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RESEARCH PAPERS

Genetic diversity among phytopathogenic Sclerotiniaceae, based on retrotransposon molecular markers

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Summary. Molecular marker systems have been widely used for determination, discrimination and population structure analysis in the Sclerotiniaceae. The usefulness of a new marker system iPBS, based on the sequences of reverse transcriptase primer binding sites in long terminal repeats retrotransposons, was investigated with 34 isolates of six species confirmed by species-specific markers. Six of the iPBS primers were found to produce highly polymorphic (98%) and very distinct species-specific band patterns. Each primer amplified, on average, 33.5 polymorphic bands that was sufficient for species differentiation. The polymorphism information content was 0.896, indicating better discriminating power of markers, , the Shannon's information index was 0.438, and the genetic distance was 0.707 as average values. UPGMA cluster analysis based on retrotransposons divided all the isolates into three cluster and six sub-clusters in accordance with their species. Principal co-ordinate analysis also strongly confirmed this cluster pattern. The iPBS marker system was therefore a useful tool for evaluation of genetic variation at intra- and inter-species, and at the population levels for members of the Sclerotiniaceae. Furthermore, the iPBS markers could provide easy discrimination of *Botrytis cinerea* from *Botrytis pseudocinerea*.

Key words: iPBS, retrotransposons.

Introduction

Species of the Sclerotiniaceae (Ascomycota: Helotiales) are characterized by stalked apothecia with inoperculate asci produced on the living plant tissues or saprotrophically on dead tissues. Plant pathogenic members of this family, such as *Sclerotinia* spp., *Botrytis* spp. and *Monilinia* spp., cause economically important diseases with wide host ranges. Three white mold pathogens in the *Sclerotinia* genus infect many plant hosts; these are *Sclerotinia trifoliorum* Erikks, *Sclerotinia sclerotiorum* Lib. de Bary and *Sclerotinia minor* Jagger (Kohn *et al.*, 1988). *Botrytis cinerea*, the causal agent of gray mold, has more than 200 host plant species, while *Botrytis pseudocinerea*, newly separated from *B. cinerea* and identified as a new species, causes losses in several fruit and vegetable crops (Walker *et al.*, 2011). *Monilinia fructigena* causes severe brown rot on fruits (Côté *et al.*, 2004). These fungi have been studied intensively because of their economic importance. However, their classification at species level based on morphological characters poses difficulties (Ekins *et al.*, 2005; Hirschhäuser and Fröhlich, 2007; Plesken *et al.*, 2015).

Molecular techniques have been widely used in recent decades to overcome bottlenecks and reveal the genetic variation at intra- and interspecies levels. Internal transcribed spacer (ITS) region or 18S rDNA sequence analyses are most commonly used for identification of filamentous fungi. However, these sequences cannot be used to evaluate genetic diversity in Sclerotiniaceae due to high similarity (Freeman *et al.*, 2002). Therefore, researchers have focused on

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different molecular marker techniques. Walker *et al.* (2011) described a method for discrimination of *B. cinerea* from *B. pseudocinerea* using the PCR-RFLP technique relying on the Bc-*hch* locus. Plesken *et al.* (2015) differentiated *Botrytis* species by sequence analysis of the *g3pdh*, *tubA*, and *ms547* genes. Hirschhäuser and Fröhlich (2007) showed that analysis based on sequences of the laccase 2 gene (*lcc2*) situated in the genome of Sclerotiniaceae genomes provided useful information for detection of *S. sclerotiorum* and *S. minor*. Vleugels *et al.* (2012) discriminated *S. trifoliorum* from *S. sclerotiorum* and *S. minor* in a study by using the AFLP technique to reveal genetic diversity. Andrew and Kohn (2009) identified *S. minor*, *S. sclerotiorum* and *S. trifoliorum* by using a single nucleotide polymorphism. Abd-Elmagid *et al.* (2013) developed a multiplex assay to differentiate four *Sclerotinia* species in a single PCR reaction, with species-specific primers for the *Aspr* gene of *S. sclerotiorum*, the *Cad* gene of *S. trifoliorum*, the *Ef1-α* gene of *S. homoeocarpa*, and the *lcc2* gene of *S. minor*.

Retrotransposons, which is a main class of transposable elements, have been identified as an excellent source of molecular markers due to their structures and transport mechanisms. These transposons can move from one location to another within a genome, while the original copy remains in its original locus (Muszewska *et al.*, 2011). Many fungal transposable elements (TEs) have been identified, especially in Ascomycota species (Daboussi and Capy, 2003). Based on TEs, various PCR based marker systems, such as retrotransposon-microsatellite amplified polymorphism (REMAP) and inter-retrotransposon amplified polymorphism (IRAP), have been developed to reveal genetic diversities in fungi (Chadha and Gopalakrishna, 2007; Jawhar and Arabi, 2009). These marker systems have some restrictions, such as the requirement of sequencing data to be universal for each examined organism. Kalendar *et al.* (2010) demonstrated inter primer binding site (iPBS) retrotransposons as an "*Universal Retrotransposon Markers*" system for molecular characterization of plants and animals. To date, studies performed in plants revealed that iPBS was a powerful DNA fingerprinting technique not requiring previous knowledge of sequencing of long terminal repeat retrotransposons. Recently, Pourmahdi and Taheri (2015) and Özer *et al.* (2016) indicated that this marker system also provided useful information for the genetic differentiation of fungi at both intra- and interspecies levels.

The aim of the present study was to evaluate the genetic diversity among members of the Sclerotiniaceae using iPBS markers.

Materials and methods

Fungal material

Fungal isolates were obtained from infected plants or provided by different researchers (Table 1). The isolates were retrieved from different diseased plants, and isolation of pathogens was carried out according to procedures described previously (Boehm *et al.* 2001; Fournier *et al.*, 2003; Ekins *et al.*, 2005; Vleugels *et al.* 2012). Surface sterilized plant tissues were placed in Petri dishes containing potato dextrose agar (PDA), and the plates were incubated at 23°C for 7 d under 12 h light/ 12 h dark cycle. Cultures were then maintained on PDA at 8°C.

DNA isolation and molecular identification

Fungal mycelium of each isolate was recovered by scraping with a sterile scalpel from the surface of medium and grinding in liquid nitrogen. DNeasy Plant Mini Kit (Qiagen, Cat No./ID: 69106) was used for DNA extraction according to manufacturer's instructions. The quantity of resulting DNA was determined using a DS-11 FX+ spectrophotometer (Denovix) and diluted to 10 ng μL^{-1} with ddH_2O. PCR was performed (with species-specific primer sets) to identify *S. minor* (SMLcc2 F-SMLcc2 R), *S. trifoliorum,* (STCad F-STCad R), *S. sclerotiorum* (SSaspr F-SSaspr R) and *M. fructigena* (ITS1-Mfg-R2) (Table 2; Hughes *et al.*, 2000; Abd-Elmagid *et al.*, 2013). The identification of *Botrytis* species was confirmed using the PCR-RFLP method based on the Bc-*hch* locus (Fournier *et al.*, 2003).

iPBS PCR assays

All iPBS primers (Kalendar *et al.*, 2010) were screened to evaluate their ability to produce sharp banding profiles among the isolates. The PCR assays were performed using two biological replicates with selected iPBS primers (Table 3) in 25 μL reaction volume containing 25-50 ng template DNA, 1× Dream Taq Buffer, 0.6 μM for 18 nt primers or 1 μM primer for 12-13 nt primers, 0.2 mM dNTPs, and 1.5 units of Dream *Taq* DNA polymerase (Thermo Scientific)

Table 1. Thirty-four fungal isolates, representing six species in Sclerotiniaceae.

No.	Species	Host plant	Location
Sc_01	*Botrytis cinerea*	Cornelian cherry	Bolu-Turkey
Sc_02	*Botrytis cinerea*	Cornelian cherry	Bolu-Turkey
Sc_03	*Botrytis cinerea*	Cornelian cherry	Bolu-Turkey
Sc_04	*Botrytis cinerea*	Cornelian cherry	Bolu-Turkey
Sc_05	*Botrytis cinerea*	Hawthorn	Bolu-Turkey
Sc_06	*Botrytis cinerea*	Artichoke	Bursa-Turkey
Sc_07	*Botrytis cinerea*	Strawberry	Mersin-Turkey
Sc_08	*Botrytis cinerea*	Vineyards	France
Sc_09	*Botrytis pseudocinerea*	Vineyards	France
Sc_10	*Sclerotinia minor*	Jerusalem artichoke	Ankara-Turkey
Sc_11	*Sclerotinia minor*	Jerusalem artichoke	Ankara-Turkey
Sc_12	*Sclerotinia minor*	Jerusalem artichoke	Ankara-Turkey
Sc_13	*Sclerotinia minor*	Jerusalem artichoke	Ankara-Turkey
Sc_14	*Sclerotinia minor*	Jerusalem artichoke	Ankara-Turkey
Sc_15	*Sclerotinia minor*	Jerusalem artichoke	Ankara-Turkey
Sc_16	*Sclerotinia minor*	Jerusalem artichoke	Ankara-Turkey
Sc_17	*Sclerotinia minor*	Lettuce	Ankara-Turkey
Sc_18	*Sclerotinia trifoliorum*	Red Clover	Belgium
Sc_19	*Sclerotinia trifoliorum*	Red Clover	Belgium
Sc_20	*Sclerotinia trifoliorum*	Red Clover	Belgium
Sc_21	*Sclerotinia trifoliorum*	Red Clover	Belgium
Sc_22	*Sclerotinia sclerotiorum*	Jerusalem artichoke	Ankara-Turkey
Sc_23	*Sclerotinia sclerotiorum*	Jerusalem artichoke	Ankara-Turkey
Sc_24	*Sclerotinia sclerotiorum*	Jerusalem artichoke	Ankara-Turkey
Sc_25	*Sclerotinia sclerotiorum*	Jerusalem artichoke	Ankara-Turkey
Sc_26	*Sclerotinia sclerotiorum*	Jerusalem artichoke	Ankara-Turkey
Sc_27	*Sclerotinia sclerotiorum*	Jerusalem artichoke	Ankara-Turkey
Sc_28	*Sclerotinia sclerotiorum*	Jerusalem artichoke	Ankara-Turkey
Sc_29	*Sclerotinia sclerotiorum*	Sunflower	Bursa-Turkey
Sc_30	*Monilinia fructigena*	Quince	Bolu-Turkey
Sc_31	*Monilinia fructigena*	Quince	Bolu-Turkey
Sc_32	*Monilinia fructigena*	Quince	Bolu-Turkey
Sc_33	*Monilinia fructigena*	Quince	Bolu-Turkey
Sc_34	*Monilinia fructigena*	Quince	Bolu-Turkey

Table 2. Details of the species-specific primers used in this study.

Species	Primer code	Primer sequence 5′–3′)	Product size bp)	Reference
Botrytis cinerea	262	AAGCCCTTCGATGTCTTGGA	with HhaI: 517, 287, 155, 119, 84 and 9 bp	Fournier *et al.*, 2005
	520L	ACGGATTCCGAACTAAGTAA		
Botrytis pseudocinerea	262	AAGCCCTTCGATGTCTTGGA	with HhaI: 601, 287, 155, 119, 84 and 9 bp	
	520L	ACGGATTCCGAACTAAGTAA		
Sclerotinia minor	SMLcc2F	CCCTCCTATCTCTCTTCCAAACA	264	Ahmed Abd-Elmagid *et al.*, 2013
	SMLcc2R	TGACCAATACCAATGAGGAGAG		
Sclerotinia trifoliorum	STCadF	TCCTAGATCGACTCTCCTCCTTT	97	
	STCadR	CGTGTTATTGCCTCCTTGTTG		
Sclerotinia sclerotiorum	SSasprF	CATTGGAAGTCTCGTCGTCA	171	
	SSasprR	TCAAACGCCAAAGCTGTATG		
Monilinia fructigena	ITS1	TCCTCCGCTTATTGATATGC	460	Hughes *et al.*, 2000
	Mfg-R2	GGTCAACCATAGAAAATTGGT		

Table 3. Primer names, their sequences, annealing temperatures, numbers of amplified and polymorphic bands, and some diversity parameters of iPBS retrotransposons primers, used in the study.

iPBS primers	Sequences (5′–3′)	Tm (°C)	Number of bands		Diversity parameters				
			Total	Polymorphic	P%	Ne	h	I	PIC
iPBS2221	ACCTAGCTCACGATGCCA	57	41	41	100	1.493	0.307	0.471	0.909
iPBS2237	CCCCTACCTGGCGTGCCA	55	28	27	96.43	1.437	0.274	0.426	0.909
iPBS2239	ACCTAGGCTCGGATGCCA	55	53	53	100	1.455	0.288	0.450	0.918
iPBS2242	GCCCCATGGTGGGCGCCA	57	24	24	100	1.505	0.312	0.478	0.909
iPBS2390	GCAACAACCCCA	57	30	28	93.33	1.414	0.272	0.426	0.853
iPBS2395	TCCCCAGCGGAGTCGCCA	53	29	28	96.55	1.320	0.217	0.358	0.876
Total			205	201					
Average			34.17	33.50	98.04	1.441	0.280	0.438	0.896

Tm, annealing temperatures; *P%*, polymorphism percentage; *Ne*, number of effective alleles; *h*, Nei's (1973) gene diversity; *I*, Shannon's Information index; PIC, polymorphism information contents.

(Kalendar *et al.*, 2010). The amplifications were conducted with a Bio-Rad T100™ thermocycler under the following temperature profiles: one cycle initial denaturation at 95°C 3 min, 30 cycles of 95°C 15 sec, 50–65°C 1 min annealing (depending upon primers), and 68°C 1 min; and then at 72°C 5 min (Baloch *et al.*, 2015). The amplified products were separated on a 1.5% (w/v) agarose gel with 1× TAE buffer for 2.5 h and visualized on using an Imager Gel Doc XR+ system (Bio-Rad), after staining with ethidium bromide.

iPBS data analyses

To construct a binary matrix, reproducible fragments were scored as presence (1) or absence (0). Genetic distance among the isolates was evaluated us-

ing R version 3.3.1 according to the Jaccard's index. Shannon's information index and gene diversity for each primer were calculated using POPGENE ver. 1.32 software (Yeh *et al.*, 1999). Principal coordinate analysis (PCoA) and the unweighted pair-group method with arithmetic mean (UPGMA) phenogram were carried out using the VEGAN *package* in R version 3.3.1 and MEGA ver.7 software (Kumar *et al.*, 2016). The mean polymorphism information contents (PIC) of each primer were estimated using the following formula: PIC = Σ (1-pi^2)/n, where "pi" is the frequency of presence 1 for each band, and "n" is the number of bands for each primer (Weir, 1990).

Results

Molecular identification of fungal isolates

Specific primer pairs amplified the predicted size DNA fragments, and the classification of 34 fungal isolates, representing six species from two genera, was verified. SMLcc2F/R amplified a 264 bp fragment for *S. minor* isolates, STCadF/R amplified a 97 bp fragment for *S. trifoliorum* isolates, and SSasprF/R amplified a 171-bp fragment for *S. sclerotiorum* isolates. The isolates of *M. fructigena* produced the expected 460 bp DNA fragment with the primer set ITS1/Mfg-R2. The 262/520L primer pair amplified a 1171 bp DNA fragment corresponding to the region of the Bc-*hch* gene for all *Botrytis* spp. isolates. After digestion of this PCR product with the *Hha*I restriction enzyme, two polymorphic restriction patterns were generated, allowing separation of the isolates (Table 2).

Genetic diversity among members of the Sclerotiniaceae

Six iPBS primers, iPBS2221, iPBS2237, iPBS2239, iPBS2242, iPBS2390, and iPBS2395, were determined to amplify distinct species-specific band patterns among the 34 fungal isolates examined. These markers produced 205 bands, of which 201 were polymorphic with 98% polymorphism among all isolates (Table 3). The number of amplified fragments with iPBS

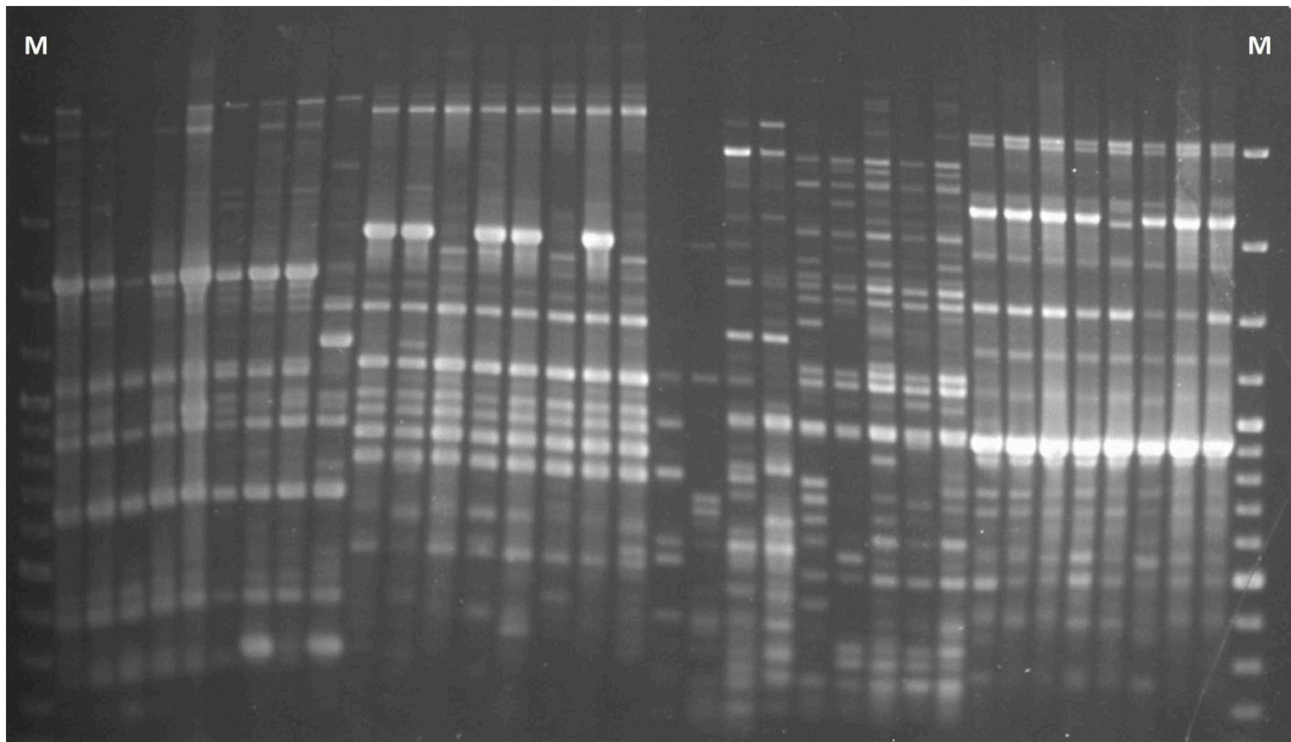

Figure 1. A representative gel of reproducible profiles with iPBS2239 primer. Sample order (from left to right) is as listed in Table 1. The DNA marker (M) is GeneRuler 100 bp plus (Thermo Scientific).

primers varied from 24 (iPBS2242) to 53 (iPBS2239), with an average of 34.2 bands per primer (Figure 1). The number of polymorphic bands ranged from 24 to 53, while the average number of polymorphic bands being 33.5.

PIC values for each primer ranged from 0.853 to 0.918 with an average of 0.896, and these were less than 0.9 for two primers including iPBS2390 and iPBS2395. The Nei gene diversity was the least (0.217) for iPBS2395 and the greatest (0.312) for iPBS2242. The same trend was observed for the Shannon's information index which varied from 0.358 (iPBS2395) to 0.478 (iPBS2242), with a mean value of 0.438.

Intraspecies differentiation was observed at different levels for each species (Table 4). The rates of polymorphism were calculated to be 28% for *B. cinerea*, 26% for *S. minor*, 58% for *S. trifoliorum*, 30% for *S. sclerotiorum*, and 32% for *M. fructigena*. This analysis could not be performed for *B. pseudocinerea* because only one isolate was included in the study.

The evaluation of Jaccard distance among all pairs of isolates revealed that the average pairwise genetic distance was 0.707. The greatest genetic distance for isolates was 0.906 between Sc_05 and Sc_21, while the least distance was 0.026 between Sc_12 and Sc_15. The greatest genetic distance among the species was 0.884, between *B. cinerea* and *M. fructigena*, while the least genetic distance was 0.533, between *B. cinera* and *B. pseudocinerea* (Table 5).

UPGMA cluster analysis grouped all isolates into three major groups (Figure 2). Cluster I included isolates belonging to three *Sclerotinia* species (*S. trifoliorum*, *S. sclerotiorum*, and *S. minor*), which cause white mold diseases, and subdivided isolates into three distinct subgroups depending on their species. *Monilinia fructigena* isolates constituted cluster II. *Botrytis* isolates were grouped in cluster III, which was further subdivided into two subgroups, and the *B. pseudocinerea* isolate differentiated from eight isolates of *B. ci-*

Table 4. Intraspecies genetic variation among isolates belonging to five fungal species, based on iPBS retrotransposons data.

Species	NI	TAB	NPB	PWS (%)
Botrytis cinerea	8	67	19	28
Sclerotinia minor	8	47	12	26
Sclerotinia trifoliorum	4	66	38	58
Sclerotinia sclerotiorum	8	74	22	30
Monilinia fructigena	5	53	17	32

NI, number of isolates; TAB, total amplified bands; NPB, number of polymorphic bands; PWS%, percent polymorphism within species.

Table 5. Genetic distance matrices among six Sclerotiniaceae species, based on iPBS retrotransposons data.

	Bc	Bpc	Sm	St	Ss	Mf
B. cinerea	***					
B. pseudocinerea	***0.533***	***				
S. minor	0.868	0.816	***			
S. trifoliorum	0.877	0.855	0.793	***		
S. sclerotiorum	0.863	0.870	0.769	0.774	***	
M. fructigena	***0.884***	0.880	0.837	0.819	0.836	***

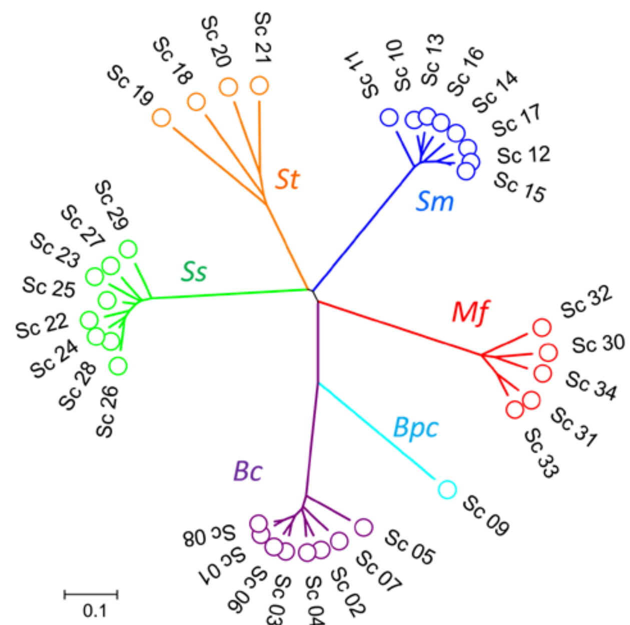

Figure 2. UPGMA dendrogram of 34 Sclerotiniaceae isolates examined in this study, constructed with retrotransposon data. Isolate identification codes as indicated in Table 1. *St* = *Sclerotinia trifoliorum*, *SM* = *Sclerotinia minor*, *Ss* = *Sclerotinia sclerotiorum*, *Mf* = *Monilinia fructigena*, *Bc* = *Botrytis cinerea*, *Bpc* = *Botrytis pseudocinerea*. The scale bar (0.1) indicates 10% genetic distance.

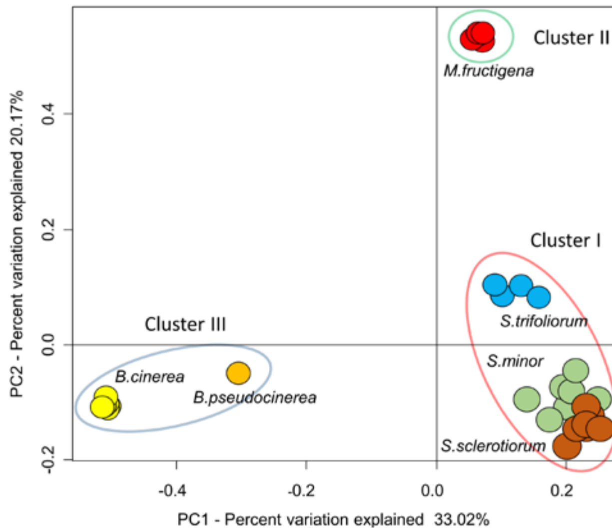

Figure 3. Association matrix for six fungal species, based on principal coordinate analysis.

nerea. The PCoA analysis strongly confirmed the cluster pattern of UPGMA. The isolates were separated in accordance with their species, and three main groups were formed and plotted (Figure 3).

Discussion and conclusions

Members of the Sclerotiniaceae can infect many plant species, including vegetables, fruits, ornamentals, and field crops, and cause economically important diseases and severe losses, both in the field and postharvest (Agrios, 2005). Rapid and accurate identification and monitoring of population structure of the pathogens are important for achieving effective control strategies for these diseases. Many molecular marker techniques have been developed and widely used in studies conducted for these purposes, because there are limitations in classical diagnostic methods based on morphological characteristics of the fungi. These limitations include that the classical methods are culture-based, labour-intensive, time-consuming and require extensive experience. In the present study, the efficacy of iPBS markers has been investigated for evaluating intra- and interspecies genetic relationships among the Sclerotiniaceae.

Six of 83 iPBS markers provided very distinct species-specific band profiles, allowing unambiguous discrimination of the fungal species. In the reproducible repeated experiments carried out for each primer, there were no changes in the banding patterns obtained for each isolate. A total of 201 polymorphic bands were amplified with an average of 33.5 bands per primer, which is sufficient for species identification. iPBS markers produced a greater proportion of polymorphic bands when compared to results from previous studies of other retrotransposon marker systems for fungi, such as IRAP for *Fusarium* spp. (Arabi and Jawhar, 2010).

In the present study, the discriminating power of the retrotransposon primers was calculated with PIC analysis, and was 0.86 on average. This is comparable to the results of Andeden *et al.* (2013) and Yıldız *et al.* (2015). This value confirmed that the iPBS primers produced highly informative polymorphic loci. The average of gene diversity was 0.28, and the average Shannon's information index was 0.438. These values are comparable to the results obtained in previous studies (Baloch *et al.*, 2015).

The UPGMA analysis revealed that iPBS markers were very effective for grouping the studied isolates at the species level. The phylogenetic tree divided the 34 isolates into three clusters. The PCoA also distinguished the same clustering.

iPBS retrotransposon markers could be a simpler method for discriminating *B. pseudocinerea* from *B. cinere*a than the previously published fenhexamid/fenpropidine sensitivity test and PCR-RFLP with Bc-*hch* locus (Walker *et al.*, 2011). *Sclerotinia* species were also differentiated easily with retrotransposons without any species-specific marker. Intraspecies genetic variation was highly polymorphic among *S. trifoliorum* isolates from Belgium, which can be associated with any sub-species differentiation. This study has demonstrated that this marker system, which provides high polymorphism rate at the intraspecies level, could be used to identify species/sub-species, and could be beneficial for studying populations of fungal isolates. The iPBS technique based on the retrotransposons has considerable potential for studying intra- and interspecies genetic diversity among Sclerotiniaceae members without genome sequencing data.

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