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Development of microsatellite markers for the pine needle blight pathogen, *Dothistroma pini*

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Summary

Dothistroma needle blight of *Pinus* spp. is a serious disease that can be caused by two distinct fungal species: *Dothistroma septosporum* and *D. pini*. *Dothistroma septosporum* has a broad pine host range, a worldwide distribution and has caused many serious epidemics. In contrast, *D. pini* has a more limited distribution; known only from Europe and the USA. Unlike the situation for the better-known *D. septosporum,* the population biology of *D. pini* has not been intensively studied. Microsatellite markers developed for *D. septosporum* either do not amplify in *D. pini* or they are uninformative. The aim of this study was to develop a robust set of microsatellite markers that amplify across different *D. pini* isolates from a range of geographical locations and different host species. The genome of an isolate from Ukraine was sequenced using Mi-Seq Illumina sequencing. Thirty-two primer pairs were designed to amplify microsatellite regions targeting a mixture of di-, tri-, tetra-, penta- and hexa- nucleotide repeat regions. The results yielded 16 polymorphic markers that amplified across 32 isolates from seven countries and four hosts. Screening of the polymorphic markers on a population of *D. pini* from France and Slovenia revealed that the Slovenian isolates were clonal but that those from France were genetically more diverse. The markers developed in this study will provide a useful tool with which to study the population structure and genetic diversity of *D. pini* populations in countries where it is emerging as an important pathogen.

1 Introduction

Dothistroma needle blight (DNB), also known as red band needle blight is a devastating foliar disease of *Pinus* spp. that has caused several global tree disease epidemics (Gibson 1972; Ivory 1987; Bradshaw 2004; Barnes et al. 2008b; Welsh et al. 2014; Rodas et al. 2015). The disease is characterized by red bands or spots surrounding black fruiting structures on infected pine needles (Peterson and Walla 1978). The infected needles become necrotic with time and are prematurely shed (van der Pas 1981). Like many foliar diseases of trees, infection spreads from the base of trees and moves upwards within the canopies. In younger trees or more susceptible pine species, this can result in stunted growth or even tree death (Gibson et al. 1964; Peterson and Graham 1974; Ivory 1987).

Dothistroma needle blight can be caused by either of two closely related, but phylogenetically distinct, haploid ascomycete fungal species; *D. septosporum* (G. Dorog.) M. Morelet and *D. pini* Hulbary (Barnes et al. 2004). *D. septosporum* has been documented to infect more than 42 different *Pinus* spp. (Drenkhan et al. 2016), as well as non-*Pinus* species such as *Larix decidua* and *Picea abies* (Watt et al. 2009). In contrast, *D. pini* has been recorded on only 10 *Pinus* spp. (Barnes et al. 2004, 2008b, 2014a; Piškur et al. 2013; Piou and Ioos 2014). While *D. septosporum* has a worldwide distribution, *D. pini* is known only from 12 Northern Hemisphere countries in Europe and in the north-central states of the USA (Drenkhan et al. 2016).

The first report of *D. pini,* confirmed based on DNA sequence data, was from material collected in Michigan and Minnesota from the United States (Barnes et al. 2004). In 2008, *D. pini* was confirmed to be present in Europe, in this case, based on collections from Russia and Ukraine (Barnes et al. 2008b). A few years later, the pathogen was recorded in France (Ioos et al. 2010) and Hungary (Barnes et al. 2011). As additional studies are being conducted using molecular markers, reports of *D. pini* in Europe are increasing in number. For example, in the last 3 years, there have been seven new country reports including those from Belgium, the Czech Republic and Slovenia in 2013 (Piškur et al. 2013; Schmitz et al. 2013; Bergová and Kryštofová 2014), Montenegro and Switzerland in 2014 (Lazarević et al. 2014; Queloz et al. 2014) and from Romania and Serbia in 2015 (Drenkhan et al. 2016).

A set of 12 microsatellite markers were previously developed for *D. septosporum* (Barnes et al. 2008a). These markers have been successfully used to determine the genetic diversity of the pathogen on a global scale, to trace the movement of *D. septosporum* in parts of Europe and to consider patterns of its introduction into the Southern Hemisphere (Barnes et al. 2008a, 2014b; Drenkhan et al. 2013; Mullett et al. 2015). Unfortunately, these markers either do not amplify in *D. pini*, produce multiple bands or they are uninformative (Barnes et al. 2008a).

The growing numbers of new host and country reports for *D. pini* show that the fungus is emerging as a serious pathogen. This demands a better understanding of the possible origins and patterns of spread of the pathogen. The aim of this study was, therefore, to develop a set of microsatellite markers for *D. pini* that can be used to amplify across isolates from various geographical regions and different pine hosts, ultimately to be used in population genetic studies.

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2 Materials and methods

2.1 Fungal isolates, DNA extraction and identification

The isolates used in the study were obtained from infected needles collected from La Ferté-Imbault and Souesmes in France. Collections of needles were also made in Dutovlje, Hruševica, Pivka and Škocjan in Slovenia and Hola Prystan, Kinbun Peninsula and Tsjurupinsk in Ukraine (Table 1). In addition, some isolates were used from previous studies including isolates from Csabrendek and Diszel in Hungary (Barnes et al. 2011), Pivka in Slovenia (Piškur et al. 2013), Krasnosulinsky and Tarasovsky in Russia, Tsjurupinsk in the Kherson region of Ukraine (Barnes et al. 2008b) and Michigan in the USA (Barnes et al. 2004). The single isolate from the Czech Republic was supplied by Eva Bergová. Twenty-four isolates each from La Ferté-Imbault in France, and Pivka in Slovenia, were used for genetic diversity analyses.

All needles were collected from trees showing characteristic symptoms of DNB. Infected needles were stored at −70°C to preserve the viability of the spores until processing. Fungal conidiomata were excised from the infected needles and spread on agar plates to dislodge individual conidia. The conidia were allowed to germinate on Dothistroma Sporulating Medium (DSM), made up of 20 g malt extract, 5 g yeast extract and 15 g agar (BioRad, Johannesburg, South Africa) supplemented with 10 ml of 100 mg/l streptomycin (Sigma-Aldrich, St. Louis, MO, USA) per litre of water. The cultures were grown at 21°C for approximately 3–4 weeks and exposed to natural day/night intervals of light. Mycelium was then scraped off from the surface of the agar plates, placed in 1.5 ml Eppendorf tubes (Axygen, Union City, CA, USA) and freeze-dried overnight using the Vir Tris advantage lyophilizer (SP Scientific, PA, USA). The mycelium was crushed with stainless steel beads using a Retsch GmbH MM301 homogenizer (Haan, Germany) and DNA was extracted using the Zymo Research Fungal DNA MiniPrep kit (Irvine, CA, USA) at an elute volume of 100 μl. DNA concentration was measured using the Nanodrop 1000 spectrophotometer and diluted to a final concentration of 30 ng/μl.

To confirm the identity of the cultures, DNA for all isolates was PCR-amplified using the ITS-1 and ITS-4 primer pair (White et al. 1990) according to the methods described in Barnes et al. (2004). The PCR products were run on a 2% agarose gel (Merck, Darmstadt, Germany) and visualized using the GelRed application of the GelDoc™ EZ Imager (BioRad). The PCR products were cleaned using Sephadex solution made up of 3 g Sephadex G50 powder (Sigma-Aldrich) and 45 ml distilled water filtered through Centrisep spin columns (Princeton Separations, Foster City, CA, USA). Cleaned PCR products were amplified for sequencing using the Big Dye® (Life Technologies, Carlsbad, CA, USA) Reaction Kit as specified in the manufacturer's instructions. The sequencing products were run on the ABI PRISM™ 3500xl Autosequencer (Thermo Fisher Scientific, Carlsbad, CA, USA) and the resulting reads were assembled into consensus sequences using CLC MAIN WORKBENCH v6.0 (CLC Bio-Qiagen, Aarhus, Denmark). The consensus sequence was used to perform a BLASTn analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to confirm that the isolates were those of *D. pini*.

Table 1. Details of country, location, host species and number of isolates of *Dotistroma pini* used in this study.

Country	Locality	*Pinus* host	No. of isolates	Date collected	Collectors
Czech Republic	Chodská Lhota	*P. jeffreyi*	1	September-2013	Bergová E
France	La Ferté-Imbault	*P. nigra* subsp. *laricio*	24	August-2013	Barnes I, Mullett M
	Souesmes	*P. nigra* subsp. *laricio*	5	August-2013	Barnes I, Mullett M
Hungary	Csabrendek	*P. nigra* subsp. *nigra*	2	May-2007	Barnes I
	Diszel	*P. nigra* subsp. *nigra*	3	May-2007	Barnes I
Russia	Krasnosulinsky	*P. nigra* subsp. *nigra*	1	May-2007	Timur SB, Barnes I
	Krasnosulinsky	*P. nigra* subsp. *pallasiana*	1	June-2007	Timur SB, Barnes I
	Krasnosulinsky	*P. mugo turra* subsp. *mugo*	1	June-2007	Timur SB, Barnes I
	Rostov region, Tarasovsky	*P. nigra* subsp. *pallasiana*	2	June-2007	Timur SB, Barnes I
Slovenia	Dutovlje, Karst	*P. nigra* subsp. *nigra*	1	June-2013	Jurc D, Hauptman T
	Hruševica, Karst	*P. nigra* subsp. *nigra*	1	October-2013	Jurc D, Hauptman T, Ogris N
	Pivka	*P. nigra* subsp. *nigra*	24	May-2014	Sadiković D, Hauptman T
	Pivka	*P. nigra* subsp. *nigra*	1	June-2012	Jurc D, Piškur B
	Škocjan	*P. nigra* subsp. *nigra*	2	December-2013	Jurc D, Hauptman T
Ukraine	Kherson region, HolaPrystan	*P. nigra* subsp. *pallasiana*	2	September-2013	Davydenko K, Siziba V
	Mykolaiv, Kinbun Peninsula	*P. nigra* subsp. *pallasiana*	1	September-2013	Davydenko K, Siziba V
	Kherson region, Tsjurupinsk	*P. nigra* subsp. *pallasiana*	2	September-2013	Davydenko K, Siziba V
	Kherson region, Tsjurupinsk	*P. nigra* subsp. *pallasiana*	1	November-2004	Usichenko AC, Barnes I
USA	Michigan, Missaukee County, Macbain	*P. nigra* subsp. *nigra*	2	August-2001	Adams G, Barnes I
	Michigan, Montcalm County, Crystal	*P. nigra* subsp. *nigra*	1	August-2001	Adams G, Barnes I
	Michigan, Montcalm County, Stanton	*P. nigra* subsp. *nigra*	2	August-2001	Adams G, Barnes I

2.2 Genome sequencing and primer design

An isolate of *D. pini* from Tsjurupinsk, in the Kherson district of Ukraine was chosen for genome sequencing and microsatellite discovery. The isolate was obtained from an infected needle of a *P. nigra* sub-species *pallasiana* tree approximately 25–30 years old. The needle collections were made in November 2004 by AC Usichenko, and the isolate (CMW 23767; CBS 121011) was deposited in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI) and the Centraalbureau voor Schimmelcultures collection (CBS) (Barnes et al. 2008b).

The isolate (CMW 23767) was grown on 2% MEA (Malt Extract Agar) (BioRad) for genomic DNA extraction. Mycelial plugs were transferred to cellophane disks (BioRad) placed on the surface of fresh 2% MEA agar plates and grown at 21°C for 4 weeks. Mycelium was pulled off from the cellophane, freeze-dried overnight and crushed into a fine powder using a pestle and mortar. DNA was extracted using a phenol-chloroform method (Goodwin et al. 1992). The quality of the DNA was verified by running the DNA extraction products on a 1% agarose gel and visualized using the GelRed application of the GelDoc™ EZ Imager. The concentration of the DNA was determined with a Qubit fluorometer (Life Technologies) using the Qubit DNA assay kit (Life Technologies).

DNA of the *D. pini* isolate (CMW 23767) was submitted to InqabaBiotec™, Pretoria, South Africa, for genome sequencing using the Mi-Seq Illumina sequencing platform. The raw sequencing reads were categorized into Q20 and Q30 reads based on sequence quality. The Q30 reads, which had higher sequence quality, were assembled into contigs using CLC GENOMICS WORKBENCH v6.0 (CLC In.) and analysed for the presence of microsatellite regions using MSATFINDER v2.0.9 (Thurston and Field 2006). The parameters for MSATFINDER were set to search for di-, tri-, tetra-, penta- and hexanucleotides with a repeat motif higher than seven. Mononucleotides were excluded from the search.

Primers were designed on the contigs containing microsatellite regions using CLC MAIN WORKBENCH v6.0. Contigs chosen for primer design did not contain any compound microsatellites and had no other repeats or indels in the flanking regions of the microsatellite repeat. From the selected contigs, each primer pair was designed to contain a GC content of at least 45%, an annealing temperature of at least 55°C and produce an amplicon size of between 100–450 bps. A BLASTn analysis with the designed primers was performed against the *D. septosporum* genome (Joint Genome Institute, http://genome.jgi-psf.org/Dotse1/Dotse1.home.html) to ensure that the primers amplified on different scaffolds to reduce the chance of generating linked loci.

2.3 Primer testing

The designed primers were tested for PCR amplification success and polymorphism on a subset of isolates (CMW 10926, CMW 10944, CMW 26424, CMW 29366 and CMW 43910) from different geographical locations. The PCR reaction mixture was made up to a 25 μl volume that included 17 μl of distilled autoclaved water, 2.5 μl of a 10$\times$ reaction buffer (Roche Diagnostics, Indianapolis, IN, USA), 2.5 μl of 0.2 mM dNTPs, 0.25 μl of 200 nM of each of the forward and reverse primers, 0.5 μl of 250U FastStart Taq (Roche Diagnostics), 1 μl of 25 mM $MgCl_2$ (Roche Diagnostics) and 1 μl of 30 ng/μl DNA. PCR cycle conditions were as follows: 96°C for 10 min, 10 cycles of 96°C for 30 s, 45 s at the appropriate annealing temperature for each primer (Table 2) and 72°C for 1 min. This was followed by 30 cycles of 96°C for 30 s, 45 s at the appropriate annealing temperature for each primer with a 5 s expansion after each cycle and 72°C for 1 min. The cycle was completed by a 60°C step for 30 min. The PCR products were run on a 2% agarose gel to verify amplification success and approximate size of the PCR product. The PCR products were cleaned using Sephadex G50.

PCR products for all five isolates were sequenced using the methods described above to ensure the regions amplified by the primers were polymorphic. Consensus sequences were generated from the forward and reverse sequences for each microsatellite primer pair using CLC MAIN WORKBENCH v6.0. Consensus sequences from the five different isolates were aligned in MEGA v5.2 (Tamura et al. 2011) using MUSCLE (Edgar 2004). Polymorphism was determined by comparing the different amplicon lengths created by indel differences within the microsatellite region between each of the isolates. The alignments were also used to ensure that the polymorphism observed was as a result of only insertions or deletions in the microsatellite repeat unit. All primer pairs that failed to amplify, produced multiple bands, or that amplified regions where indels were present in the flanking regions of the microsatellite motif were discarded.

2.4 Fragment analysis

The program MULTIPLEX MANAGER v1.2 (Holleley and Geerts 2009) was used to determine the colour of the fluorescent dye and panel position for each polymorphic primer pair. The polymorphic primers were labelled on the 5′ end of the forward primer using one of four fluorescent dyes according to the G5 Filter set (Life Technologies, Johannesburg, South Africa): NED™ (yellow), FAM™ (blue), PET® (red) or VIC® (green). Each panel was designed to separate primers that were most likely to form primer dimers for future PCR multiplex purposes. Primers amplifying the same allelic ranges were either assigned different fluorescent labels, or were split into different panels for fragment analysis.

The labelled microsatellite primers were used to amplify 32 isolates using the PCR conditions provided above to determine allele sizes and allelic range for each primer pair (Tables 2 and 3). PCR products belonging to the same panel (either Panel 1 or Panel 2, see Table 2) were pooled and 1 μl of the pooled dilutions was mixed with 0.14 μl GENESCAN™ -500 LIZ® 500 (Life Technologies, Applied Biosystems, Warrington, UK) size standard and 10 μl formamide. The pooled mixture was run on the ABI PRISM™ 3500xL Genetic Analyzer (Applied Biosystems). GENEMAPPER v4.1 was used to score the alleles

Table 2. Primer specifications of the 16 polymorphic microsatellite markers developed for *Dothistroma pini* in this study.

Primer name	Primer sequence 5'–3'	GenBank accession no.	Repeat motif	Fragment length	Optimum annealing temp (°C)	Fluorescent label	Dilution ratio (in μl) for fragment analyses[1]	Panel No.	Number of alleles[2]	Allelic range	h^3
DP-MS1	F-GTGGGGTTTGTTGTTTCTGT R-CTCAAGTGGTATCCGTCCGT	KM206750	(CAGCA)8	229	60	NED	1.5/100	1	6	209–294	0.75
DP-MS2	F-CCGTGCGGCATCTCTAAT R-CGCGTCGAGGATAATGTC	KM206751	(AG)14	201	57	PET	2.0/100	1	3	201–203	0.34
DP-MS4	F-TGGCGCTCGGAAGATAGT R-TGCTCTCCCTCCGTTTGT	KM206752	(AT)11	185	60	VIC	1.5/100	1	2	169–185	0.22
DP-MS5	F-GGTGTGCGTTTCTTGTCGT R-CGCGTGATTTGTGGCTTGT	KM206753	(GATGCT)8	352	58	VIC	1.0/100	1	4	340–364	0.6
DP-MS6	F-CAGCGGAAACAACTCGATATAC R-GACCAAGAAGACGAACAGCA	KM206754	(TGTGCC)7	203	58	FAM	1.6/200	2	2	187–205	0.43
DP-MS7	F-ACAAGCATACAACTTACCACCA R-CAAGAACGCCACCACACAACCA	KM206755	(GTT)17	397	62	PET	1.6/200	2	6	392–404	0.77
DP-MS8	F-CCACTCCACCGACATCGAA R-ACCCGCATAGCATCGACAA	KM206756	(CAT)10	350	60	PET	2.0/200	2	3	349–352	0.31
DP-MS9	F-GCCTGCTGGTGATCCTGTCT R-TGCTCGTCTTGGCCTGGTT	KM206757	(TTG)15	359	60	NED	1.4/200	2	2	341–347	0.44
DP-MS10	F- GCTGAGATCCACCGAACGA R-CACTACTCCTCCTCCTCTTTCC	KM206758	(GAT)13	256	58	VIC	0.8/100	1	2	250–256	0.26
DP-MS11	F-CTCGCACCCTCTCTTTCATT R-GCTCGGGGTCCATTTTCA	KM206759	(ACA)18	325	58	FAM	1.0/100	1	7	298–328	0.79
DP-MS12	F-GCGCTTTCTTCGCCTCATT R-CGACCCCGACATACTACC	KM206760	(CTT)17	389	60	FAM	1.4/200	2	10	285–396	0.82
DP-MS13	F-CGTTGCTGGGAAGTAGGA R-GAAGAGAGTGAGAGCGGGT	KM206761	(TGA)10	344	58	PET	1.5/100	1	9	342–363	0.75
DP-MS15	F-CGTCGTCCTTCTCCCATT R-GATCTTGGGGCATTCTTTGT	KM206762	(ATC)14	270	60	PET	2.0/200	2	2	259–271	0.54
DP-MS16	F-GCTCTTTGCCTGGGGTTT R-GGTGGTTGGAGGTGCTTGGA	KM206763	(TTC)13	325	58	VIC	1.4/200	2	2	324–327	0.28
DP-MS17	F-ACCAGGGAAGAGGACAGCA R-TGGTCGAGGTAAAGAGAGGG	KM206764	(TCGTAG)8	240	60	NED	1.2/200	2	4	226–250	0.45
DP-MS18	F-ATGGGGAAGTGTGGAGAGG R-GCAGGAGACAGGAAGCAGA	KM206765	(AG)13	217	58	VIC	1.2/200	2	2	213–215	0.17

[1]Optimal dilution for the amplicon produced per primer set for fragment analysis.
[2]Number of alleles per marker after analysis of 32 isolates (see Table 3).
[3]*h*, Nei's (1973) gene diversity.

Table 3. Mating type and allele sizes of 32 *Dothistroma pini* isolates from various geographical regions screened using the 16 microsatellite markers developed in this study. Fragment analysis was performed on the ABI PRISM ™ 3500xL genetic analyzer and allele sizes were determined using GENEMAPPER v4.1.

			Microsatellite loci																
			Panel 1								Panel 2								
Location	CMW	Mating type	Doth_A[1]	DP_MS_4	DP_MS_1	DP_MS_10	DP_MS_11	DP_MS_5	DP_MS_13	DP_MS_2	DP_MS_6	DP_MS_18	DP_MS_17	DP_MS_15	DP_MS_16	DP_MS_9	DP_MS_8	DP_MS_12	DP_MS_7
Czech Republic																			
Chodská Lhota	43394	MAT-1	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	406
France																			
Souesmens	43910	MAT-1	111	169	264	250	301	352	396	204	203	217	234	267	319	353	350	386	385
Souesmens	41506	MAT-2	111	169	264	250	301	352	399	204	203	217	234	267	319	353	350	386	406
Souesmens	41495	MAT-2	111	169	264	250	307	352	390	204	197	219	234	270	319	353	350	398	397
Souesmens	41499	MAT-2	111	169	264	250	307	352	402	204	197	217	234	270	322	353	350	386	406
Souesmens	41494	MAT-2	111	169	264	250	307	352	405	204	197	219	234	270	322	356	353	398	403
Hungary																			
Csabrendek	26407	MAT-1	111	169	295	250	307	352	364	204	203	217	234	267	319	353	350	425	394
Csabrendek	26422	MAT-1	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	403
Diszel	26412	MAT-1	111	169	295	250	307	352	364	204	197	217	222	267	319	353	350	440	394
Diszel	26424	MAT-1	111	169	295	250	307	352	364	204	197	219	234	267	319	353	350	425	394
Diszel	26405	MAT-1	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	428	394
Russia																			
Tarasobsky	29366	MAT-1	111	185	284	250	292	340	358	212	197	217	222	267	322	353	350	404	394
Tarasobsky	29367	MAT-1	111	169	229	256	322	352	344	204	203	217	222	270	319	359	350	389	394
Krasnosulinsky	29368	MAT-1	111	185	249	250	331	340	361	206	203	217	234	267	319	353	350	410	400
Krasnosulinsky	29369	MAT-1	111	169	249	250	310	340	364	206	197	217	222	267	319	353	350	410	394
Krasnosulinsky	29370	MAT-1	111	169	295	250	307	352	364	204	203	217	234	267	319	353	350	425	394
Slovenia																			
Panovec	39746	MAT-2	111	169	229	250	325	352	344	204	197	217	240	270	319	359	350	389	409
Pivka	43423	MAT-2	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	409
Pivka	43424	MAT-2	111	169	249	250	331	360	344	204	197	217	240	270	319	353	350	380	406
Pivka	43425	MAT-2	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	409
Škocjan	43426	MAT-2	111	169	249	250	325	360	344	204	197	217	240	270	319	353	350	380	406
Ukraine																			
Tsurupynsk	42947	MAT-2	111	169	249	250	331	340	361	206	197	217	222	267	319	353	350	410	394
Tsurupynsk	23769	MAT-1	111	185	229	256	322	352	344	204	203	217	234	270	319	359	350	389	400
Tsurupynsk	42955	MAT-2	111	185	229	256	322	352	344	204	203	217	234	270	319	353	350	389	403
Hola Prystan	42939	MAT-2	111	185	295	256	322	352	396	204	203	217	234	270	319	359	350	389	400
Hola Prystan	42940	MAT-2	111	169	264	250	301	352	344	204	203	217	234	267	319	353	350	386	406
Kinbun Peninsula	42941	MAT-2	111	169	229	250	301	352	396	204	203	217	234	267	319	353	350	386	400
USA																			
Crystal	10926	MAT-2	111	169	295	250	307	352	364	204	197	217	234	267	319	353	350	425	394
Macbain	10954	MAT-1	111	169	229	250	307	352	381	204	197	217	234	270	319	353	365	425	397
Macbain	4953	MAT-2	111	185	229	256	322	352	381	204	203	217	234	270	319	359	350	389	406
Stanton	10939	MAT-2	111	169	295	250	307	364	344	206	197	217	246	270	319	353	365	398	400
Stanton	10944	MAT-2	111	169	295	250	307	364	381	206	197	217	246	270	319	353	365	398	397

[1]Doth_A diagnostic marker developed by Barnes et al. (2008a,b) to differentiate *D. septosporum* isolates from *D. pini*. The diagnostic marker confirmed all isolates used in this study as *D. pini*.

based on the fragment lengths of the PCR products. Primers were individually optimized for the best annealing temperature, choice of polymerase and dilution ratio for fragment analyses.

The polymorphic microsatellite primers were used to amplify a larger collection of 24 isolates each from La Ferté-Imbault in France and from Pivka in Slovenia in order to test the efficacy of the markers for population genetic analyses. The isolates were amplified and alleles scored using the methods described above. In addition to the microsatellite markers developed here, all isolates were screened with the species-specific microsatellite marker Doth_A (Barnes et al. 2008a) as a control for *D. pini* in the fragment analyses. All *D. pini* isolates should produce an allele size of 111 bp.

2.5 Data analysis

Adequacy in terms of the number of microsatellite markers designed for population genetic analyses was tested using MULTILOCUS version 1.3 (Agapow and Burt 2001). Using 24 isolates from La Ferté-Imbault, France, mean genotypic diversity was plotted against the number of loci to assess whether genotypic diversity reached a plateau with the number of microsatellite markers developed. Pairwise linkage disequilibrium between loci was tested using MULTILOCUS VERSION 1.3 with 1000 randomizations on a dataset comprising one representative haplotype from all the isolates used in this study (Tables 1 and 3). Allelic frequency for each of the microsatellite loci was calculated using POPGENE VERSION 1.32 (Yeh et al. 1999). Gene diversity (Nei 1973) and total number of alleles were calculated overall for the 32 isolates from different geographical regions, and separately for each of the two populations from France and Slovenia.

2.6 Mating type

The mating type of *D. pini* isolates used in this study was determined by amplifying the mating type idiomorphs, MAT1-1-1 and MAT1-2 using primers developed by Janoušek et al. (2013). The isolates were assigned as MAT1-1 (634 bp) or MAT-2 (323 bp) depending on the size of the PCR amplicon obtained. The PCRs were carried out as described in Janoušek et al. (2013). The PCR amplicons were run on a 2% agarose gel to determine the size of the amplicons.

3 Results

3.1 Fungal isolates and identification

Isolations from infected needles collected in France resulted in five isolates from Souesmes and 24 from La Ferté-Imbault. From Slovenia, four isolates were made from needles collected in Dutovlje, Hruševica, Škocjan, and 24 from Pivka. A further five isolates were obtained from Hola Prystan, Kinbun Peninsula and Tsjurupinsk in Ukraine. Details regarding the location and hosts of isolates obtained for each of these regions are provided in Table 1.

A total of 32 isolates from various geographic regions were obtained and verified as representing *D. pini* based on 100% sequence homology between the ITS sequence data and BLASTn searches in GenBank (Accession Number AF21197 from France, AY808301 from USA, DQ926964 from Ukraine, EF450254 from Russia and KC149561 from Slovenia) as well as using the diagnostic microsatellite marker (Doth_A). In addition, the identity of the 24 isolates from La Ferté-Imbault in France and Pivka from Slovenia, used for the population studies, was also confirmed as *D. pini* using the same methods. All isolates produced the *D. pini* allele size of 111 bp for marker Doth_A.

3.2 Genome sequencing and primer design

Partial genome sequencing of *D. pini* with Illumina sequencing produced 26 748 reads. The reads assembled into a total of 3404 contigs. Of these contigs, 348 contained microsatellite regions containing seven repeats or more of di- to hexanucleotides. The microsatellites were made up of 148 dinucleotide, 169 trinucleotide, five tetranucleotide, 10 pentanucleotide and six hexanucleotide repeats (Fig. 1). Thirty-two contigs, containing a mixture of different repeat motifs, were chosen for primer design.

3.3 Primer testing

All 32 primer sets designed using CLC WORKBENCH successfully amplified PCR products of the expected length in all isolates tested. Of these, 56% were shown to be polymorphic. The 14 monomorphic markers (Table S1) mostly contained trinucleotide repeat regions. These were discarded from further analyses. In addition, two of the polymorphic primers were discarded due to extreme stutter that could not be resolved with PCR optimization. This made scoring of allele sizes for these markers extremely difficult as they produced multiple peaks. Ultimately, a total of 16 polymorphic markers were obtained (Table 2). Sequencing the amplified products from each locus revealed the polymorphism observed was as a result of differing repeat numbers in the microsatellite region and not due to indels in the flanking regions. At least one representative allele per locus has been submitted to GenBank with accession number KU254135–KU254150 and can be compared against accession numbers KM206750–KM206765 (Table 2).

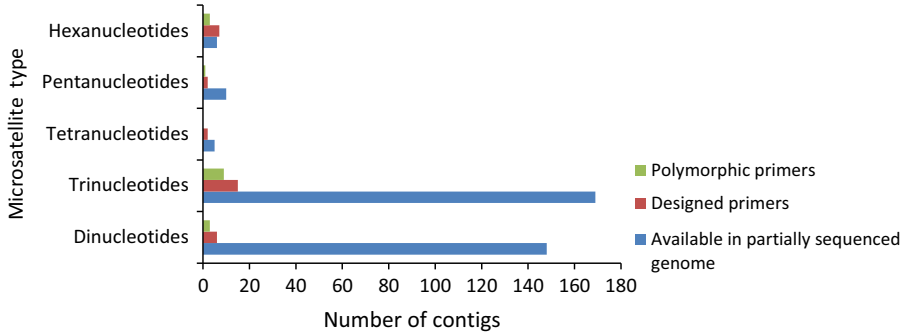

Fig. 1. Bar plot showing the number of microsatellite regions in the 348 contigs obtained from the partially sequenced genome of *Dothistroma pini*. The contigs are shown without duplicate regions and these only represent microsatellites with seven repeats or more.

3.4 Data analysis

Comparison of genotypic diversity values against number of microsatellite loci generated a line graph that plateaued at 12 loci (Fig 2) for the 24 isolates from La Ferté-Imbault, France. The mean genotypic diversity per locus ranged from 0.51 to 0.99. This suggested that the 16 markers designed in this study were sufficient enough for population and genetic diversity analysis. Based on the levels of genotypic diversity per locus, markers DP_MP_12, DP_MP_13, DP_MP_11, DP_MP_7, DP_MP_1, DP_MP_5, DP_MP_17, DP_MP_2, DP_MS_4 and DP_MP_6 would provide sufficient information for a population structure and genetic diversity study in *D. pini*. Analysis of linkage disequilibrium between pairs of loci showed that 58 out of 120 pairs (48%) were linked.

BLAST analysis of the designed primer sets against the *D. septosporum* genome showed that 12 of the 16 markers had sequence identity >80% (Table S2). Four of the primer sets and flanking sequences (DP_MS_1, DP_MS_2, DP_MS_17 and DP_MS_18) could not be located on the *D. septosporum* genome. Some of the primers were located on the same scaffold: DP_MS_7, DP_MS_10 and DP_MS_13 were identified on scaffold 1 with a minimum distance between the loci of 235 023 bp; DP_MS_4 and DP_MS_6 were both identified on scaffold 6 with a distance of 1 646 793 bp between the two loci and DP_MS_15, DP_MS_8 and DP_MS_5 were identified on scaffold 4 with a minimum distance of 826 464 bp between the closest two loci. The detailed report of the BLAST analysis is provided in Table S2.

From the 16 selected microsatellite loci, a total of 66 alleles were obtained from 32 isolates. The number of alleles ranged from two alleles for markers DP_MS_4, DP_MS_6, DP_MS_9, DP_MS_10, DP_MS_15, DP_MS_16 and DP_MS_18 to ten alleles for primer DP_MS_12. The gene diversity for each marker ranged from 0.17 for DP_MS_18 to 0.82 for DP_MS_12. The number of alleles and gene diversity obtained for the isolates from different geographic locations (countries) are shown in Table 4.

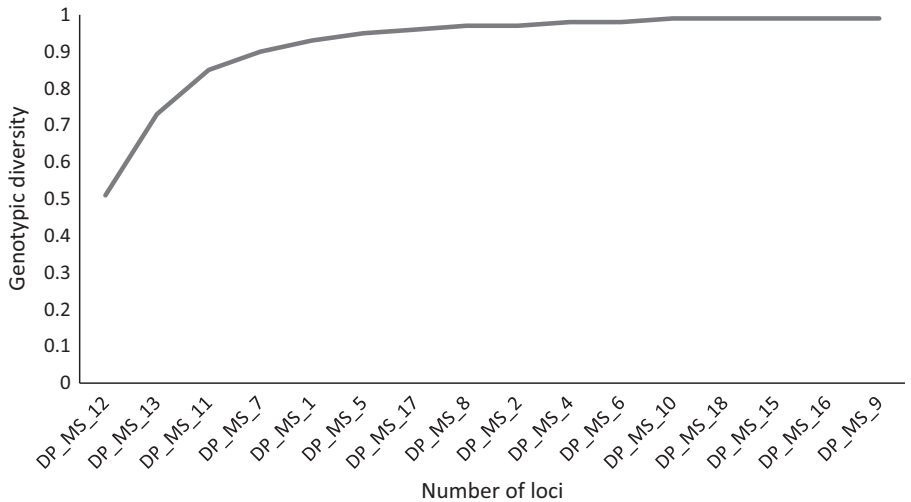

Fig. 2. A line graph showing the mean genotypic diversity against the number of microsatellite markers obtained using MULTILOCUS v1.3 used for the population and genetic diversity study of 24 *Dothistroma pini* isolates from France. The graph reaches a plateau at 12 microsatellite loci (DP_MS_18), which shows that an adequate number of microsatellite markers were developed for this study.

Table 4. Summary statistics of gene diveristy and number of alleles per country for *Dothistroma pini* isolates used in this study.

Country	Number of isolates	na[1]	h^2
USA	5	34	0.39
France	5	32	0.31
Ukraine	6	25	0.21
Hungary	5	19	0.07
Slovenia	5	18	0.04
Russia	5	40	0.45
Czech Republic	1	16	0.00
France (La Ferté-Imbault)	24	41	0.35
Slovenia (Pivka)	24	16	0.00

[1]na, number of observed alleles.
[2]*h*, Nei's (1973) gene diversity.

The population from La Ferté-Imbault in France had a gene diversity of 0.35 and a total of 41 alleles. Twelve of the 16 loci were polymorphic. In contrast, the population from Pivka collected in Slovenia in 2014 was completely clonal with only one allele obtained per locus.

3.5 Mating type

Both MAT1-1-1 and MAT1-2 mating types were found in isolates from the USA, France and Ukraine. Isolates from Russia and Hungary contained only the MAT1-1-1 idiomorph while all the isolates from Slovenia were MAT1-2. The mating types for the isolates from the various countries are presented in Table 3.

4 Discussion

An important outcome of this study was the production of a set of microsatellite markers for *D. pini*. The 16 polymorphic markers amplified *D. pini* isolates from seven different geographical regions, four hosts and isolates representing both mating types of the fungus. The efficacy of the microsatellite markers for population genetic analyses studies was demonstrated on two populations of *D. pini* from Europe.

Previous studies considering the abundance of microsatellites in fungi have shown that tri- and hexanucleotides make up the greatest percentage of repeat motifs in fungal genomes (Toth et al. 2000; Li et al. 2009; Simpson et al. 2013). In the present study, tri- and dinucleotides were the most abundant repeat motif while hexanucleotides were the least common. Markers with trinucleotide repeats also generated the greatest number of alleles and showed the highest allelic diversity. Even though dinucleotide repeats were most abundant in the genome, they generated the lowest number of alleles and were not informative. The results are consistent with those showing that the distribution of microsatellites is similar amongst fungi but where the abundance of motifs is unique to each organism (Toth et al. 2000; Lim et al. 2004; Karaoglu et al. 2005).

Analyses showed that 12 microsatellite markers were sufficient to analyse genetic diversity in *D. pini*. Previous studies have shown that increasing the number of microsatellite markers from a minimum number, as determined by genotypic diversity, leads to more reliably supported results that correspond to the geographical origins (Koskinen et al. 2004). Thus, using only 12 of the 16 microsatellite loci developed in this study would be sufficient to study the population structure and genetic diversity of *D. pini*.

Tests for linkage disequilibrium between pairs of loci were high (48%). However, this is most likely as a result of the small sample size tested. Tests for linkage through BLAST analysis of the microsatellite flanking regions showed that the markers were on different scaffolds of the *D. septosporum* genome. The loci that were on the same scaffold were at least 235 023 bp apart. This demonstrates that none of the loci were physically linked and ensures their reliability to detect patterns of population structure as suggested by Selkoe and Toonen (2006).

The microsatellite markers developed in this study easily amplified *D. pini* isolates of both mating types. Although only a relatively small number of isolates were screened, initial analyses showed that both mating types of *D. pini* are present in USA, France and Ukraine. This implies that sexual recombination could be occurring in *D. pini* in those countries (Groenewald et al. 2006, 2007; Kuck and Poggeler 2009).

The low gene diversity in the five Slovenian isolates from Dutovlje, Hruševica, Pivka and Škocjan, as well as the clonal nature of the Pivka population can be attributed to founder effects. This could also reflect the slow rate of population growth after a genetic bottleneck (Goodwin et al. 1994; Sakai et al. 2001). The presence of only one mating type and a single haplotype in a population of isolates from Pivka provides strong evidence that *D. pini* has been introduced into this region and that it is reproducing asexually. This is further supported by the fact that the pathogen has only recently been reported from Slovenia (Piškur et al. 2013).

The *D. pini* population from La Ferté-Imbault in France was genetically diverse and included both mating types, despite the small collection size. Such high levels of diversity might suggest that the pathogen is either native to the area or that it has been present for an extended period of time (Hamelin and Lecours 1998; Sakai et al. 2001; Allendorf and Lundquist

2003; Dlugosh and Parker 2008). The long-term presence of *D. pini* in France is supported by the detection of this pathogen in herbarium material dating back to 1907 (Fabre et al. 2012). Studies in France have also shown high levels of genetic diversity and allelic richness in *D. septosporum* (Mullett et al. 2015). Thus, all available evidence suggests that the more recent epidemics observed in that country could be linked to changes in climate (Fabre et al. 2012) as opposed to newly introduced populations of either pathogen.

The microsatellite markers developed in this study have not only been shown to amplify in *D. pini* isolates from different geographical areas, different hosts and both mating types, but that they will be useful in genetic and population diversity analyses. Ongoing and future research will utilize these markers to study the population structure and genetic diversity of *D. pini* populations from a wide selection of countries in an effort to trace patterns of spread at a global scale.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. List of designed microsatellite primers excluded from this study.

Table S2. Blast results of microsatellite flanking regions including the primer sequence of *Dothistroma pini* on the *D. septosporum* genome.