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First microsatellite markers developed and applied for the genetic diversity study and population structure of *Didymella pisi* associated with ascochyta blight of dry pea in Montana

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ABSTRACT

Didymella pisi is the predominant causal pathogen of ascochyta blight of dry pea causing yield losses in Montana, where 415 000 acres were planted to dry pea in 2018. Thirty-three microsatellite markers were developed for dry pea pathogenic fungus, *Didymella pisi*, these markers were used to analyze genetic diversity and population structure of 205 isolates from four different geographical regions of Montana. These loci produced a total of 216 alleles with an average of 1.63 alleles per microsatellite marker. The polymorphic information content values ranged from 0.020 to 0.990 with an average of 0.323. The average observed heterozygosity across all loci varied from 0.000 to 0.018. The gene diversity among the loci ranged from 0.003 to 0.461. Unweighted Neighbor-joining and population structure analysis grouped these 205 isolates into two major sub-groups. The clusters did not match the geographic origin of the isolates. Analysis of molecular variance showed 85 % of the total variation within populations and only 15 % among populations. There was moderate genetic variation in the total populations (PhiPT = 0.153). Information obtained from this study could be useful as a base to design strategies for improved management such as breeding for resistance to ascochyta blight of dry pea in Montana.

1. Introduction

The production of dry pea (*Pisium sativum* L) in the Northern Great Plains of the United States is rapidly increasing. Montana leads in the production of dry pea in the US, where 415 000 acres were planted in 2018, accounting for 47 % of the total production in the US. Also, in the last five years, the area planted with pulse crops in Montana increased from 686 000 acres in 2014 to 1.2 million acres in 2018, with dry pea accounting for 50.5 % of the increase (United States Department of Agriculture National Agricultural Statistics Service USDA-NASS, 2018). Being a high-water-use efficiency crop, it is of great importance to the semi-arid region like Montana. In addition, dry pea provide nitrogen credit to subsequent crops because of their capacity to fix atmospheric nitrogen. Thus, Montana growers include dry pea in rotation with cereals such as wheat and barley. This increase in the production of dry pea in Montana is associated with increasing export demands due to the high quality of dry pea seeds from the United States and increasing domestic interest in pulse crops as a ‘clear label’ source of protein in food and drink manufacturing. Furthermore, the Food and Agriculture Organization of the United Nations (FAO) declared 2016 as the International Year of Pulses (FAO, 2016). This initiative promoted public awareness of the nutritional benefits of pulse crops and encouraged increased consumption of pulses. Finally, fractionation of dry pea into components desired by consumers, especially its protein content, will drive the future demand for dry pea production.

As the acres planted to dry pea increase, so does the disease risk. Ascochyta blight (AB) has been reported to cause 15 % yield loss under favorable conditions (Skoglund et al., 2011). All parts of the dry pea plant and every growth stage can be infected by the fungus. Symptoms of the disease can develop on leaves, stems, and pods and can also cause pre-emergence seed rot. AB is a seed- and residue-borne. The lesions on pods are purplish-brown and infected seeds may be small and discolored (Gossen et al., 2011). Ascochyta blight of dry pea is often referred to as a disease complex because it is caused by a few closely related seed-borne fungal pathogens which can exist together on a host or independently of each other (Davidson et al., 2009; Tivoli and Banniza, 2007). Prior to

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2009, three fungal pathogens were associated with this disease: *Didymella pisi* (Barilli et al., 2016), *Peyronellaea pinodes* and *Peyronellaea pinodella* (Aveskam et al., 2010). Davidson et al. (2009) characterized a fourth causal agent, *Phoma koolunga*. This pathogen and other species such as *Phoma herbarum* (Li et al., 2011), and *Phoma glomerata* (Tran et al., 2014) were both shown to be pathogenic on dry pea and have been associated with ascochyta blight only in Australia.

Seed infection is one of the major survival mechanisms of fungi species causing ascochyta blight of dry pea and an important mode of transmission into previously uninfected areas. The impact of seed-borne inoculum is determined by climatic factors such as precipitation and temperature. Thus, areas with low precipitation often produce disease-free seeds in the field (Sivachandra-Kumar and Banniza, 2017). In addition, some species can also produce significant wind-borne inoculum (Sivachandra-Kumar and Banniza, 2017; Tivoli and Banniza, 2007). In addition to seed as a source of inoculum, *D. pisi*, and *P. pinodes* also can overwinter on crop residue as pycnidia and pseudothecia, which produce conidia and ascospores, respectively (Chilvers et al., 2009; Tivoli and Banniza, 2007). Generally, primary inoculum (asexual conidia or sexual ascospores) of all the pathogens are dispersed by rain and wind onto emerging dry pea crops and cause outbreaks of disease (Schoeny et al., 2008). However, ascospores are more efficient than conidia at disseminating the fungus because the ascospores are airborne (Chilvers et al., 2009; Tivoli and Banniza, 2007; and Wise et al., 2011).

Management of AB on dry pea requires an integrated approach, this often includes cultural practices, chemical treatment, and use of resistant cultivars (Kinane and Lyngkjaer, 2002; Wise et al., 2011). The use of resistant varieties is the best efficient approach for AB management. However, no single gene resistance to AB of dry pea has been found (Kraft et al., 1998). In addition, this difficulty in securing gene resistance to AB of dry pea may be due to the genetic variability that exists in the pathogen (Darby and Lewis, 1986).

Didymella pisi tends to be more prevalent in Montana compared to other species of fungus that cause Ascochyta blight in dry pea. This is based on seed tests of 130 dry pea seed lots submitted by growers from 23 Montana counties to the Regional Pulse Crop Diagnostic Laboratory (RPCDL) in Bozeman, MT during the 2015 and 2016 growing seasons. About 98 % of the ascochyta isolates detected and characterized were *D. pisi* (Owati et al., 2017). However, *P. pinodes* and *P. pinodella* are the prominent species in other dry pea producing regions of North America such as North Dakota and Saskatchewan (Sivachandra-Kumar and Banniza, 2017; Wallen et al., 1967).

D. pisi is heterothallic, the formation of sexual spores enables genetic diversity due to sexual recombination, which is associated with increased aggressiveness, development of fungicide resistance, and loss of resistant cultivars (Chilvers et al., 2009). *D. pisi* secretes ascochitine, a metabolite toxic to *Pisum* species and associated with pathogenicity (Foremska et al., 1990; Marcinkowska et al., 1991). An epidemic in Spain was attributed primarily to *D. pisi* (Chilvers et al., 2009; Kaiser et al., 2008). Thus, it is important to understand the genetic diversity and population structure of the fungus and define the spatial distribution of the pathogen. This information is vital in the development and implementation of effective disease management strategies. For instance, in Brazil, the development of effective disease management strategies was achieved for the control of sugarcane brown rust after understanding the population genetic variability of *Puccinia melanocephala* its causal pathogen (Peixoto-Junior et al., 2014).

DNA markers have been widely adopted for analyzing the population dynamics of plant pathogens due to their high levels of precision and accuracy (Milgroom and Peever, 2003; Moges et al., 2016; and Peixoto-Junior et al., 2014). They are rapid, highly specific, and have a low detection limit (Milgroom and Peever, 2003; Moges et al., 2016). Microsatellites also referred to as simple sequence repeats (SSRs), are among the most variable types of DNA sequences in eukaryotic genomes and are widely used for the study of plant pathogens (Ellegren, 2004). Simple sequence repeats (SSRs) are tandemly repeated tracts of DNA with about 1–6 base pair (bp) long units (Tóth et al., 2000). They are ubiquitous and relatively abundant in prokaryotes and eukaryotes (Moges et al., 2016; Peixoto-Junior et al., 2014; and Tóth et al., 2000).

Although SSRs are ubiquitous and evenly distributed in eukaryote genomes, fungal genomes are known to contain fewer SSR sequences than other eukaryotes (Dutech et al., 2007; Huntley and Golding, 2000; Katti et al., 2001; Lenz et al., 2014; and Moges et al., 2016). However, in the presence of polymorphic loci, SSRs are useful for population genetic studies. In this light, SSRs have been used for the study of the genetic diversity of various plant pathogenic fungi including *Didymella rabiei* (Baite et al., 2017; Bayraktar et al., 2007) *Sclerotinia subarctica* and *S. sclerotiorum* (Winton et al., 2007), *P. melanocephala* (Peixoto-Junior et al., 2014), *Colletotrichum* spp (Marulanda et al., 2014), and *Colletotrichum gloeosporioides* (Moges et al., 2016). Pathogen diversity studies offer information about the organism's future evolutionary potential, which is useful not only in the screening for resistance genes but also in defining fungicide use (Ciampi et al., 2011; Marulanda et al., 2014; and McDonald and Linde, 2002). In addition, temporal and spatial information on genetic diversity and population structure of plant pathogen are highly important to understand the evolutionary adaptability and the pathogen's potential to overcome the potential resistance of the host plant (Ciampi et al., 2011; McDonald and Linde, 2002).

Despite the importance of *D. pisi* on dry pea as the predominant pathogen associated with AB of dry pea in Montana, its population genetic diversity has not been determined. Thus, the objective of this study was to develop microsatellite markers and analyze the genetic diversity and population structure of *D. pisi* populations from the major dry pea production regions of Montana.

2. Materials and methods

2.1. Fungal isolate collection

Two hundred and five isolates of *D. pisi* were previously obtained from two sources (Owati et al., 2017) (Table 1). A total of 171 isolates were collected in 2016 and 34 isolates were collected in 2017 (Table 1). About 90 % of the isolates were obtained from AB contaminated seed lots submitted by growers in 15 Montana counties to the Regional Pulse Crop Diagnostic Laboratory (RPCDL) in Bozeman, MT, for seed testing intended for planting in 2016 and 2017 (Tables 1 and S1). The second set of isolates were obtained in 2016 and 2017 during a survey of dry pea production fields in Montana (Tables 1 and S1).

2.2. Fungi isolation and DNA extraction

A total of 205 *D. pisi* isolates were recovered on PDA from AB contaminated dry pea seed and infected foliage collected from major dry pea production areas of Montana from 2016 to 2017. The isolates were assigned to four populations based on their geographic origin (Table 1).

The total genomic DNA was extracted from a 10-day-old culture grown on PDA using the DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instruction. The quality and concentration of extracted DNA were estimated using the NanoDrop 2000c

Table 1
Geographic origins of *Didymella pisi* population on *Pisium sativum*, the number of isolates from seed and plant sources and year of collection (Owati et al., 2017).

Region	No. of isolates	No. of isolates from seed	No. of isolates from plants	Counties represented	Year
Central	29	26	3	Cascade, Teton, and Judith Basin	2016
	7	7	0	Cascade, Teton, and Judith Basin	2017
North Central	32	30	2	Philips, Glacier, Hill, and Chouteau	2016
	9	9	0	Philips and Chouteau	2017
North East	77	67	10	Valley, Daniels, Sheridian, Garfield, Roosevelt, Richland, and Dawson	2016
	18	16	2	Daniels, McCone, Garfield, and Roosevelt	2017
South West	31	23	8	Gallatin	2016
	2	2	0	Gallatin	2017
Total	205	180	25		

Spectrophotometer at 260 nm (Thermo Fisher Scientific, Walthum, MA) and visualized in 1 % agarose gel stained with SYBR Safe DNA gel stain under ultraviolet-light (Bio-Rad Universal Hood II Gel Doc systems, Hercules, CA). DNA was stored at −20 °C until further use.

2.3. Genome sequencing and assembly

Whole genome DNA libraries were constructed using next-generation Illumina MiSeq sequencing technology (MCLAB) for one representative *D. pisi* isolate. The libraries were sequenced and 250 bp × 2 paired-end reads were obtained. The quality and nucleotide distribution of the sequences were determined using FastQC version 0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and Galaxy (https://usegalaxy.org/).

The removal of the indexed adapters, trimming of poor-quality sequence from the end of each sequence, and genome assembly was done using Galaxy (https://usegalaxy.org/) and CLC Genomic Workbench 10 (https://www.qiagenbioinformatics.com/products/clc-genomics-workbench/). SSR markers were developed from the assembled sequences.

2.4. SSR identification and primer design

A genome-wide SSR marker database was developed for *D. pisi*. *De novo* genome assembly was performed to obtain long contigs followed by a microsatellite search and primer design. SSR motifs were identified using simple sequence repeat locator (SSRLocator) (Da Maia et al., 2008) and an online simple sequence repeat identification tool (SSRIT) (Moges et al., 2016). The results obtained using these two search tools were compared and validated using tandem repeats finder (TRF) version 4.09 (https://tandem.bu.edu/trf/trf.basic.submit.html) and primers were designed for selected

Table 2
Characteristics of 33 polymorphic microsatellite markers developed in this study for population genetic diversity analysis of *Didymella pisi* isolates.

Locus	Forward primer Sequence (5′-3′)	Reverse primer Sequence (5′-3′)	Tm (°C)[a]	Allele size (bp)	Repeat motifs
AJO-1	CAGCACACTCAACATAGCAG	CTTAACCACTCACCTGACAAC	55	253	(AG)11
AJO-2	AAAAGAGAGAGGAACCAAGC	AAGGGAGGGTGAAGGTAGT	55	304	(GA)11
AJO-3	TCGTACGATTACTCTCCTCAC	GAGAAATAGCAGTAGGGTGCT	54	214	(CT)8
AJO-4	ACAGGGTGACCTTTGCTT	GACCATTGAAGGACAGAGG	55	368	(CA)12
AJO-5	AGCAGGCATTACGTTTAACT	GGTAAGATGCGAGTACGAAT	54	249	(AGC)6
AJO-7	ATAACAACCAACCTCTGACG	GGAGCAATAGGTGATCTTCTC	55	486	(ACC)6
AJO-8	CGAGCTCTACAACTACCTCCT	GCCTCCTCTTTGAGTATCTTC	55	402	(CGA)6
AJO-9	GGTGGACTGAGTTCTGTGTAG	TAGCGTGCTCTTGAGGATTA	55	199	(TTC)7
AJO-10	CCTCTGTCCTTCAATGGTC	GTTAGCAGGGTAGCGAAAG	55	332	(GTC)8
AJO-11	CTACGGAGTGCTCTACAAAGA	AGCTTGCTCACAGCATAAAG	55	354	(CTG)7
AJO-12	CTAGAATCGTGCTTGTTGC	GAGTCTCCCTGTCTTTGTCC	55	406	(TCG)7
AJO-13	AAGAGCTCAACCTAACACCTC	CTCTCTCTCTTCACACCCAAT	55	248	(GGT)8
AJO-14	CCAGTGTAACTCCTGTAATGC	GTCTCCATACAGACGACAGG	55	290	(CAG)10
AJO-15	GCACGACGAAACAGTAGTTC	AGGAGGTTGAAAGTTCGAG	55	255	(TGAG)6
AJO-16	GTACCCATTCATCGTACAGG	AGCCCAAAAGAGCAGAGT	55	209	(AATC)7
AJO-17	GAGTTGAGTTGAGTTGAGTCG	GAAAGACAGGGTAGGTAGGTG	55	385	(GGAT)5
AJO-18	GTACAGTGCAACACAGCAGTA	ACTCGAGACCACATAGCATT	55	405	(AAGG)6
AJO-19	CATGAGTCGGACTTGTTTTC	GTACGTCCATATCTGCGATT	55	401	(AATC)7
AJO-20	GTTGTCAGGTGAGTGGTTAAG	GTAGGTAGGCGTGTATCTGTG	54	230	(AGTG)7
AJO-22	AAGATGAGACTCGGAGAAGAG	CTTATATCCTGAACGCCTTG	55	235	(TGAGA)5
AJO-23	GATAGCCTGGTCATCATTTC	ATCCACAGTGATAGCGAATC	54	316	(ACAGT)5
AJO-25	CTTAATCTCAACCCACATCG	TGCTGTAAGCACACAGACAC	55	298	(CATCT)5
AJO-26	GGTTGGATTGTGGTGTAGTAG	GAGGGTGCTAGGTGACTTACT	54	199	(ATCGC)6
AJO-27	CGTAGTATGTGCAGGAAGAAG	GAGGGTGCTAGGTGACTTACT	55	390	(ATCGC)6
AJO-28	CACCCTACTGCTATTTCTCCT	TCTGATCTCCTTCTTGCAGT	55	211	(TACTC)5
AJO-29	ACAGTGTGTCTGTCTCGTCTC	GAAGTCATGAACAGGAGGAA	55	269	(TTCTT)4
AJO-30	TAGTGGGAGTGTGCTAGTGTC	GTGGATTAGGTTGAGGGTAGT	55	172	(GGTGA)4
AJO-31	CGAGACTCACACACACAATC	GTAGATACCCAACCCGACTAC	55	354	(AAGTCA)4
AJO-32	ACATGACCTCGACTGCTG	GCTGGACGTCGAGTTGTC	56	206	(CCACGC)8
AJO-33	CCATACGTTGACGTAGACAG	ACATTCCATACCCTCTGTACC	54	378	(TGGTAC)5
AJO-34	CCCCTAACACTTTCTCTCTCT	ACTTTTCAACCTACCGTCCT	54	398	(TAACTC)4
AJO-35	GTCCAGAAGGGACAAAGAC	ACTACGGGGTAGTTGCTTACT	54	396	(CCTGCA)6
AJO-36	AGGTGAGTGGAGGTGATAAGT	GTTTGTCGACACAGACAGAG	54	237	(TGTGCT)5

[a] Tm = Melting Temperature.

SSR loci using BatchPrimer version 3 (https://probes.pw.usda.gov/cgi-bin/batchprimer3/batchprimer3.cgi). The primers were validated using OligoAnalyzer version 3.1 (http://www.idtdna.com/calc/analyzer). The parameters for primer design included product sizes of 180–500 bp; primer size of 18–22 bp with optimal length 20 bp; primer melting temperature (Tm) of 50 °C–60 °C with an optimum at 55 °C; the GC % of 45 %–65 % with an optimum of 50 % and primers were at least 5 bp away from the SSR locus.

2.5. PCR amplification and genotyping

Standard gradient PCR was performed to determine the optimal annealing temperature for the PCR amplification for each SSR locus. The PCR assay was optimized in a final volume of 20 µl containing 10 µl of EconoTaq Plus 2x master mix according to manufacturer's recommendations (Lucigen Corporation, Middleton, WI, United States), 10 pM of each forward and reverse primer, and 2.5 µl DNA (50 ng). Cycling parameters were 4 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 56.6 °C, 30 s at 72 °C, and a final extension at 72 °C for 5 min. To reveal polymorphisms and for allele identification, the PCR products were analyzed on SYBR Safe-stained 2.5 % (w/v) agarose gels run in the 1x sodium-borate buffer (Brody and Kern, 2004) and exposed to UV light to visualize DNA fragments. The amplicon sizes were estimated using a 100-bp DNA ladder (Thermo Fisher Scientific, Walthum, MA). The SSR markers were scored for the presence or absence of the corresponding bands among the test isolates (Fig. S1) (Moges et al., 2016).

2.6. SSR polymorphism and genetic diversity

SSR markers were used to analyze the genetic diversity of 205 *D. pisi* isolates from Montana. Basic statistics, including the major allele frequency, the number of alleles per locus, heterozygosity, polymorphic information content (PIC), and gene diversity were estimated using GenAlEx version 6.503 (Peakall and Smouse, 2012). For individual SSR marker, the amount of polymorphism estimated by gene diversity was calculated for all 205 isolates (Nei, 1987). The PIC for each locus, which estimates the discriminatory power of the markers, was computed by $PIC = 1 - \sum Pi2$, where Pi^2 referred to the sum of the ith allelic frequency of each microsatellite locus for the genotypes (Anderson et al., 1993; Botstein et al., 1980).

In addition, the number of effective alleles per locus, number of different alleles per locus, number of private alleles, observed heterozygosity, expected heterozygosity, unbiased heterozygosity, banding pattern across the population, and Shannon's Information Index were estimated for each population using GenAlEx version 6.503 (Peakall and Smouse, 2012).

2.7. Population structure and gene flow

To assess the statistical distribution of gene diversity and estimate the variance components of the populations, analysis of molecular variance (AMOVA) based on co-dominant SSR loci was estimated using GenAlEx version 6.503 (Peakall and Smouse, 2012). In addition, population differentiation, PhiPT of the total populations and pairwise PhiPT among all pairs of populations were determined, and significance was tested based on 1000 bootstraps. Furthermore, the Principal Coordinate Analysis (PCoA) was computed using GenAlEx version 6.503 (Peakall and Smouse, 2012) to show the pattern of genetic variation of the populations of *D. pisi* isolates. An unweighted neighbor-joining dendrogram for the 205 isolates belonging to the four populations of *D. pisi* was constructed based on the Euclidean distances using Paleontological Statistics (PAST) version 3 (Hammer et al., 2001). The tree was bootstrapped with 1000 replicates.

Table 3
Diversity indices of the 33 microsatellite loci used in the study.

Locus	MAF	Na	GD	He	Ho	uHe	PIC	Ne	SI
AJO-1	0.273	2.00	0.232	0.034	0.017	0.236	0.956	1.360	0.378
AJO-2	0.248	2.00	0.226	0.015	0.007	0.229	0.954	1.308	0.380
AJO-3	0.956	2.00	0.314	0.085	0.043	0.317	0.114	1.472	0.491
AJO-4	0.934	1.75	0.296	0.168	0.084	0.299	0.177	1.502	0.436
AJO-5	0.965	1.75	0.250	0.134	0.067	0.252	0.086	1.378	0.386
AJO-7	0.992	1.25	0.073	0.127	0.064	0.073	0.029	1.103	0.117
AJO-8	0.906	1.75	0.293	0.238	0.119	0.296	0.271	1.508	0.432
AJO-9	0.984	1.25	0.094	0.177	0.088	0.095	0.058	1.151	0.141
AJO-10	0.880	1.75	0.320	0.233	0.117	0.323	0.328	1.569	0.463
AJO-11	0.675	1.75	0.322	0.250	0.125	0.325	0.663	1.587	0.463
AJO-12	0.987	1.25	0.088	0.162	0.081	0.089	0.048	1.137	0.135
AJO-13	0.791	1.75	0.369	0.239	0.119	0.373	0.450	1.726	0.514
AJO-14	0.912	2.00	0.390	0.118	0.059	0.394	0.203	1.667	0.575
AJO-15	0.955	1.75	0.275	0.149	0.074	0.278	0.104	1.436	0.415
AJO-16	0.956	1.50	0.188	0.237	0.119	0.189	0.141	1.321	0.279
AJO-17	0.912	1.75	0.313	0.203	0.102	0.316	0.238	1.554	0.455
AJO-18	0.961	1.50	0.202	0.241	0.120	0.204	0.095	1.338	0.296
AJO-19	0.731	2.00	0.440	0.163	0.081	0.445	0.553	1.797	0.631
AJO-20	0.921	1.25	0.123	0.362	0.181	0.124	0.271	1.242	0.171
AJO-22	0.831	1.75	0.314	0.254	0.127	0.318	0.457	1.542	0.457
AJO-23	0.938	1.50	0.194	0.268	0.134	0.196	0.203	1.344	0.285
AJO-25	0.804	1.75	0.339	0.242	0.121	0.342	0.486	1.621	0.483
AJO-26	0.970	1.50	0.172	0.205	0.103	0.174	0.095	1.275	0.263
AJO-27	0.941	1.75	0.276	0.191	0.096	0.278	0.168	1.460	0.413
AJO-28	0.968	1.50	0.175	0.211	0.106	0.177	0.104	1.285	0.266
AJO-29	0.003	0.50	0.003	0.000	0.000	0.003	0.990	1.003	0.008
AJO-30	0.632	2.00	0.461	0.065	0.032	0.467	0.674	1.874	0.653
AJO-31	0.995	1.25	0.062	0.105	0.052	0.062	0.019	1.082	0.104
AJO-32	0.493	2.00	0.401	0.038	0.019	0.406	0.794	1.685	0.589
AJO-33	0.992	1.25	0.073	0.127	0.064	0.073	0.029	1.103	0.117
AJO-34	0.897	2.00	0.365	0.172	0.086	0.369	0.280	1.614	0.547
AJO-35	0.765	1.75	0.329	0.250	0.125	0.332	0.566	1.601	0.471
AJO-36	0.985	1.50	0.143	0.157	0.079	0.144	0.039	1.199	0.230
Mean	0.832	1.64	0.246	0.170	0.085	0.248	0.323	1.420	0.365

MAF = Major allele frequency; **Na** = Number of different allele; **GD** = Genetic diversity; **He** = Expected heterozygosity; **Ho** = Observed heterozygosity; **uHe** = Unbiased heterozygosity; **PIC** = Polymorphic information content; **Ne** = Number of effective allele; and **SI** = Shannon's information index.

Using the SSR loci data, the detection of admixture and pattern of population structure were inferred using a Bayesian model-based clustering algorithm designed in STRUCTURE version 2.3.4 (Falush et al., 2003, 2007; Hubisz et al., 2009; Moges et al., 2016; and Pritchard et al., 2000). For this, two independent analyses were computed with and without prior information about the populations (Moges et al., 2016). The first run assigned the collection site as the putative population origin for individual isolates while the second run was without location assignment and letting the STRUCTURE software assign individuals to a population (Moges et al., 2016). To determine the most appropriate number of populations (K), a burn-in period of 25 000 was used in each run, and data were collected over 100 000 Markov chain Monte Carlo (MCMC) replications from K = 1 to K = 10 (Moges et al., 2016). This procedure group individuals into populations and estimates the

Table 4
Summary of the population diversity indices averaged over 33 microsatellite loci for isolates of *D. pisi* from four geographic regions in Montana.

Population	Na	Ne	PL	Ho	He	I
Southwest	1.212	1.161	24.24	0.385	0.094	0.139
Central	1.697	1.452	72.73	0.033	0.268	0.401
Northcentral	1.636	1.403	66.67	0.150	0.242	0.363
Northeast	2.00	1.662	100	0.076	0.379	0.557
Overall	1.636	1.420	65.91	0.161	0.246	0.365

Na = Number of different allele; **Ne** = Number of effective allele; **PL** = Percentage of polymorphic loci; **Ho** = Observed heterozygosity; **He** = Expected heterozygosity; and **SI** = Shannon's information index.

Table 5
Analysis of molecular variance among and within populations of *D. pisi* populations from Montana based on 33 SSR loci.

Source	Degree of freedom	Sum of squares	Mean squares	Estimated Variance	Variation (%)	P-value
Among populations	3	90.744	30.248	0.575	15	<0.001
Within population	201	641.608	3.192	3.192	85	<0.001
Total	204	732.351		3.767	100	<0.001

proportion of membership in each population for individuals (Moges et al., 2016; Pritchard et al., 2000). The K value was determined by the log probability of data (Ln P(D)) based on the rate of change in LnP(D) between successive K (Earl and von Holdt, 2012; Moges et al., 2016). The optimum K value was predicted following the simulation method of (Evanno et al., 2005) using the web-based software STRUCTURE HARVESTER version 0.6.92 (Earl and von Holdt, 2012).

3. Results

3.1. De-novo assembly of sequence and marker development

De novo assembly of the sequence data produced 4283 contigs covering 33.8 Mb with an average N50 of 25.2 Kb and an average contig length of 9.2 Kb. Each of the thirty-three SSR primer pairs successfully produced a clear single amplicon at annealing temperatures of 54 °C or 55 °C (Table 2). Multiplex PCR amplifications were performed using three to five SSR primer pairs in each PCR based on annealing temperatures.

3.2. SSRs polymorphism and gene diversity

The polymorphism and diversity of the different SSR loci are presented in Table 3. The 33 polymorphic SSR markers detected a total of 216 alleles with an average of 1.63 alleles per marker. The PIC values for the markers ranged from 0.02 (AJO-7) to 0.99 (AJO-29), with an average of 0.323 per marker. Seven SSR loci were highly polymorphic (PIC $\geq$ 0.5), six were moderately polymorphic (0.5 < PIC >0.25), and twenty were slightly polymorphic (PIC < 0.25).

The frequency of the major alleles in each locus ranged from 0.003 to 0.995, with an average of 0.831. The number of effective alleles was in the range of 1.003–1.874, with an average of 1.420. Gene diversity, defined as the probability that an individual from a population will be heterozygous at a given locus (Moges et al., 2016), ranged from 0.003 (AJO-29) to 0.461 (AJO-30), with an average of 0.246. Furthermore, a low level of heterozygosity (0.000–0.180) was observed in the *D. pisi* isolates used in this study. In addition, one SSR locus had no heterozygosity while one displayed less than 0.01 heterozygosity. The expected heterozygosity ranged from 0.000 to 0.362 (Table 3). Based on the Hardy–Weinberg equilibrium (HWE) exact test for all populations, 32 loci (96.9 %) exhibited significant deviation from HWE corrected for multiple comparisons (P < 0.001), by having less expected levels of heterozygosity. However, one locus (AJO-29) did not show significant departure from HWE. These results are not surprising, especially when most of the *D. pisi* isolates used in the study were assumed to be from the asexual population.

3.3. Population genetic diversity

A summary of the genetic diversity estimates for the four populations of *D. pisi* is presented in Table 4. The number of different alleles (Na), and effective alleles (Ne) averaged across all loci ranged from 1.212 to 2.00 and 1.161 to 1.662, respectively for the four populations (Southwest, Northcentral, Central, and Northeast). The Northeast population had the highest while the Southwest population had the lowest Na, and Ne values. Average observed heterozygosity (Ho) was in the range of 0.033–0.385, with a mean of 0.161 across all loci. Genetic diversity was the lowest in Southwest population (He = 0.094) and the highest in Northeast population (He = 0.379), and its value averaged over all populations and loci was 0.246 (SE = 0.017). The percentage of polymorphic loci (PL) ranged from 24.24 % in the Southwest to 100 % in the Northeast, with an average of 65.91 %. Based on the Shannon's Index (I), a higher diversity was observed in the Northeast (I = 0.557) population and was least in the Southwest (I = 0.139) population.

3.4. Population genetic structure and gene flow

Analysis of molecular variance (AMOVA) showed that the differences among isolates within the population accounted for 85 % and the differences among the different populations accounted for 15 % of the total genetic variation detected (Table 5). There was significant genetic variation among the populations based on the randomization test (PhiPT = 0.153 at P < 0.001). The pairwise PhiPT values of the genetic distance among all populations were significant (P < 0.01, Table 6). The PhiPT value between northcentral and central populations was the smallest (0.000) while the PhiPT value between northeast and southwest populations was the largest (0.254). A similar pattern of differentiation among subgroups was observed using Nei's genetic distance. The Nei's genetic distance value between northcentral and central populations was the smallest (0.015) distance while the genetic distance value between northeast and southwest populations was the largest (0.237, P < 0.001, Table 7).

The unweighted Neighbor-joining dendrogram grouped the 205 isolates of the four populations into two major clusters (Fig. 1A and B). From the 205 isolates, 106 and 99 isolates were grouped together in Cluster I and II respectively. The overall topology of the dendrogram indicated the presence of two clades in *D. pisi* associated with dry pea in Montana. Several sub-clades were observed for the populations indicating genetic variability within and among isolates in each population.

Table 6
Pairwise genetic distance based on PhiPT matrix, a measure of divergence among the *D. pisi* populations from Montana.

Population	SW	NE	NC
NE	0.254		
NC	0.085	0.131	
C	0.130	0.103	0

PhiPT matrix is related to variance in allele frequency among the population.

Table 7
Pairwise genetic distance based Nei's Genetic Distance among the *D. pisi* populations from Montana.

Population	SW	NE	NC
NE	0.237		
NC	0.050	0.097	
C	0.065	0.079	0.015

Nei's Genetic Distance measures genetic differences over time due to mutation and genetic drift.

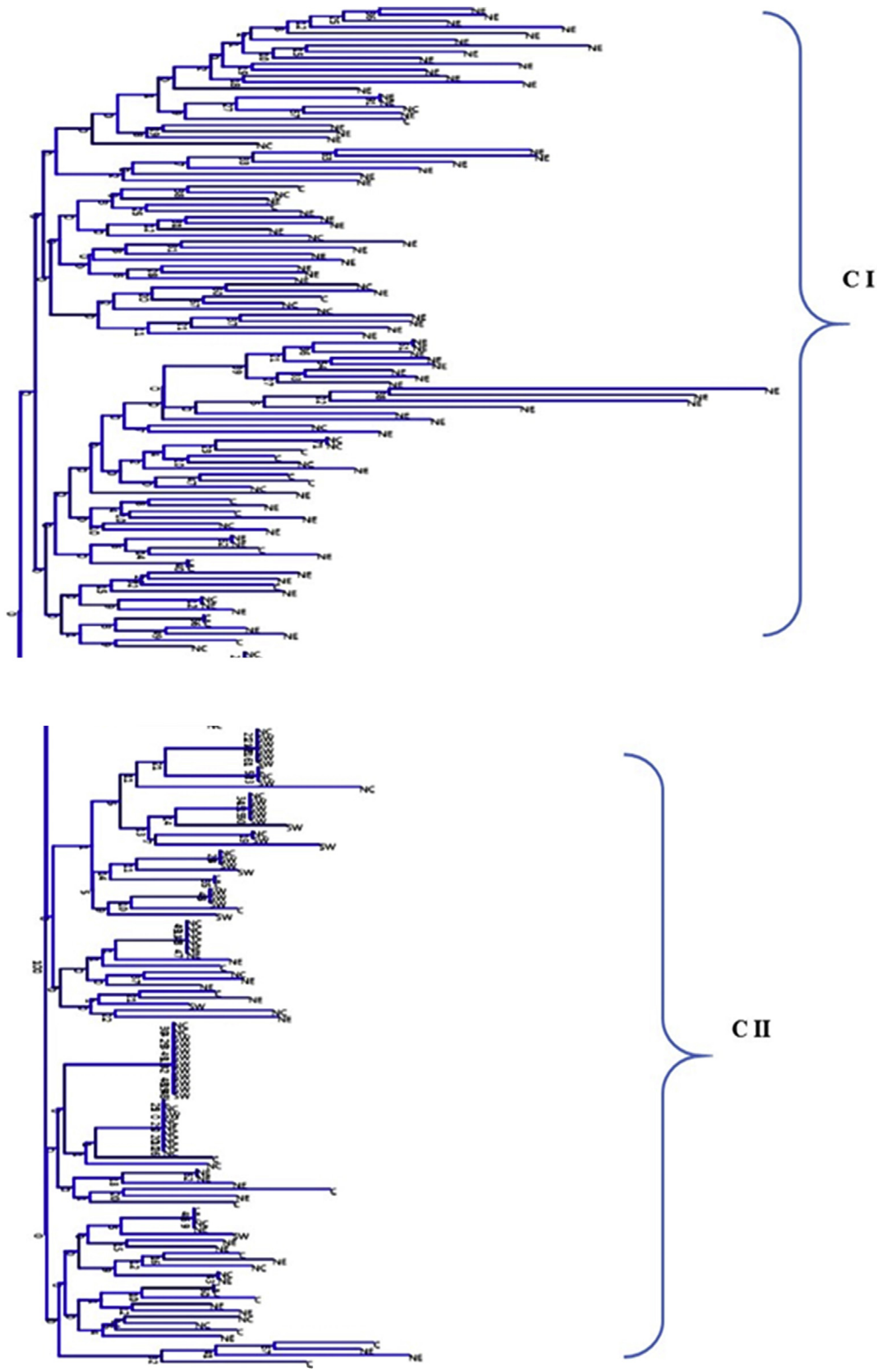

Fig. 1. (**A**) Unweighted Neighbor-joining tree using the simple matching similarity coefficient based on 33 microsatellite markers for the 205 isolates of *Didymella pisi* isolated from dry pea in Montana. The tree shows the clustering pattern of isolates (C I = cluster 1) from the four *D. pisi* populations. (**B**) Unweighted Neighbor-joining tree using the simple matching similarity coefficient based on 33 microsatellite markers for the 205 isolates of *Didymella pisi* isolated from dry pea in Montana. The tree shows the clustering pattern of isolates (C II = cluster 2) from the four *D. pisi* populations.

A similar pattern of clustering was observed in the Principal Coordinate Analysis (PCoA) based on the 33 microsatellite loci (Fig. 2). Based on the Evanno et al. (2005) method on STRUCTURE outputs, it predicted K = 2 to be the most likely number of clusters (Fig. 3A and B).

4. Discussion

Ascochyta blight of dry pea is caused by a complex of fungal pathogens. *D. pisi* is the predominant causal agent in Montana and has also been reported to cause an epidemic in Europe (Kaiser et al.,

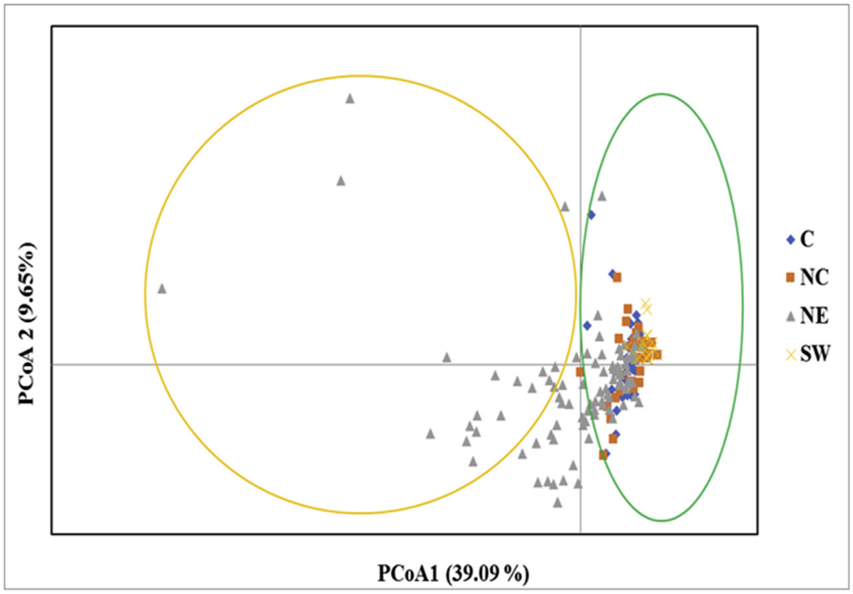

Fig. 2. Principal coordinates analysis (PCoA) bi-plot showing the clustering of the 205 *D. pisi* isolates based on 33 microsatellite loci. The four populations are color coded as follows: C, Central region (blue); NC, Northcentral region (orange); NE, Northeast region (gray); SW, Southwest region (gold). Percentages of variation explained by the first 3 axes (1, 2, and 3) are 39.09, 9.65, and 8.08 %, respectively.

2008). However, SSR markers have only been developed for *D. rabiei*, the causal pathogen of ascochyta blight of chickpea (Baite et al., 2017). The present study is the first report of *D. pisi* SSR markers development and their use for a genetic diversity and population structure study. This study has increased the number of population genetic studies on pathogenic fungi. Currently, population genetic studies of fungal pathogens are low when compared with other organisms (Dutech et al., 2007; Zane et al., 2002). Information on pathogen genetic diversity and population structure on the landscape scale are important to understand the potential of pathogen populations to spread, increase aggressiveness, develop fungicide resistance, and overcome host resistance (Ciampi et al., 2011; Marulanda et al., 2014).

In this study, 33 polymorphic markers were developed and used to evaluate the genetic diversity of *D. pisi* isolates from dry pea. The polymorphism detected by the markers ranged from slightly informative to highly informative. The PIC value of a marker, which refers to the discriminatory power of a locus while accounting for the number and relative frequency of the alleles.

The AMOVA results supported the presence of genetic diversity in the *D. pisi* population in Montana. The highest percentage of variation (85 %) was within populations of *D. pisi* isolates. However, the gene diversity observed among the Montana *D. pisi* populations was low. This might be associated with the introduction of a few genotypes of the fungus into Montana as well as low level of seeds coming from outside Montana. Also, the incidence of *D. pisi*, unlike *P. pinodes* is very low in North Dakota and Canada, which are neighboring regions to Montana with high production of dry pea (Gossen et al., 2011; Sivachandra-Kumar and Banniza, 2017). High genetic diversity was detected in the northeast region of Montana while genetic diversity was lowest in the southwest. Additionally, Shannon's index value was highest in the northeast and lowest in southwest Montana. This is an information statistic index, which assumes all types are represented in a sample and that they are randomly sampled (Heip et al., 1998; Moges et al., 2016; and Morris et al., 2014). The genetic variability between these two regions is expected because the northeast region is the epicenter of dry pea production in Montana and might be due to the early introduction of the fungus in this region. This allows time for genetic mutation, genetic drift, and recombination which results in greater genetic diversity (McDonald, 1997; Moges et al., 2016). This can be associated with the high incidence of ascochyta blight recorded on seed lots sent to the RPCDL for seed test from NE region than from other regions of Montana. These differences may also be due to environmental conditions, geography, and differences in alternative host species diversity that may be associated with generating variability within populations (McDonald, 1997; Moges et al., 2016). Several factors such as single nucleotide mutation and recombination, gene gain and gene loss, horizontal gene transfer, loss of heterozygosity, genome rearrangement, and conditionally dispensable chromosomes have been associated with genetic variation in fungi (McDonald, 1997; Taylor et al., 2017). *Didymella pisi* is a heterothallic fungus. It is known that a heterothallic fungal pathogen that exhibits sexual recombination poses a greater threat when they inbreed due to the emergence of new genotypes (McDonald, 1997; Taylor et al., 2017). However, genetic diversity may not be affected by the mating system because fungi that reproduce exclusively through asexual reproduction may have a similar number of alleles at individual loci as those that reproduce sexually (McDonald, 1997; Moges et al., 2016). Thus, gene diversity is affected by the age of a population, population size, and selection processes (McDonald, 1997). For instance, populations that have evolved over a long time at one location are expected to have more alleles than newly introduced populations, because there has been more time for mutation to introduce new variants and for genetic drift to increase the frequencies of new alleles to detectable levels (McDonald, 1997).

Information on the population structure of *D. pisi* populations from different locations improves the understanding of the biology of the pathogen, evolutionary, and potentially adaptive genotypic diversity in the species (Marulanda et al., 2014). The *D. pisi* isolates from the four geographic regions of Montana are closely related as reflected by the high genetic identity among populations. Furthermore, based on the population genetic analyses of the Montana *D. pisi* isolates, the population was categorized into two sub-populations. STRUCTURE analysis, PCoA, and the unweighted Neighbor-joining algorithm supported and indicated admixture among the two populations. Evidence of admixture among isolates

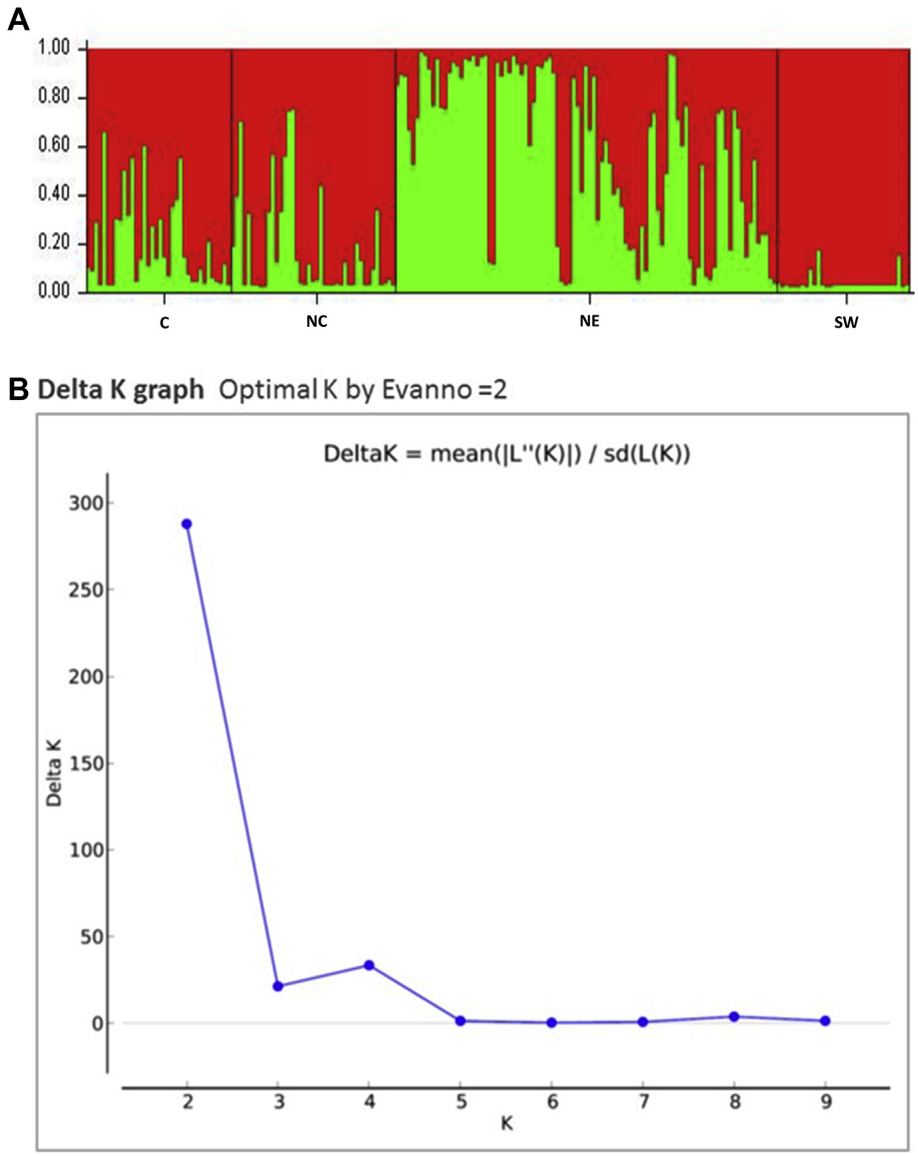

Fig. 3. (**A**) Bayesian model-based estimation of population structure (K = 2) for the 205 *D. pisi* isolates in four pre-determined populations (x-axis): Central Montana (C), Northcentral Montana (NC), Northeast Montana (NE), and Southwest Montana (SW). Each group is separated by a black vertical line. Numbers in the y-axis show the coefficient of assignment. (**B**) The relationship between K and Delta K based on the Evanno method (Evanno et al., 2005). K = appropriate number of subpopulations.

from the different regions does not support geographical separation of isolates into distinctly isolated sub-populations. The PhiPT value (0.153) between the *D. pisi* populations evaluated in this study indicated moderate differentiation among the groups that might be attributed to gene flow among regions. The moderate degree of diversity in populations of *D. pisi* shown in this study may be attributed to the dispersal of the clonal inoculum over long distances that may allow for pathogen spread in dry pea-growing areas in Montana. This dispersal may be associated with the exchange of dry pea germplasm between growers, seed elevators, and researchers.

The microsatellite markers developed in this study were used to understand the genetic diversity and population structure of *D. pisi* isolates from dry pea growing regions of Montana. Despite regional variations, the observed genetic diversity in all four populations was lower than expected, suggesting inter-regional exchanges of planting materials and dispersal of inoculum among the regions. This study generated information that can be used to further understand the pathogen biology and its evolutionary potential and provide the basis for other studies on disease development, host–pathogen interactions, and development of disease management strategies which includes development and use of resistant dry pea varieties. In addition, information generated from this study can be used to design novel specific primers for characterization of *D. pisi*.

Authors contribution

AO and BA conceived and designed the experiments. AO performed the experiment, analyzed the data, and wrote the manuscript. MB and BA reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2019.02.004.

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