Mycopathologia (2011) 171:361–371
DOI 10.1007/s11046-010-9362-x

Genetic Diversity of *Cercospora kikuchii* Isolates From Soybean Cultured in Argentina as Revealed by Molecular Markers and Cercosporin Production

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Received: 23 October 2009 / Accepted: 31 August 2010 / Published online: 12 September 2010
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Abstract Leaf blight and purple seed, caused by the fungal pathogen *Cercospora kikuchii* (Matsumoto & Tomoyasu) M. W. Gardner are very important diseases of soybean (*Glycine max* L. Merr.) in Argentina. The aims of this work were: (a) to confirm and to assess the genetic variability among *C. kikuchii* isolates collected from different soybean growing areas in Santa Fe province using inter simple sequence repeats (ISSR) markers and sequence information from the internal transcribed spacer (ITS) region of rDNA and (b) to analyze the cercosporin production of the regional *C. kikuchi* isolates in order to assess whether there was any relationship between the molecular profiles and the toxin production. Isolates from different regions in Santa Fe province were studied. The sequence of the ITS regions showed high similarity (99–100%) to the GenBank sequences of *C. kikuchii* BRCK179 (accession number AY633838). The ISSR markers clustered all the isolates into many groups and cercosporin content was highly variable among isolates. No relationship was observed between ITS region, ISSR groups and origin or cercosporin content. The high degree of genetic variability and cercosporin production among isolates compared in this study characterizes a diverse population of *C. kikuchii* in the region.

Keywords *Cercospora kikuchii* · Genotypic diversity · ITS sequence · ISSR markers · Cercosporin

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Introduction

Some species of *Cercospora* are etiological agents of leaf spot disease in a wide range of crop plant and wild plant species [1, 2]. Leaf blight and purple seed, caused by the fungal pathogen *Cercospora kikuchii* (Matsumoto & Tomoyasu) M. W. Gardner, is one of the most important diseases of soybean (*Glycine max* L. Merr.) worldwide [3]. High yield losses because of leaf blight disease are currently being recorded in Argentina on soybean. Incidence has increased up to 80% in the east-central region of Santa Fe province [4, 5].

C. kikuchii produces the non-host-specific phytotoxin cercosporin, which is very important for the pathogen infecting soybean [6–8]. Cercosporin is a photosensitizer and uses light energy to produce the activated oxygen species superoxide and singlet

oxygen. This perylenequinone toxin is photoactivated and lack toxicity in the dark [9–12].

Cercospora leaf spot diseases show no obvious effect on the vigor and/or quality of the host plant at the time of infection. Exposure of plant cells and tissues to cercosporin results in peroxidation of the membrane lipids, leading to membrane breakdown and death of the cells [9]. Membrane damage allows for leakage of nutrients into the leaf intercellular spaces, allowing fungal growth and sporulation [8, 13–15]. Reduction in vigor, quality and yield becomes apparent on the appearance of necrotic lesions commonly found on leaves [16]. The diagnosis of *Cercospora* infection is possible only from sporulating necrotic lesions or cultures. Traditionally, the identification of *Cercospora* species has been based mainly on conidial characters and host association [2, 17, 18].

Molecular markers have proven to be powerful tools for the characterization and identification of several plant pathogenic fungi [19]. Several molecular typing methods including ribotyping, randomly amplified polymorphic DNA pattern (RAPD pattern), simple sequence repeats (SSR), inter simple sequence repeats (ISSR), restriction fraction length polymorphism (RFLP), have been successfully used for the identification and epidemiological characterization of different organisms [20–22]. The ribosomal DNA (encoding ribosomal RNA) unit has component sequences that evolve at different rates and that can be used for systematic studies at different taxonomic levels. The internal transcribed spacer (ITS) region within the rDNA units evolves rapidly, but it tends to be uniform in sequence with a particular species, and it differs between species. Therefore, the region separating 18S and 26S rDNA and the 5.8S coding region sequence can be used for a comparison of closely related species and subspecies as well as to characterize across interspecific and intergenic level divergences [23–25]. ISSR consists of the amplification of DNA sequences between SSR by means of anchored or non-anchored SSR homologous primers [26, 27]. ISSR-PCR has also been described as microsatellite-primed PCR in the literature because it specifically amplifies regions of the genome between microsatellites. The method of comparing inter-repeat profiles from the whole genome was first used in the identification of different eukaryotic species because of its accuracy and reproducibility [28].

Preliminary studies with few Argentinian isolates of *C. kikuchi* demonstrated phenotypic variability [29] and high levels of polymorphisms [30, 31]. While the basis for variability has not been resolved, the utilization of this variability as an exploitable tool has become the major focus of molecular ecology.

The aims of this work were: (a) to confirm and to assess the genetic variability among *Cercospora kikuchii* isolates collected from different soybean growing areas in Santa Fe province (Argentina) using ISSR markers and sequence information from the internal transcribed spacer (ITS) region of rDNA and (b) to analyze the cercosporin production of the regional *C. kikuchi* isolates in order to assess whether there was any relationship between the molecular profiles and the toxin production.

Materials and Methods

Fungal Isolates

Samples with characteristic *C. kikuchii* lesions were collected from soybean growing in different regions of Santa Fe province (Argentina), in years 2005, 2006 and 2007.

Petioles were surface–disinfected by dipping into 3% commercial sodium hypochlorite (55 g Cl l^{-1}) for 3 min and then rinsed twice in fresh sterilized distilled water. Finally petiole pieces (0.5–1 cm) were placed in each wet chamber. Plates were incubated at 26 ± 0.5°C with alternating light–dark cycles of 8 h, for stimulating sporulation [32]. After the third and up to fifth day of incubation, tissues were observed with a stereo zoom microscope (BOECO Germany, BTB 3-C). *C. kikuchii* conidia from sporulating lesions were picked with the tip of a sterilized dissecting needle, placed onto Potato-Dextrose Agar (PDA) plates and incubated 3–7 days under the same temperature and lightning regimens [29, 30]. In order to obtain monosporic cultures of each *C. kikuchii* isolate, mycelium from individual germinated conidia was transferred to PDA and cultured fourteen days at 26 ± 0.5°C. Isolates were identified as *C. kikuchii* by their mycelial aspect and the size and shape of their conidia.

Each isolate was identified with a code, combining letters and numbers. They were maintained on PDA slants and stored at 4°C for daily work.

Strains from the NITE Biological Resource Center (NBRC) Collection (Japan) were used as control.

All studied fungi and their origin are listed in Table 1.

DNA Extraction

DNA extraction was performed as described by Di Conza et al. [33]. In brief, each isolate was subcultured onto a PDA slant, and the slant was incubated at 25°C for 4 days. Conidia suspension in water (10^5–10^6 UFC ml^{-1}) was inoculated into 100 ml of *Colletotrichum* broth [34], which was in turn incubated at 28 ± 0.5°C for 48 h at 180 rpm. Cultures were harvested by filtration, washed with sterile water, blotted dry and finally powdered. The mycelium (0.06 g) was treated with 600 µl lysis buffer [Tris–HCl (pH 7.2) 0.05 M, EDTA 0.05 M, SDS 3%, 2-mercaptoethanol 1%] and 60 µl sarkosyl (10%). The suspension was incubated at 65°C for 1 h and 200 µl of 5 M potassium acetate and 100 µl 4 M sodium chloride were added. After a gentle ten times inversion, the suspension was allowed to rest on ice for 10 min. Following a centrifugation at 15,000 rpm. for 15 min, the upper phase was carefully transferred into a new tube and an equal volume of phenol–chloroform–isoamilic alcohol solution (25:24:1) was added. The suspension was gently mixed by inversion, pelleted by centrifugation for 5 min at 15,000 rpm and the aqueous phase was then carefully removed. Phenol–chloroform–isoamilic alcohol extraction was performed twice. An equal volume of chloroform–isoamilic alcohol solution (24:1, v/v) was added and emulsified by inversion 5 times, followed by centrifugation for 5 min. The aqueous phase was recovered and DNA was precipitated by adding 0.7 volumes of isopropanol. The tube was kept at room temperature for 20–30 min and the DNA was collected by centrifugation. The pellet was washed with 500 µl of 70% ethanol (previously cooled at −20°C) and completely dried at 55°C. Finally, DNA was solubilized in 100 µl of milliQ water, treated with 1 µl RNAsa (Promega, 10 mg ml^{-1}) and stored at −20°C.

DNA concentrations were determined by measuring the A_{260} with a spectrophotometer and by gel electrophoresis [35].

PCR of Ribosomal DNA. Analysis of the Nucleotide Sequences

Primers designed to amplify the ITS region between 18S and 5.8S and part of the 28S rDNA were used in this case. Each fungal rDNA was amplified by PCR with primers ITS-4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS-5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′) [2, 36–39] obtained from FAGOS/Ruralex (Argentina).

The 50 µl PCR reaction mixture contained 5 µl 10× buffer (InbioHighway, Argentina), 5 µl each of 10 mM dNTP (InbioHighway, Argentina), about 2.5 µl each of ITS-4 and ITS-5 (10 µM), 50 ng of genomic DNA, 0.5 µl 5,000 U ml^{-1} *Taq*DNA Polymerase (InbioHighway, Argentina) and 4 µl 25 mM $MgCl_2$. The mixture was gently vortexed and centrifuged briefly to collect the sample at the bottom, then overlaid with 15 µl of sterile mineral oil. The amplification reaction was performed in an MJ Research Thermal Cycler. The following cycling parameters were used: initial denaturation at 95°C for 5 min, primer annealing at 55°C for 1 min, enzyme chain extension at 72°C for 1 min, and cycled 34 times with a final extension at 72°C for 10 min.

Control experiments were performed without template DNA.

DNA concentration was determined by horizontal submerged electrophoresis (1× TBE buffer at 100 V) [35] on 0.8% agarose gels by comparison with the 100-bp DNA Ladder (InbioHighway, Argentina) used as molecular weight marker.

For sequencing purposes, amplified PCR products were excised from agarose gels and purified with the Wizard SV Gel & PCR Clean Up System (Promega, Cat.#A9281) and sent to be sequenced at the Biotechnology Institute (Unidad de Genómica) belonging to Instituto Nacional de Tecnología Agropecuaria (INTA), Argentina.

Bioinformatics analyses of sequences with Chromas Lite 2.01 program were carried out. Then, the sequences were aligned using the Vector NTI.9. Align X (http://bioinformatics.unc.edu/software/nti/index.htm) and Blast (http://www.ncbi.nlm.nih.gov/BLAST/) programs and compared with those available in the GenBank data base for *Cercospora* species (accession number AY633838).

Table 1 *Cercospora* species isolated from soybean and their origin, genotypes based on internal transcribed spacer regions (ITS) and inter simple sequence repeats (ISSR) analysis and cercosporin production

Isolate	Cultivar-varietal	Regions[a]	ITS sequences	ISSR clusters	Cercosporin production[b] (nmol cyl^{-1}) ± SD
C8	8000 RG	La Capital[c]	TAC	B_1	NP[d]
C20	TJ2070	RiiA Region 1	TAC	B	NP[d]
C15	A 5409	RiiA Region 4	CAC	B	3.93 ± 0.39
C22	TJ2070	RiiA Region 1	CAC	B_3	11.07 ± 1.70
C26	A 5409	RiiA Region 4	TAC	B_2	13.04 ± 0.07
C21	TJ2070	RiiA Region 1	CAC	B_3	23.84 ± 1.91
C17	A5409	RiiA Region 4	CAC	B_7	26.19 ± 1.36
C27	A 8000	RiiA Region 2	CAC	B_4	30.74 ± 0.25
C9	8000 RG	La Capital[c]	CAC	B_1	31.44 ± 3.82
C30	A7636	RiiA Region 5	CAC	B_6	32.55 ± 0.40
C29	A7636	RiiA Region 5	CAC	B	35.25 ± 1.49
C19	A 5409	RiiA Region 4	CAC	B_5	38.42 ± 1.90
C28	A7636	RiiA Region 5	CAC	B_6	38.90 ± 6.03
C7	A 7118	RiiA Region 5	TAC	B_1	53.20 ± 5.88
C16	A 5409	RiiA Region 4	CAC	B	55.04 ± 5.30
C6	A 7118	RiiA Re gion 5	TAC	B_1	56.66 ± 5.23
C36	RA 626	RiiA Region 5	CAC	B_5	67.42 ± 1.73
C18	A5409	RiiA Region 4	CAC	B_7	69.68 ± 7.05
C0	A 8000 Nidera	Las Colonias[c]	TAC	B_1	73.59 ± 3.80
C24	TJ2070	RiiA Region 1	CAC	B	74.62 ± 8.45
C23	A4910	RiiA Region 3	TAC	B_1	85.07 ± 14.88
C31	A7636	RiiA Region 5	CAC	B	91.97 ± 8.59
C14	A 5409	RiiA Region 4	TAT	B_4	101.26 ± 5.05
C38	ASGROW 8000	RiiA Region 1	CAT	B_3	101.61 ± 4.43
C40	ASGROW 8000	RiiA Region 1	TAT	B_2	106.40 ± 9.56
C25	A 8000	RiiA Region 2	TAT	B_3	114.52 ± 12.39
C37	ASGROW 8000	RiiA Region 1	CAC	B	124.10 ± 1.63
C35	RA 514	RiiA Region 3	CAT	B_3	127.13 ± 14.85
C39	A 6401	RiiA Region 6	TAT	B_3	131.20 ± 16.06
C3	A5409	RiiA Region 5	TAC	A	131.96 ± 5.40
C32	A7636	RiiA Region 5	TAC	B_2	148.51 ± 3.98
C41	A6401	RiiA Region 6	TAC	B_2	148.70 ± 14.42
C4	ACA530	RiiA Region 3	TAC	A	160.35 ± 15.13
C2	3700	RiiA Region 6	TAC	A	191.33 ± 6.83
C5	ACA530	RiiA Region 3	TAC	A	233.30 ± 8.00
CK6711		*C. kikuchii* NBRC 6711[e]	TAC	B_2	243.30 ± 11.60
CS6715		*C. sojina* NBRC 6715[e]	TAC	B_2	553.08 ± 5.36

[a] RiiA regions: six regions in which the central-east zone of Santa Fe province, Argentina, is divided; [b]Mean of two experiments expressed as nmol cyl^{-1} (nanomol $cylinder^{-1}$) ± SD (standard deviations); [c]particular producers; [d]*NP* Non producer, [e]*NBRC* Nite Biological Resource Center Collection (Japan)

ISSR-PCR DNA Fingerprint Profiling

ISSR PCR-assay proposed by Longato and Bonfante [40] was followed. Briefly, primer (5′-GTG GTG GTG GTG GTG-3′) obtained from FAGOS/Ruralex (Argentina) was used. The reaction mixture (final volume: 50 µl) consisted of approximately 20 ng of DNA and 5 µl adequate buffer (InbioHighway, Argentina) with 1.5 mM $MgCl_2$, 1 mM each of dNTPs (InbioHighway, Argentina), 100 pmol of primer and 1 U of *Taq*DNA Polymerase (InbioHighway, Argentina) (5 U μl^{-1}). Mineral oil (15 µl) was added to the top of the reaction. Control experiments were performed without template DNA. The amplification reaction was performed in an MJ Research Thermal Cycler programmed as follows: the starting annealing temperature of 70°C, was first decreased to 55°C by 2°C per cycle (repeating this cycle twice), and then 25 extra cycles were run at 55°C. The extension temperature was of 72°C.

The amplification products were analyzed by electrophoresis in 1.3% agarose gel, run in 1× TBE buffer [35] at 100 V and subsequently stained with ethidium bromide. Lambda DNA/*Eco*RI + *Hind* III (Promega) and 100-bp DNA Ladder (InbioHighway, Argentina) were used as molecular weight markers.

Penicillium spp. and *Cladosporium* spp. were included as differentiating genera.

Each assay was performed at least twice.

Gel images were photographed and analyzed with Gel Doc XR system (Bio Rad Life Science Cat.# 170–8170) using the Quantity One Software. Banding pattern similarities were scored by the Dice coefficient (D). The relationships among the different isolates studied were portrayed graphically in the form of dendograms. Bootstrap analysis (1,000 replication) was performed on the resulting tree with WinBoot Program to test the statistical support for each branch. Less than 50% bootstrap values do not represent statistically support and >70% values were considered a strong bootstrap support.

Cercosporin Production Assay

Cercosporin concentration was determined spectrophotometrically according to Jenns et al. [41] modified by González et al. [30]. Briefly, isolates were grown on PDA and incubated at 26 ± 0.5°C for eleven days under 16 h light. Three mycelium plugs (10 mm diameter) were cut from the border of the colonies, transferred to tubes containing 6 ml of 5 N KOH and kept in the dark for 3 h. After centrifugation at 7,500 rpm during 20 min, cercosporin was corroborated by the presence of characteristic peaks at 480, 595 and 640 nm in a spectrophotometer Perkin Elmer Lambda 20 UV/VIS (960 nm min^{-1} every 1 nm) and analyzed with OriginPro 7 program. A commercial cercosporin toxin (Sigma, lot 35082-49-6) was processed in parallel. Toxin concentration in a spectrophotometer at A_{480} nm using a molar extinction coefficient of 23,300 [41] was determined. *C. kikuchii* NBRC 6711 and *C. sojina* NBRC 6715 were used as control strains. Experiments were repeated twice. Results were reported as nanomoles per agar plug (nmol cyl^{-1}) and standard deviations (SD) were calculated.

Results

ITS Sequence Analysis

The ITS-4 and ITS-5 primers uniformly amplified a fragment of approximately 518 bp from Argentinian isolates and NBRC strains. Amplified rDNA concentrations ranged between 100 and 150 ng μl^{-1}.

Fifteen fungi presented complete sequence identity (100% identical) with *C. kikuchii* BRCK179 (GenBank, accession number AY633838), *C. kikuchii* NBRC 6711 and *C. sojina* NBRC 6715 included. Four isolates were 99% identical to *C. kikuchii* BRCK179, the difference consisting 1 substitution at position 476 (T instead of C). Two isolates were 99% identical to *C. kikuchii* BRCK179, the difference consisting of two substitutions at positions 474 and 476 (C and T instead of T and C, respectively). Finally, 16 isolates were 99% identical to *C. kikuchii* BRCK179 but, in these fungi, C (474 position) substituted T (Fig. 1). The four ITS sequences obtained have been deposited in the GenBank sequence database under accesion numbers HM631725, HM631727, HM631728 and HM631726.

ISSR-PCR DNA Fingerprint Profiling

Clearly detectable amplified ISSR ranged from 159 to 2,450 bp in size. The average number of clear bands generated was 8, with a maximum of 22 and a

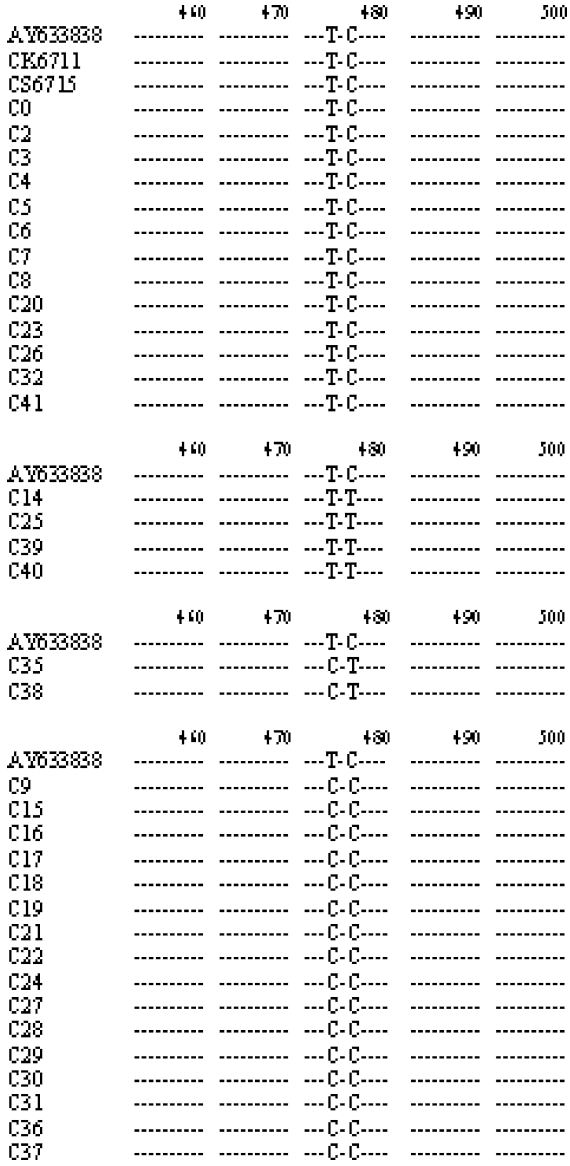

Fig. 1 Computer alignment of the internal transcribed spacer region (ITS) of *Cercospora kikuchii* isolates and NBRC strains. AY633838: *Cercospora kikuchii* BRCK179 (Gen-Bank, accession number AY633838); CK6711: *Cercospora kikuchii* NBRC 6711; CS6715: *Cercospora sojina* NBRC 6715; C0, C2, C3, C4, C5, C6, C7, C8, C20, C23, C26, C32, C41, C14, C25, C39,C40, C35, C38, C9, C15, C16, C17, C18, C19, C21, C22, C24, C27, C28, C29, C30, C31, C36, C37: regional isolates

minimum of two. Figure 2 shows the ISSR patterns of *C. kikuchii* isolates and NBRC strains.

Cluster analysis generated a dendogram (Fig. 2) with two branches: A and B, with a statistical support between them of 56.9% (not so strong). Branch "A" included four isolates, C2, C3, C4 and C5 (99.7% similarity) from different origin (Table 1) and the same ITS sequence (TAC). Cluster "B" included seven subclusters (B_1, B_2, B_3, B_4, B_5, B_6 and B_7). B_1 included six isolates, C23, C9, C8, C7, C6 and C0 (100% similarity), all of them with ITS sequence TAC, except C9 (CAC). C6 and C7 were isolated from the Riia Region 5 and A7118 varietal/cultivar, and C8 and C9 were obtained from soybean cultured by particular producers (Table 1). Subcluster B_2 included six fungi. C40 presented 61.9% similarity with respect to the other five fungi. Both strains NBRC (C6711 and C6715) presented 88.3% similarity between them and 71.1% with C41. ITS sequence TAC was present in these fungi (B_2), except C40 (TAT); C32 and C26, from different origin and cultivar/varietal, presented scarce genetic similarity with respect to C41 and NBRC strains. Subcluster B_3 included six isolates, C39, C25, C38, C35, C22, and C21: C39 and C25 with the same pattern bands (100% similarities and ITS sequence TAT), but with less than 50% bootstrap support (45.9% with respect to the other 4 isolates). A 100% similarity between C22 and C21 (ITS sequence CAC) and 99.9% similarity between C38 and C35 (ITS sequence CAT), were detected. Only C21 and C22 were isolated from the same cultivar/varietal and region. B_4 included C27 and C14 with the same pattern bands (100% similarity and ITS sequences CAC and TAT, respectively). B_5 included C36 and C19 (ITS sequence CAC), both of them from different origin and cultivar/varietal and a low bootstrap value (51.1%). In a similar way, B_6 included two isolates: C30 and C28 (ITS sequence CAC) from the same origin and cultivar/varietal (61.7% similarity). Finally, B_7 included C18 and C17 (99.5% similarity), both of them from the same origin and cultivar/varietal.

The remaining seven isolates in cluster B: C24, C20, C31, C29, C16, C37 and C15 (different origin), all of them with ITS sequence CAC, except C20 (TAC) (Table 1), could not be assigned to any of these subgroups; further information being needed to solve this situation.

The inclusion of *Cladosporium* spp. and *Penicillium* spp. was useful to determine that the technique allowed to differentiate fungal genera.

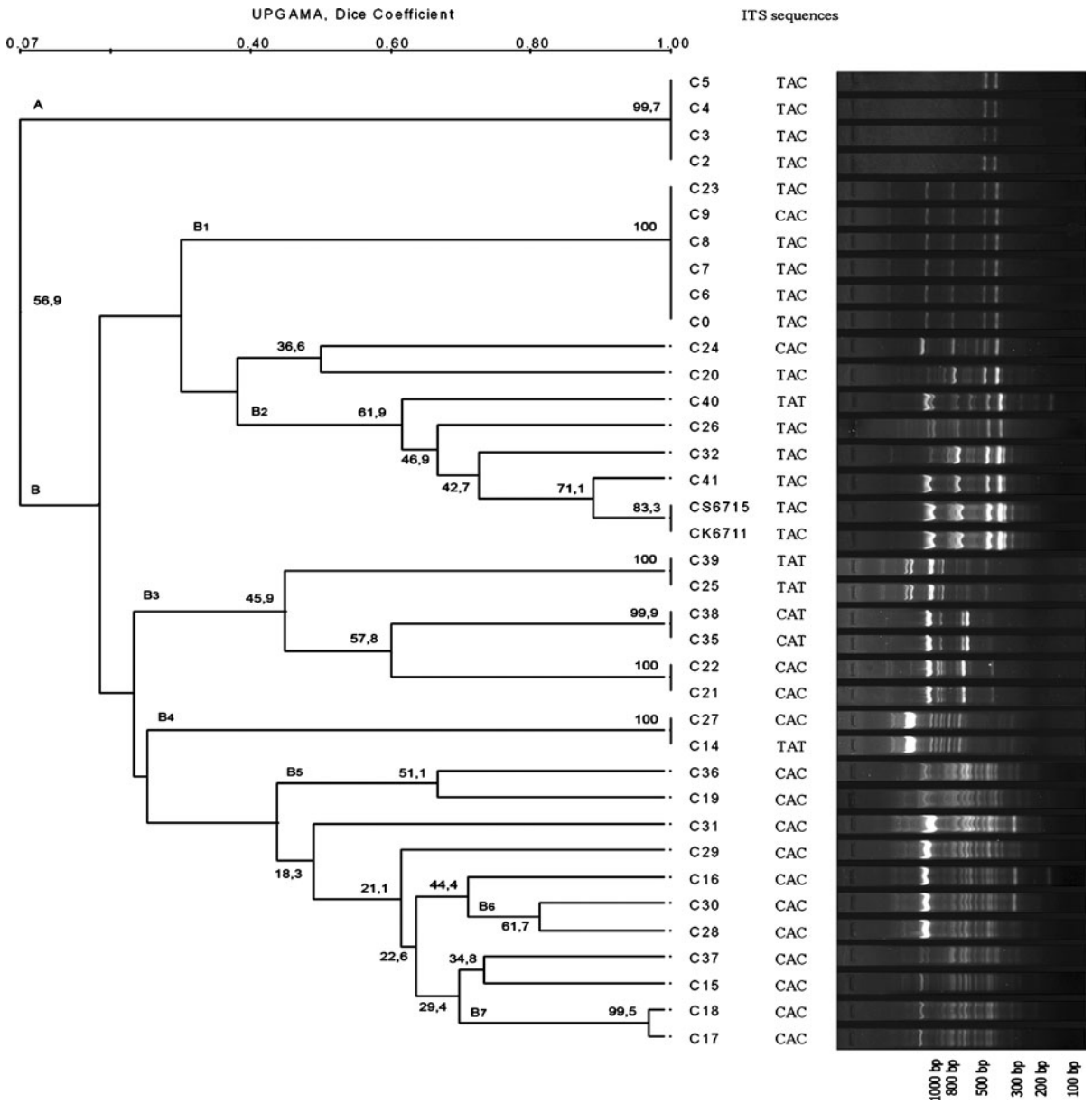

Fig. 2 Sample gel of ISSR (primer GTG_5) patterns produced by *Cercospora kikuchii* isolates and NBRC strains, their respective ITS sequences and cluster analysis dendrogram. C5, C4, C3, C2, C23, C9, C8, C7, C6, C0, C24, C20, C40, C26, C32, C41, C39, C25, C38, C35, C22, C21, C27, C14, C36, C19, C31, C29, C16, C30, C28, C37, C15, C18, C17: regional isolates; CS6715: *Cercospora sojina* NBRC 6715; CK6711:*Cercospora kikuchii* NBRC 6711; percentage bootstrap values based on 1,000 replicates are shown on the nodes

Cercosporin Production Assay

Thirty three regional isolates and the NBRC strains were cercosporin producers, the greatest concentration being from *C. sojina* NBRC 6715 and *C. kikuchii* NBRC 6711. Non-producers were C8 and C20 (all of them with TAC sequence) (Table 1).

Discussion

Soybean (*Glycine max* (L.) Merr.) is one of the most important crops in Argentina, and it has been characterized by an incredible rate of adoption and growth. In 1970–1971 the soybean production amounted to 59,000 tons, covering a crop area of

approximately 38,000 hectares. Nowadays, the crop area has increased to over 16.6 million hectares, making it the world's third largest producer, the main exporter of soybean oil (30% of the world exports) and the second exporter of soybean flour (27% of the world exports) [42, 43]. Twenty-one percent of the cultivated lands of Argentina are in Santa Fe province whose main crop, soybean, makes it the main national producer. However, soybean crops are affected by several diseases which decrease the total production. The RiiA programme, an interdisciplinary intervention carried out at the Center North of Santa Fe province, was developed by the Facultad de Ciencias Agrarias of Universidad Nacional del Litoral, the Experimental Station INTA Rafaela and some institutions linked to agricultural production. One of its aims is to detect early adverse situations to prevent or mitigate the effects so as to generate information for subsequent campaigns and identify and priorize regional problems. Monitoring, processing and communication of the soybean crop evolution are the goals aimed at by this programme.

This study contributes to the knowledge of some phenotypic and genotypic characteristics of one of the most frequent phytopathogens in order to provide information for the soybean breeding program at RiiA.

Since typing is a necessary first step in knowing pathogens [44], techniques based on DNA polymorphisms are especially valuable to enhance epidemiological studies. PCR of ITS regions and its sequence data have been studied to assess genetic diversity at intraspecific level in different species [2, 25, 45]. As Somai et al. [46] reported, ITS sequence data in this study indicated a closer relationship between *C. kikuchii* isolates than ISSR data did, even though intraspecific variation was found in the rDNA ITS region of *C. kikuchii* isolated from geographically widely distributed cultivars of soybean developed in the northern region of Santa Fe province. Therefore, it appears that four genotypes of *C. kikuchii* may be associated with and isolated from soybean exhibiting symptoms of leaf blight. It has been suggested that the spacer sequences may accumulate mutations in the form of base substitution, duplications, deletions and insertions and chromosomal rearrangements in genomes [47]. In our study, the simplest explanation for this fact is that fungi with ITS sequences CAC, CAT, TAT are genetic subgroups within *C. kikuchii* that are diverging from *C kikuchii* BRCK179 sequence (TAC) which is deposited in Gen Bank. ITS sequence analysis results showed high similarity (99–100%) with published sequences of *C kikuchii* BRCK179 (Gen Bank AY633838). Even in this investigation, CAC sequence was the most frequently detected (43.2%). The number of nucleotide differences of ITS of the rDNA among all sequences ranged from 0 to 2. However, Goodwin et al. [48], in a previous publication, mentioned that the mean number of nucleotide differences between three isolates of *C kikuchii* ranged from 2 to 7. These differences may be due to different sets of sequences (TREEEBASE) used by those authors for comparing their isolates. Another authors, studying 12 sequences of Brazilian isolates of *C. kikuchii*, detected that the differences in ITS sequences ranged from 0 to 3 [36].

Data obtained revealed a considerable degree of variation in the Argentinian population of *C kikuchii*, thus confirming previous results [30, 31]. In agreement with our study, Almeida et al. [36] and Cai [3] determined a high level of variability among isolates of *C. kikuchii* and these results show no genetic grouping of cercosporin producers or grouping for geographic location. According to Pujol Vieira dos Santos et al. [49] and Stenglein and Ballati [50] there are many factors that could have been affecting polymorphism analysis, e.g. the intraspecific variants of a pathogen, the number of samples selected for analysis, genetic flow between populations, environmental adaptation and selective pressure and migration.

The role of cercosporin in the pathogenicity of *C. kikuchii* was first demonstrated by Upchurch et al. [8], who considered it crucial for the infection of soybean plants. Ability to produce cercosporin allowed the ancestral *Cercospora* species to expand its host range. This would explain the occurrence of a large number of closely related species, some with identical ITS sequences, on widely divergent hosts [44, 51].

In this study, isolates that produced high cercosporin concentration belonged to ITS sequence group TAC, as well as non producers did (C8 and C20). It is important to consider that C8 and C20 were isolated from ill soybean. Under our working conditions, *C. sojina* NBRC 6715 produced a high concentration of cercosporin, unlike Goodwin et al.'s study in which *C. sojina* isolate was non-producing [48]. It was not clear if the lack of colour in the extract for

colorimetric assay observed in some isolates could be due to the sensitivity of the method used since isolates with very low amounts of cercosporin were also able to cause lesions when inoculated on soybean leaves. Upchurch et al. observed that spontaneous and UV-induced mutants which did not produce cercosporin were not able to cause infection when inoculated on soybean leaves [8].

There was no association between the groups of isolates based on ISSR markers and cercosporin content. Isolates that produced high contents of cercosporin were clustered together with those from low cercosporin producers. For example, isolates C14 (101.26 nmol cyl^{-1}) and C25 (114.52 nmol cyl^{-1}) were high producers of cercosporin and were separated in clusters B_4 and B_3, respectively. Isolates C27 (30.74 nmol cyl^{-1}) and C14 were clustered together but cercosporin content was significantly higher in the latter.

In all analyses it was shown that *C. kikuchii* isolates from the same geographic region appeared in different groups. Cercosporin production and molecular analyses showed intraspecific variability within *C. kikuchii* isolates recovered from soybean collected in different RiiA regions from Argentina, so it was difficult to establish a relationship between this variability and the soybean cultivars from which *C. kikuchii* isolates were obtained. Similar results were previously reported by González et al. and Almeida et al. [30, 36]. According to the obtained results, Argentinian populations of *C. kikuchii* are phenotypically, genotypically and geographically variable. In agreement with Almeida et al. [36], who consider that this pathogen is easily transmitted by seeds, it is not surprising to find the same haplotypes in different regions. The present study expands on the previous one by using another molecular marker (ISSR) and ITS sequence analysis on a larger collection of isolates. Many groups were present among the 37 studied fungi; however, the groups could not be correlated with cercosporin content or geographic origin of the isolates.

In Argentina, there has been a rapid increase in the soybean producing area since 1970 [42]; therefore, the traffic of seeds from traditional areas to new areas could be responsible for the geographical variability since *C. kikuchii* is a seed borne pathogen. Unfortunately, an insufficient number of isolates was obtained from each area to permit the evaluation of gene flow among populations more accurately.

For countries with large soybean areas like Argentina, it is very important to know in advance the variability of the pathogen in order to avoid resistant cultivars when sown in different areas. The level of variability among isolates as identified in this work may help to define the breeding method for effective resistance.

Acknowledgments This work was funded by grants from *Universidad Nacional del Litoral*, Argentina, CAID 2009. The authors would like to thank Monitors belonging to RiiA Program for their kind help in providing samples and Ing. Lello Herzog for his very useful opinions.

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