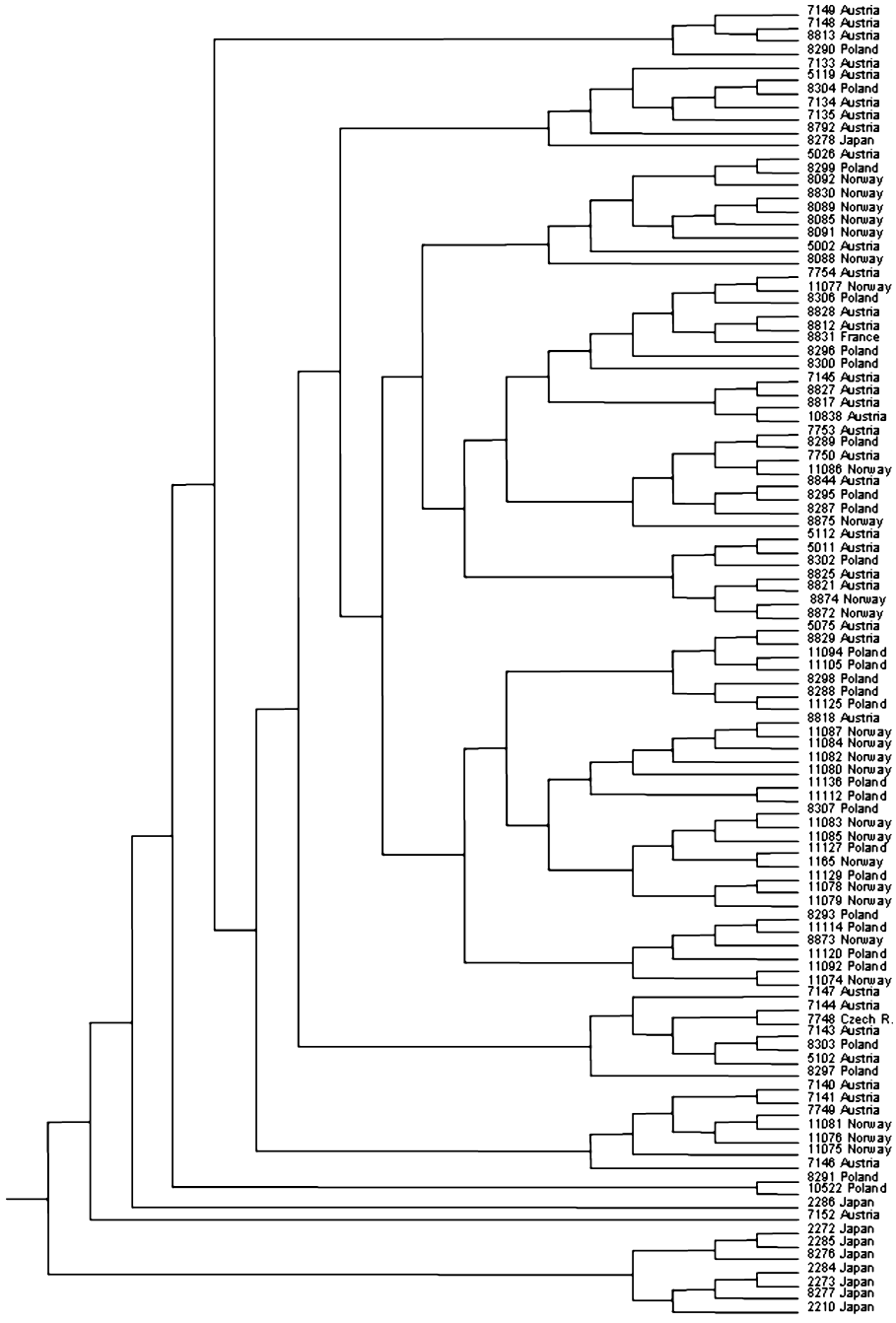
7149 Austria
7148 Austria
8813 Austria
8290 Poland
7133 Austria
5119 Austria
8304 Poland
7134 Austria
7135 Austria
8792 Austria
8278 Japan
5026 Austria
8299 Poland
8092 Norway
8830 Norway
8089 Norway
8085 Norway
8091 Norway
5002 Austria
8088 Norway
7754 Austria
11077 Norway
8306 Poland
8828 Austria
8812 Austria
8831 France
8296 Poland
8300 Poland
7145 Austria
8827 Austria
8817 Austria
10838 Austria
7753 Austria
8289 Poland
7750 Austria
11086 Norway
8844 Austria
8295 Poland
8287 Poland
8875 Norway
5112 Austria
5011 Austria
8302 Poland
8825 Austria
8821 Austria
8874 Norway
8872 Norway
5075 Austria
8829 Austria
11094 Poland
11105 Poland
8298 Poland
8288 Poland
11125 Poland
8818 Austria
11087 Norway
11084 Norway
11082 Norway
11080 Norway
11136 Poland
11112 Poland
8307 Poland
11083 Norway
11085 Norway
11127 Poland
1165 Norway
11129 Poland
11078 Norway
11079 Norway
8293 Poland
11114 Poland
8873 Norway
11120 Poland
11092 Poland
11074 Norway
7147 Austria
7144 Austria
7748 Czech R.
7143 Austria
8303 Poland
5102 Austria
8297 Poland
7140 Austria
7141 Austria
7749 Austria
11081 Norway
11076 Norway
11075 Norway
7146 Austria
8291 Poland
10522 Poland
2286 Japan
7152 Austria
2272 Japan
2285 Japan
8276 Japan
2284 Japan
2273 Japan
8277 Japan
2210 Japan

Table 5. **PCR amplification of DNA from different *Ceratocystis* species using SSR primer pairs developed for *Ceratocystis polonica* in this study.**

Species	P1	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	Total[1]
Ceratocystis coerulescens	+	+	+	+	+	+	+	−	M	+	+	+	11
C. pinicola	+	+	+	+	+	+	+	−	M	+	+	+	11
C. resinifera	−	+	+	+	−	+	+	−	M	+	+	+	9
C. rufipenni	+	+	+	+	+	+	+	−	+	+	+	+	11
C. laricicola	+	+	+	+	+	+	+	+	+	+	+	+	12
C. douglasii	+	+	+	+	+	+	+	−	M	+	+	+	11
C. polonica	+	+	+	+	+	+	+	+	+	+	+	+	12
Thielaviopsis australis	−	−	−	−	−	−	−	−	+	−	−	M	2
T. neocaledoniae	−	−	−	−	−	−	−	−	−	−	+	+	2
C. virescens	−	−	−	−	+	−	−	−	−	−	−	M	2
C. eucalypti	−	+	−	−	+	−	−	−	−	−	+	+	4
C. radicicola	−	−	−	−	−	−	−	−	−	−	−	+	1

+, successful PCR amplification; −, no PCR amplification; M, amplification of multiple bands.
[1]Total represents the number out of the 12 SSR primer pairs that amplified DNA from each *Ceratocystis* species tested.

Yuan & Kile and two from *Thielaviopsis australis* (J. Walker & Kile) A.E. Paulin, T.C. Harr. & McNew, *T. neocaledoniae* (Kiffer & Delon) A.E. Paulin, T.C. Harr. & McNew and *C. virescens* (R.W. Davidson) C. Moreau. Only primer pair P13A-B was able to amplify DNA from all the tested species including *C. radicicola.* It, however, produced multiple bands in *C. virescens* and *T. australis*, even at raised annealing temperatures (66–68°C). Nine primer pairs (P1A-B, P3A-B, P4A-B, P5A-B, P6A-B, P7A-B, P8A-B, P10A-B, P13A-B) had optimum annealing temperatures between 58 and 60°C across all the species for which they amplified DNA, while primer pairs P9A-B, P11A-B and P12A-B amplified at temperatures between 54 and 56°C.

4 Discussion

In this study, we have shown that populations of *C. polonica* associated with the Eurasian spruce bark beetle *I. typographus* in Europe and its Asian form *I. typographus japonicus* in Japan have high levels of genetic diversity. *Ceratocystis polonica* and other species in the *C. coerulescens* complex are homothallic but also possess unidirectional mating type switching, in which one of the mating types (MAT-2) is capable of selfing to produce self-sterile (MAT-1) or self-fertile (MAT-2) progeny. MAT-1 and MAT-2 isolates can cross with each other, but the MAT-1 progeny is always self-sterile (Harrington and McNew 1997, 1998). The high levels of genetic diversity that we have shown in *C. polonica,* are comparable with values obtained for fungal species with predominantly sexual reproduction such as *Mycosphaerella fijiensis* (H = 0.59) (Brygoo et al. 1998) or *Venturia inaequalis* (H = 0.46) (Tenzer et al. 1999). Thus, despite the fact that it is homothallic, genetic diversity data suggest frequent occurrence of sexual outcrossing in *C. polonica*. Tests for non-random association between alleles at all SSR loci support our hypothesis, because low levels of gametic disequilibrium were found in all the populations, as would be expected for random mating populations. In contrast to *C. polonica*, low levels of genetic diversity were found in the main vector of the fungus, *I. typographus*, reflected both by results of isozyme analyses, mitochondrial DNA sequencing and microsatellite analyses (Stauffer et al. 1999; Stauffer and Lakatos 2000; Sallé et al. 2007).

A low level of geographic substructure was found in the European populations of *C. polonica*, with a high level of gene flow between populations. Migration is an important force acting against genetic divergence among subpopulations. Results of this investigation indicated that the *C. polonica* population of Europe could be treated as a single unit. This was supported by Nm values higher than 9.9 for the three European populations sampled. *Ceratocystis polonica* is exclusively disseminated by a few bark beetle species, mainly *Ips* spp. (Kirisits 2004). Spores of *C. polonica* are carried on the body surfaces or in the guts of *I. typographus* individuals (Furniss et al. 1990) and ecological studies have suggested migration of *I. typographus* over distances up to 30–40 km (Nilssen 1984; Gries 1985). We, therefore, think that active migration and passive long-distance wind-dispersal (Forsse and Solbreck 1985) of bark beetle individuals carrying *C. polonica*, together with the trade of timber between European countries, represent the principal factors associated with the high gene flow between populations on this continent.

In *I. typographus*, isozyme and microsatellite analyses revealed a lack of genetic structure across Europe and a high level of gene flow amongst populations (Stauffer et al. 1999; Sallé et al. 2007). In contrast, the phylogeography of the insect based on mitochondrial DNA sequence analyses suggested genetic differentiation between populations, as one haplotype was restricted to Russia and Lithuania and only one haplotype was detected in Northern Europe, while the highest numbers of haplotypes were found in Central and Southern Europe (Stauffer et al. 1999). The isozyme and microsatellite data for *I. typographus* (Stauffer et al. 1999; Sallé et al. 2007) are in agreement with our results found for *C. polonica*. This is consistent with the close association of *C. polonica* with its insect vector and suggests close co-evolution on their common host tree, *P. abies*. Our results and those of Stauffer et al. (1999) and Sallé et al. (2007) also reinforce the view that management measures should be directed to control both organisms, as they represent a natural biological complex.

Our results and those of Stauffer et al. (1999) and Sallé et al. (2007) are also consistent, in that the nuclear genetic structures of *I. typographus* and its fungal associate, *C. polonica* in Europe are incongruent with that of their host, *P. abies*. While low levels of geographic substructure were found in populations of *I. typographus* and *C. polonica*, populations of Norway spruce are, based on several genetic markers, geographically differentiated, reflecting the post-glacial colonization of Europe from refuge areas located in Russia, the Balkans and the Carpathians (Lagercrantz and Ryman 1990; Vendramin et al. 2000; Gugerli et al. 2001; Tollefsrud et al. 2008).

An effect of geographic isolation was strongly reflected by genetic differences observed for *C. polonica* populations from Japan and Europe. Thus, the low levels of gene flow and the elevated Gst values indicate high levels of genetic isolation between fungal populations from the two continents. In a recent phylogenetic study, isolates of *C. polonica* obtained from *Ips typographus japonicus* in Hokkaido (Japan) had virtually the same ITS, β-tubulin and MAT-2 HMG sequences as those recovered from *I. typographus* in various European countries (Marin et al. 2005). Thus, the fungi associated with geographically disparate populations of this insect are phylogenetically related and conspecific. However, the European and Japanese populations are genetically isolated, as shown in the present population study. The genetic isolation between European and Japanese isolates of *C. polonica* is in agreement with the degree of differentiation found for the main insect vector of this fungus in Europe and Asia. Stauffer and Lakatos (2000) studied the phylogenetic relationships between European (*I. typographus*) and Asian (*I. typographus japonicus*) eight-spined spruce bark beetles, using the mitochondrial cytochrome oxidase I (COI) gene. They determined that despite the low sequence divergence (1.71%) found between the European and the Japanese and Chinese populations of this insect, these populations contained geographically isolated haplotypes. Fairly strong genetic

differentiation between European and Asian populations of *I. typographus* was also shown in a recent microsatellite study (Sallé et al. 2007).

A surprising result of this study was that one isolate of *C. polonica* from Japan grouped with Austrian and Polish isolates and it was unrelated to those in the Japanese clade. Based on this result, we suspect that European genotypes of *C. polonica* have been introduced into Japan. This finding also provides indirect evidence that *I. typographus* from Europe might have been introduced into Japan and possibly has established there. The introduction of *C. polonica* and *I. typographus* could have occurred through the international trade in timber, in which Japan is a major participant. Substantially larger collections of isolates would be needed to evaluate this situation more fully. This finding highlights the need for caution in the export of coniferous lumber, where fungi such as *C. polonica* that invade the wood, are ideally suited to be transferred between countries and continents.

Analysis of allele frequencies revealed a moderate number of unique alleles among the Polish and the Austrian populations. However, those alleles were present in very low frequencies in both populations (<0.1 in all cases) and it is possible that their absence in other populations sampled in this study was because of the small size of the populations evaluated. A different situation occurs in the Japanese population, because some exclusive alleles had frequencies as high as 0.66 and none of the six unique alleles had frequency values lower than 0.1. These results provide additional evidence for genetic isolation of the Japanese population. However, a larger and geographically more diverse collection of *C. polonica* isolates from Asia would be necessary to conclusively determine the origin and geographical occurrence of these alleles.

The SSR markers developed for *C. polonica* in this study were able to amplify DNA for six species from conifers belonging to the *C. coerulescens* complex. They will certainly be useful in studies aimed at comparing populations of these fungi, particularly of the more important tree pathogens in this group. Of particular interest to us is *C. laricicola*, which is native in central Europe, but has been introduced into new areas such as Scotland and Denmark (Redfern et al. 1987; Stauffer et al. 2001). The ability of most *C. polonica* SSR primers to amplify DNA in the *Ceratocystis* species occurring on conifers but not in those affecting hardwood trees, supports the monophyletic origin of these species on conifers, as shown by Witthuhn et al. (1998, 2000) using ITS and MAT-2 DNA sequence comparisons.

The results of this study provide strong evidence that quarantine strategies in Europe should consider the entire range of genetic diversity of *C. polonica* and its vectors on this continent. The high levels of recombination found in *C. polonica* in Europe and Japan may lead to development of new genotypes of the pathogen over a short period of time. Consequently, continuous genotyping of isolates in these populations would be advantageous to detect new virulent forms of the fungus. Moreover, every effort should be made to avoid the transfer of *C. polonica* and its vectors between Europe and Japan. Even though these organisms can be considered the same species they do not belong to the same population, have been separate for some time and are possibly in the process of speciation. Introduction of *I. typographus* and its fungal associates into North America, where they do not occur naturally represents an even greater threat. The increased understanding of the population biology of this important blue-stain fungus and vascular stain pathogen on spruce derived from this study, should contribute to improved pest and pathogen management. It should also result in a reduction in the impact of this biological complex on forestry and the wood industry in the Northern hemisphere.

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