

*Genetic entanglement between Cercospora species associating soybean purple seed stain*

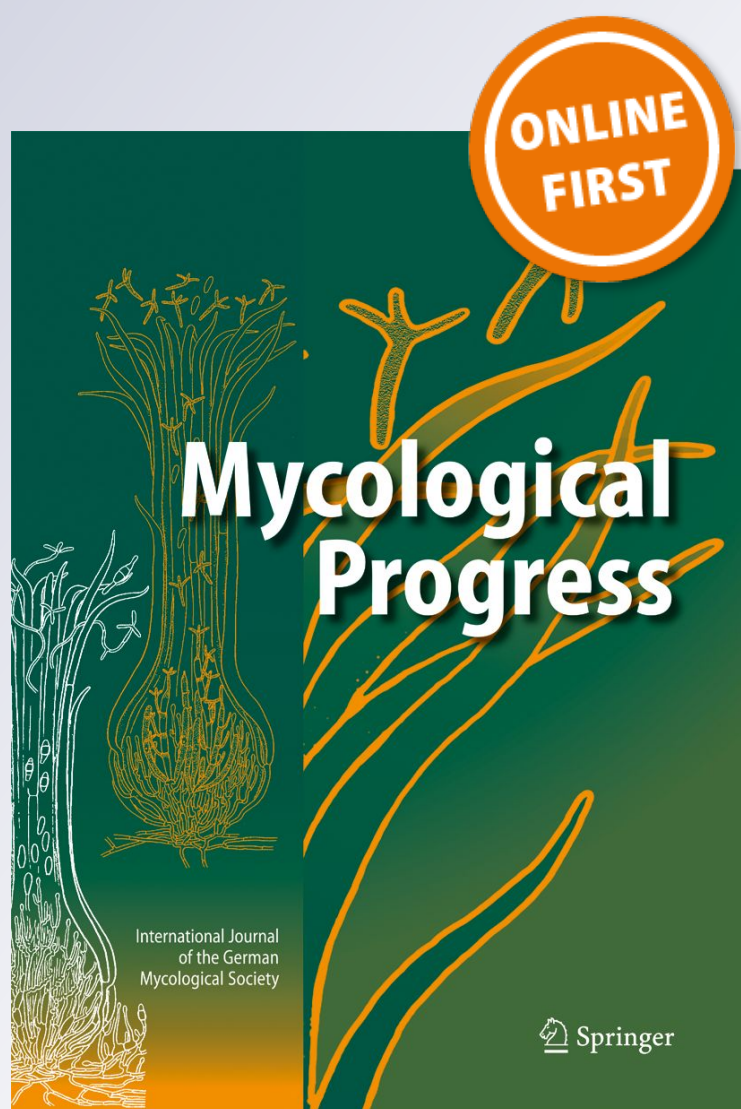
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# Genetic entanglement between *Cercospora* species associating soybean purple seed stain

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**Abstract** Soybean purple seed stain (S-PSS) is a destructive, worldwide distributed fungal disease caused by several *Cercospora* species. This work aims to shed light on the nature of the genealogical and genetic relationships amongst S-PSS causal agents. Fungal isolates were obtained from Argentina and Brazil, which belong to the leading countries in soybean production worldwide. DNA sequences were obtained from eight loci across the collection of isolates. Relationships were evaluated through Bayesian phylogenetic inferences, and distance and character-based network analyses and discriminant analyses. The occurrence of reticulate evolutionary events was tested with recombination tests. The high haplotype diversity ( $H = 1.0$ ) was arranged in four validated

haplogroups. Reticulate network topologies were evident, and 11 recombination events were validated through several tests. Five of these events occurred across species boundaries. Comparison with sequences from 70 *Cercospora* species indicated that at least five monophyletic groups of S-PSS-causing agents are currently present in South America. The provided evidence supports the hypothesis that interspecific genetic exchange plays a significant role in the evolutionary dynamics of *Cercospora* species in this region. The occurrence of interspecific recombination has implications for understanding epidemiological threats to soybean production that appear to be more serious than previously anticipated.

**Keywords** Fungal pathogens · Reticulate evolution · Genetic recombination · Epidemiology

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## Abbreviations

S-PSS Soybean purple seed stain  
GCPSR Genealogical Concordance Phylogenetic Species Recognition

## Introduction

*Cercospora* Fresen. is one of the largest genera of hyphomycetes, linked to the teleomorph genus *Mycosphaerella* (Corlett 1991; Crous et al. 2000, 2004a; Stewart et al. 1999). Fossil remains of *Mycosphaerella*-like and cercosporoid specimens have been reported in the 48.7 million-years-old Princeton Chert in British Columbia, Canada (Lepage et al. 1994). Since those times, *Cercospora* lineages have had a long history of diversification and adaptation to a vast variety of lifestyles and ecological niches.

Cercosporoid fungi have been reviewed recently (Braun et al. 2013, 2014, 2015; Groenewald et al. 2013). Traditionally, identifications and new species assessments of *Cercospora* were based on conidial characters and host association (Chupp 1954; Crous et al. 2004a; Ellis 1971; Gams et al. 1987; Siboe et al. 2000). Species of *Cercospora* are among the most destructive plant pathogens worldwide (To-Anun et al. 2011). *Cercospora kikuchii* (Matsumoto & Tomoyasu) M. W. Gardner was identified as the causal agent of soybean purple seed stain (S-PSS) and *Cercospora* leaf blight (CLB) (Cai and Schneider 2008), and usually assumed to be host-specific to soybean (Crous and Braun 2003). Symptoms associating S-PSS and CLB included reddish-purple to bronze discolouration of leaves and pods, and pink or pale to dark-purple discolourations on seeds (Almeida et al. 2005; Gams et al. 2007). A number of studies detected a great deal of widespread genetic variation within *C. kikuchii* (González et al. 2008; Latorre Rapela et al. 2011; Lurá et al. 2011) but assumed that this variation was maintained within a single coherent biological group. Groenewald et al. (2013) stated that S-PSS and CLB of *Glycine max* L. might be caused by at least two different species of *Cercospora*, and that the identification of these species should not be based on disease symptoms and host precedence alone. Recently, Soares et al. (2015) showed that at least four distinct lineages act as causal agents for S-PSS throughout the Americas and Albu et al. (2016) suggested that isolates associating S-PSS in the USA belong to *C. cf. flagellaris* and *C. cf. sigesbeckiae* but *C. kikuchii*.

We followed Soares et al. (2015) and analysed the genetic variation within S-PSS pathogens by characterising the nuclear and mitochondrial loci of 42 newly isolated strains from across soybean production areas in Argentina. These and S-PSS strains from Brazil were phylogenetically analysed with 70 other *Cercospora* species to assess species boundaries under the Genealogical Concordance Phylogenetic Species Recognition (GCPSR) criterion (O'Donnell et al. 1998; Taylor et al. 2000; De Queiroz 2007). Their dichotomous or reticulate relationships were also analysed without forcing tree-like topologies, and reticulate evolutionary events were assessed in recombination tests. The GCPSR criterion has often been applied specifically for morphologically reduced and cryptic species of species complexes (Dettman et al. 2003; Starkey et al. 2007; Peterson 2008; Quaedvlieg et al. 2014; Liu et al. 2016). Methodological approaches implemented here allowed detecting recombination events in diverse groups, such as fungi (Douhan et al. 2007; Le Gac et al. 2007; Croll and Sanders 2009), viruses (Lian et al. 2013) and bacteria (Álvarez-Pérez et al. 2013).

## Materials and methods

### Specimens used

Fungal isolates were collected from the provinces of Buenos Aires, Entre Ríos, Córdoba and Tucumán, representing the main soybean producing areas in Argentina (Supplemental Table S1). Axenic cultures were obtained from purple-stained soybean seeds; isolation techniques followed protocols described elsewhere (Almeida et al. 2005). Fungal isolates from Argentina are kept at the Facultad de Agronomía—Universidad de Buenos Aires (FAUBA) culture collection, and are available upon request. We followed the species identification and nomenclature of Groenewald et al. (2013) and Soares et al. (2015) and used “cf.” to denote uncertainty in respect to species level identifications and “aff.” for strains producing symptoms and culture characteristics resembling *C. kikuchii* (P.E. Grijalba, personal communication).

### Sequence data

We generated partial sequences of eight loci of 42 isolates associating S-PSS, hereafter referred to as *Cercospora* aff. *kikuchii*. These regions included exons and introns of protein encoding genes: actin (*act*; 151 bp), calmodulin (*cal*; 244 bp), elongation factor 1-alpha (*tef*; 236 bp), the cercosporin facilitator protein (*cfp*; 836 bp partial sequence), two non-adjacent  $\beta$ -tubulin 1 regions (*tub1* and *tub2*; 1197 bp in total) and the mitochondrial cytochrome oxidase B gene (*cyt-b*; 653 bp). Also, we generated rDNA internal transcribed spacers including the 5.8S rDNA gene (ITS; 483 bp). DNA extraction, polymerase chain reaction (PCR) amplification and sequencing procedures were carried out according to the protocols described by Soares et al. (2015). Obtained sequences were compared with (i) S-PSS fungal isolates used by Soares et al. (2015), i.e. material from Argentina (7 isolates), Brazil (11), the USA (6) and the ex-type of *C. kikuchii* (CBS 128.27 from Japan) (dataset 1; Supplemental Table S1) and (ii) representatives of 64 other *Cercospora* species identified by Groenewald et al. (2013) (dataset 2; Supplemental Table S2).

### Dataset assembly and variation estimates

Sequences of each locus were aligned in MEGA 6.0 (Tamura et al. 2013) using eight iterations of the MUSCLE subprogram (Edgar 2004), with default settings. Alignments were manually adjusted wherever necessary. After interlocus congruence tests were assessed (see below), the concatenated multilocus dataset 1 was obtained using Geneious 7.1.7 (Biomatters, available from <http://www.geneious.com>). All eight loci were used for dataset 1. The number of haplotypes (H), haplotype diversity (Hd) and the number of segregating sites were assessed using DNASP v5 (Librado and Rozas 2009).

Dataset 2 was assembled after validating interlocus congruence. It involved concatenated sequences of five loci (*act*, *cal*, *his*, *tef* and ITS) from 158 representatives of *Cercospora* species taken from Groenewald et al. (2013), Soares et al. (2015) and the isolates newly analysed here (Supplemental Tables S1 and S2). In all subsequent analyses, gaps were considered as a fifth character following criteria specified by Giribet and Wheeler (1999).

### Congruence analyses

We adopted the conditional concatenation criterion (Edwards et al. 2016) that allows the combination of different loci into a single matrix if they are significantly or at least partially congruent. This was accomplished by using the congruence amongst distance matrices (CADM) statistical test (Campbell et al. 2011), based on uncorrected p-distance matrices for each locus as input data. CADM is an extension of the Mantel test of correspondence (Mantel 1967), and can be used to test the null hypothesis of complete incongruence of the distance matrices. As a complement, CADM provides an estimate of the degree of congruence between two or more matrices, the average Kendall coefficient ( $W = 0$ , no congruence;  $W = 1$ , complete congruence).

### Bayesian phylogenetic analyses

The best-fit substitution model under the Akaike Information Criterion (Akaike 1973) was determined for each gene region with MrModeltest v.2.3 (Nylander 2004). In dataset 1, the Kimura two parameters plus gamma –G– model (K2 + G; Kimura 1980) was selected for *act*, *cal*, *cfp* and *tef*, T92 (Tamura 1992) for *cyt-b*, JC (Jukes and Cantor 1969) for *tub1*, and HKY + G + I (Hasegawa et al. 1985; plus gamma and a proportion of invariant sites) for *tub2*. In dataset 2, K2 + G was selected for *act* and *tef*; T92 + G for *cal* and *his*; and K2 for ITS. Both datasets were analysed with MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) in two simultaneous, independent runs of  $5 \times 10^6$  generations each; one cold and four heated chains were used in each run; the temperature parameter was set to 0.25 and the branch length prior was set to 0.01. Trees were sampled every 5000 generations. Convergence was assessed in Tracer v1.6 (Rambaut et al. 2014) by inspecting the trace plots and the effective sample sizes (ESS > 400) for all statistics. Thus, the first 25% of trees was discarded as burn-in. Fifty percent majority rule consensus trees were visualised using FigTree v1.4.2 (Rambaut 2006–2014). Bayesian topology derived from dataset 1 was mid-point rooted, whereas the topology from dataset 2 was rooted on *C. senecionis-walkeri* Phengs., Chuheat., McKenzie, K.D. Hyde & U. Braun, as in Groenewald et al. (2013).

### Network analyses and discriminant analysis of principal components

Genetic relationships amongst the fungal isolates of dataset 1 were visualised by constructing unrooted networks using median-joining (MJ; Bandelt et al. 1999) and Neighbor-Net (NN; Bryant and Moulton 2004) algorithms, as implemented in SplitsTree v. 4.12.3 (Huson and Bryant 2006) and by using the default settings (MJ: epsilon = 0, spring embedder iterations = 2000, label edges, show haplotypes, subdivide edges and scale nodes by taxa = false; NN: character transformation by means of uncorrected p-distance, edge fitting as ordinary least squares, equal angle as the chosen splits transformation, least squares to modify weights and four maximum dimensions as the filtering option). The MJ analysis involved 484 active sites (2864 constant characters were excluded) and NN involved 3351 sites. For the latter, fit and least square fit (LSfit) values between pairwise distances from the graph (patristic) and distance matrix were computed by SplitsTree. The MJ supplies tree and non-tree-like topologies where alternative relationships are plotted as loops, or cycles, connected by putative ancestral or missing haplotypes within reticulate topologies. NN yields a visual representation of conflicting signals by presenting them as a series of parallel edges. Tree-like topologies indicate evolutionary pathways where genetic exchange plays no significant role. Conversely, reticulate (conflicting) patterns suggest that genetic exchange events may have contributed to the evolution of analysed taxa.

A discriminant analysis of principal components was done for dataset 1 to detect the number of genetic groups and to statistically validate isolate assignment to each group. The program DAPC (Jombart 2008; Jombart et al. 2010), as implemented in the adegenet 2.0.1 package (R 3.2.4, R Core Team 2016), is a multivariate, model-free tool that maximises differences between groups while minimising variation within clusters. DAPC does not assume any a priori population structure or genetic equilibrium, a feature particularly useful for plant pathogenic organisms. The function dapc was executed by retaining ten principal components, which accounted for over 95% of accumulative variance and three discriminant functions; clusters were identified by the K-means algorithm, with n.iter =  $10^5$  and n.start = 10, evaluating a range from 1 to 40. The most useful number of clusters was estimated using the BIC value versus number of clusters scatter plot, along with the a-score algorithm. Membership probabilities were derived for each sample (isolate) and each group. These coefficients can be interpreted as a genetic proximity or assignment measure of samples to the different clusters.

### Graphical haplotype and recombination analysis

A graphical representation of the multilocus alignment for dataset 1 was obtained using Geneious. Visual haplotypes

were utilised to distinguish haplogroups that, subsequently, were related to the genetic groups defined by DAPC. Comparisons within individual graphical haplotypes were further used to identify putatively recombinant haplotypes. Global recombination within dataset 1 was subsequently tested using the quartet-based minimum recombination test (Rm; Hudson and Kaplan 1985) as implemented in DNAsp, and the non-parametric pairwise homoplasmy index test ( $\Phi$ W; Bruen et al. 2006) as implemented in SplitsTree. Both methods estimate an overall probability of recombination, while the  $\Phi$ W test assumptions do not include sample history or a given population for every sample. Therefore, the  $\Phi$ W test fits well in distinguishing recurrent mutation from recombination, irrespective of the particular evolutionary scenario under analysis. The statistical significance of  $\Phi$ W scores was estimated by 1000 random permutations of nucleotide sites while simulating the absence of recombination, then calculating whether the frequency of the non-recombinant permuted  $\Phi$ W score is less than the observed score. A threshold level of 0.05 ( $\Phi$ W < 0.05) is considered indicative of overall significant recombination within the dataset.

In order to assess the actual occurrence of individual genetic exchange events, seven recombination tests were further applied that, in conjunction, minimise the chance of false=positive claims (type II error) due to deep coalescence (incomplete lineage sorting), recurrent mutation, convergence or other vertical evolutionary events causing reticulations (character conflict) in the network topology or false-positives in the  $\Phi$ W test. The X-OVER protocol as implemented in the RDP4 program (Martin et al. 2015) was used for three triplet tests (3Seq, Boni et al. 2007; RDP, Martin and Rybicki 2000; and SiScan, Gibbs et al. 2000), three nucleotide substitution tests (GENECONV, Padidam et al. 1999; Maynard Smith 1992; Posada and Crandall 2001) and one sliding window test (BootScan, Salminen et al. 1995). Each particular recombination event was subsequently analysed using the aforementioned tests. A stringent acceptance threshold  $p$ -value <  $10^{-5}$  for at least four independent recombination tests was considered to claim each particular event as validated. Their approximate breakpoints were also recorded. The scaled recombination rate  $\rho$  ( $= 4N_c r$ ) for dataset 1 was additionally obtained using RDP4 as a graphical interface for the LDhat interval program with default settings (McVean et al. 2004).

### Species boundary delimitation

The GCPSR criterion (Taylor et al. 2000) was applied to assess species boundaries where the  $\Phi$ W test turns instrumental and concordance of gene genealogies (or lack thereof) aids in evaluating the significance of gene flow amongst groups. The analysis, however, does not inform recombination events if more than two lineages are involved. Moreover, species

boundaries among closely related taxa could be difficult to assess under the GCPSR criterion, since character conflict could be due to substantially different evolutionary processes, such as incomplete lineage sorting, recombination, selection, horizontal gene transfer and population structure (Stewart et al. 2014). Different evolutionary histories will cause inconsistency between gene trees and species trees. This phenomenon is poorly accounted for by dichotomous topologies that may cause severely biased results, and is more precisely described by non-tree like approaches (Jombart et al. 2010; Campbell et al. 2011; Morrison 2011; Wen et al. 2016; Edwards et al. 2016). Given that Soares et al. (2015) identified at least four different recently diverged S-PSS species, we articulated Bayesian phylogenetic analyses with methodologies that do not force tree-like topologies (network analyses, discriminant analyses, congruence), and further included the aforementioned (non-genealogical) recombination tests in an attempt to overcome GCPSR limitations and help define boundaries for genetic exchange.

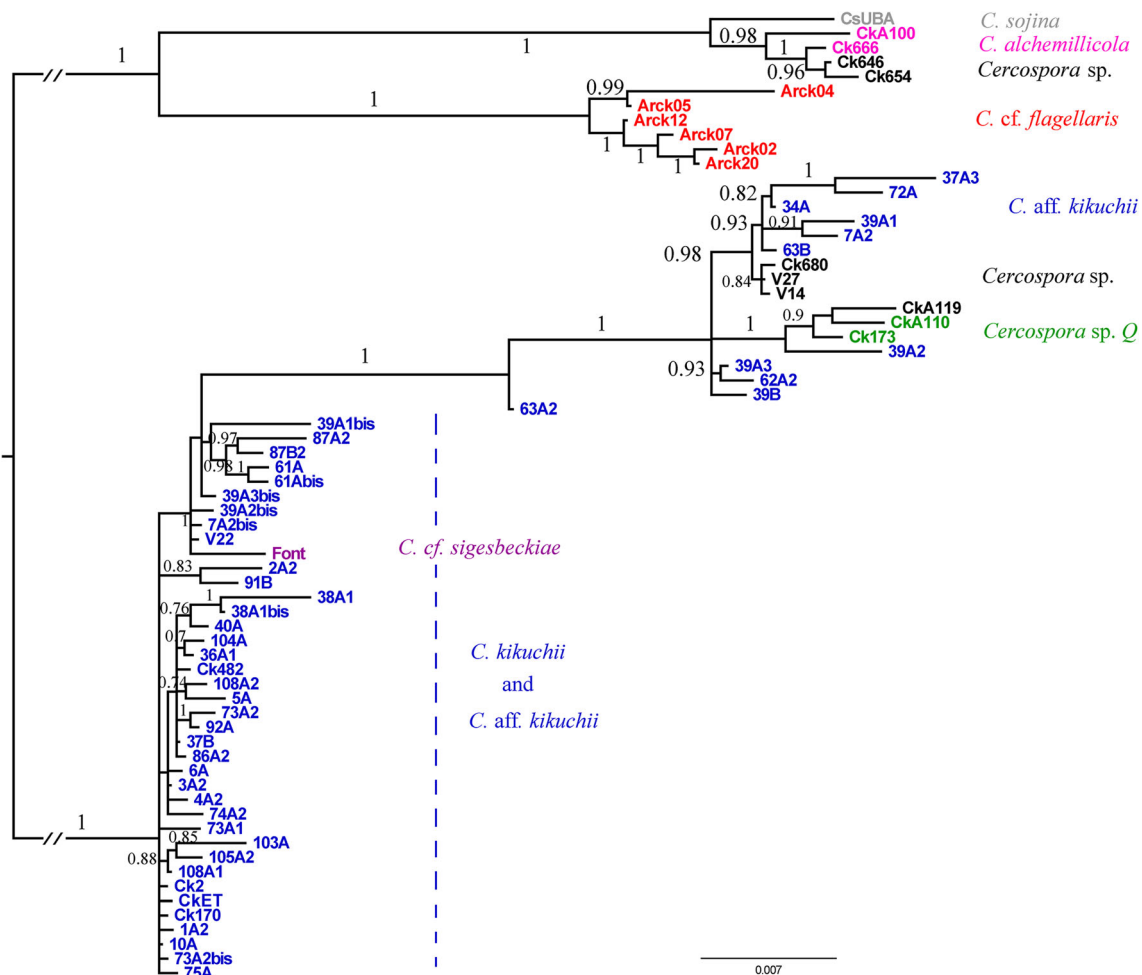
## Results

### Congruence analysis

The average Kendall coefficient value ( $W$ ) is 0.49 ( $p < 0.01$ ) for loci from dataset 1 and 0.52 ( $p < 0.01$ ) for those from dataset 2. These results indicate overall significant though partial congruence. Pairwise comparisons showed that, with the exception of *cal* and *cyt-b* (dataset 1), all other loci are partially and significantly congruent (Supplemental Tables S3 and S4). Consequently, we generated two concatenated datasets. The matrix for dataset 1 contains 3351 sites in total (448 segregating sites, 382 parsimony informative and 66 singletons), with an overall pairwise similarity of 97.1% amongst 66 terminals. The concatenated matrix for dataset 2 is 1450 bp long (423 segregating, 277 parsimony informative sites and 146 singletons), with 158 terminals. Likewise, dataset 2 yielded an overall pairwise similarity of 97%.

### Phylogenetic analyses

The Bayesian analysis derived from dataset 1 (Fig. 1) showed that isolates CkA100 and Ck666 from Brazil (as *C. alchemillicola*) formed a clade together with Ck654 and Ck646 (as *Cercospora* sp.) (posterior probability, pp = 0.98), together with *C. sojina*, showed a sister relationship (pp = 1.0) to *C. cf. flagellaris* from North America (pp = 1.0). A low topological resolution was retrieved for the other S-PSS isolates from South America. However, a strongly supported clade (pp = 1.0) accommodated ten isolates from Argentina and Brazil recognised as *C. aff. kikuchii*, *Cercospora* sp. Q (two isolates) and *Cercospora* sp. (four isolates). Most isolates



**Fig. 1** Majority rule consensus tree from Bayesian phylogeny inference for *Cercospora* species causing soybean purple seed stain (S-PSS) (dataset 1) (3351 sites; 67 terminals). Branch lengths are drawn to scale; parallel dashes replace two units of the scale bar. The numbers printed near nodes are posterior probabilities ( $\geq 0.7$ ). The scale bar corresponds to

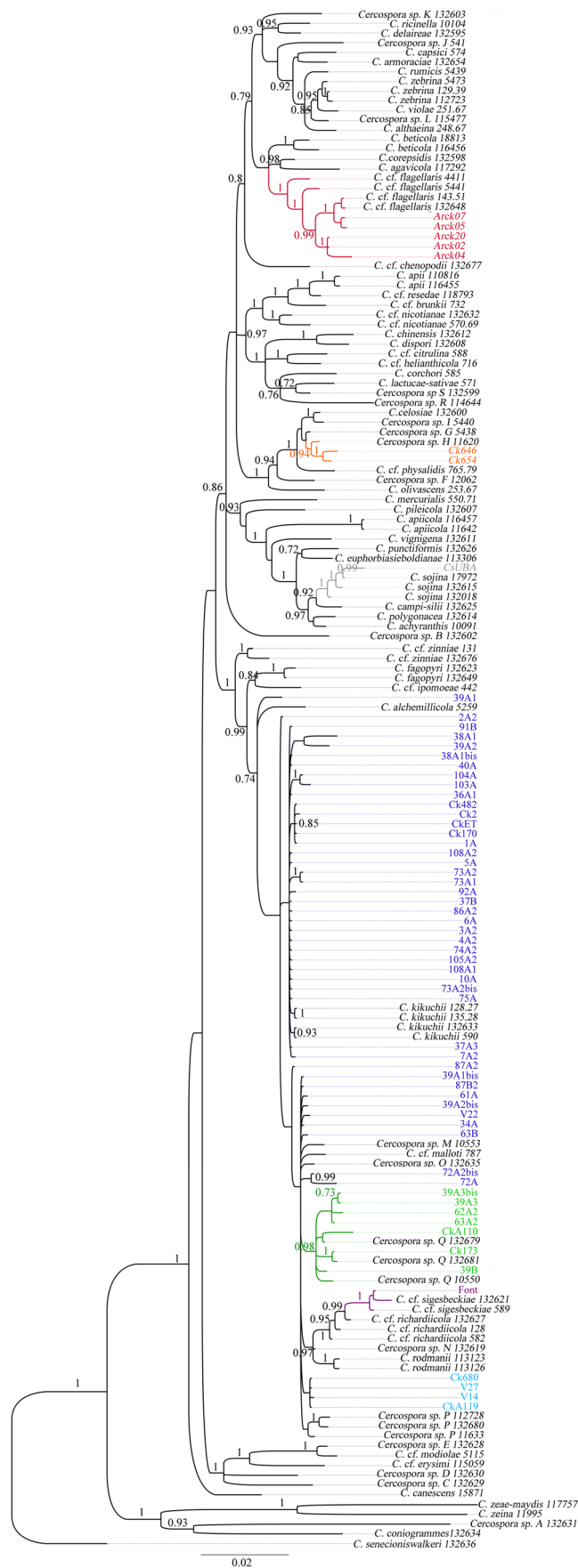
the expected number of substitutions per site. The colours in terminals indicate species identification: *C. kikuchii* and *C. aff. kikuchii* (blue); *C. alchemillicola* (pink); *C. cf. flagellaris* (red); *C. cf. sigesbeckiae* (purple); *Cercospora* sp. *Q* (green); *C. sojina* (grey); *Cercospora* sp. (black)

of *C. kikuchii* and *C. aff. kikuchii* were arranged either in small sub-clades (supported and unsupported) or as single terminals within a large polytomy, including the ex-type of *C. kikuchii* and the sole isolate of *C. cf. sigesbeckiae*.

When all these isolates were analysed together with 64 additional *Cercospora* species (dataset 2; Fig. 2), *C. cf. sigesbeckiae* ( $n = 3$ ) formed a strongly supported clade (pp = 1.0), and five Argentinean isolates (39A3bis, 39A3, 62A2, 63A2 and 39B) formed a monophyletic group together with *Cercospora* sp. *Q* ( $n = 5$ ) (pp = 0.98). Most *C. aff. kikuchii* isolates were part of a large polytomy solely including *C. kikuchii* sequences from Argentina and Japan, but this clade received no support (pp = 0.66). Isolates Ck654 and Ck646 from Brazil clustered with *Cercospora* sp. *H*. from Argentina (pp = 0.94). Sixteen isolates from South America did not cluster with any previously identified species; they appeared either scattered as single-terminal branches or forming pectinate unsupported polytomies.

### Network analyses, haplotypic variation and DAPC

The NN network depicted a clearly reticulate topology, suggesting the occurrence of character conflict within dataset 1 (Fig. 3). The heaviest edge (weight = 0.0230) split the isolates of *C. alchemillicola* and *Cercospora* sp. *H* from the rest. The second heaviest edge in magnitude (0.0198) defined a group composed by the latter two species and all the isolates of *C. cf. flagellaris*; these, in turn, were split by the third edge in weight (0.0168). The fourth edge (0.0096) separated the subset of intermingled haplotypes representing *C. aff. kikuchii*, *Cercospora* sp. *Q* and all *Cercospora* sp. Noteworthy is the intermediate position of isolate 63A2. As in the Bayesian topology (Fig. 1), *C. cf. sigesbeckiae* and isolate 39A3bis (*Cercospora* sp. *Q*) appeared to be nested among most *C. kikuchii*, including the ex-type strain, and *C. aff. kikuchii* isolates. All these isolates were involved in the central polyhedral cycle of the MJ network, where three putatively





**Fig. 2** Majority rule consensus tree from Bayesian phylogeny inference for *Cercospora* species causing S-PSS (dataset 2) (1450 sites; 158 terminals). Branch lengths are drawn to scale. The numbers printed near nodes are posterior probabilities ( $\geq 0.7$ ). The scale bar corresponds to the expected number of substitutions per site. The colours in terminals indicate species identification: *C. kikuchii* and *C. aff. kikuchii* (blue); *C. cf. flagellaris* (red); *C. cf. sigesbeckiae* (purple); *C. sojina* (grey); *Cercospora* sp. *Q* (green); *Cercospora* sp. *H* (orange); *Cercospora* sp. (light blue). The coloured branches highlight clades involving South American samples and *Cercospora* species, and with posterior probabilities  $\geq 0.94$

unsampled or extinct ancestral haplotypes were proposed (squared nodes, Supplemental Fig. S1). To either side of the central cycle, two mostly tree-like branches were apparent; the left branch comprised the group of isolates of *C. aff. kikuchii*, *Cercospora* sp. *Q* and all *Cercospora* sp., and the right branch involved isolates of *C. alchemillicola*, *Cercospora* sp. *H* and *C. cf. flagellaris*.

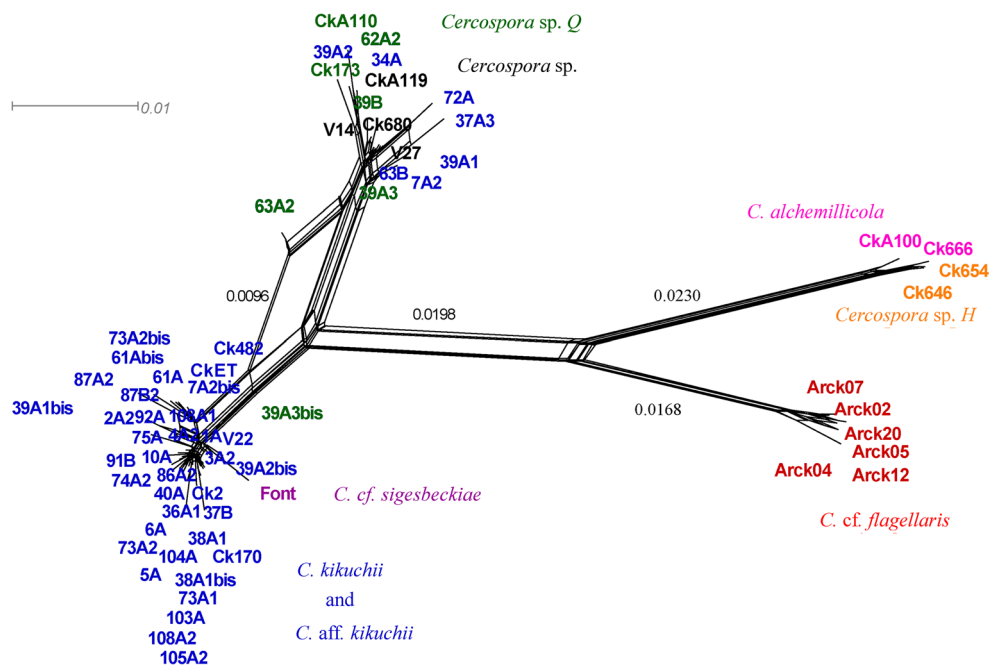
When sequence alignments for each locus were inspected, we encountered ten haplotypes for *tef1*; 17 for *act*, *cyt-b* and *tub1*, 18 for *cal*, 28 for *cfp*, 42 for *tub2* (for 66 samples) and six for ITS (for 46 samples). For the concatenated dataset 1, every fungal isolate represented a unique haplotype ( $H = 66$ ;  $Hd = 1$ ). The qualitative inspection of graphical haplotypes allowed the identification of ten putatively interlocus recombinants (*C. kikuchii*: 2A2, 5A, 37A3, 39A1bis, 39A2, 87A2, 91B; *Cercospora* sp. *Q*: CkA110, Ck173; *Cercospora* sp.: CkA119) and one intralocus recombinant (isolate 63A2) (Supplemental Fig. S2). Graphical haplotypes also permitted the recognition of four haplogroups, congruent with the splits evidenced by the NN and partially with the MJ networks. Likewise, DAPC recovered four groups (G-I to G-IV) based

on the first three principal components, accounting for 96% of genetic variation (Supplemental Fig. S3A). These groups were equivalent to the above-mentioned haplogroups. Accordingly, DAPC group assignment showed that every isolate could be univocally located into its respective group with full probability ( $p = 1.0$ ; Supplemental Fig. S3B).

### Recombination analyses and species boundary delimitation

The pairwise homoplasmy test for dataset 1 (382 informative sites) yielded a highly significant indication that recombination had occurred ( $\Phi = 0.1968 \pm 2.67 \times 10^{-5}$ ;  $p < 0.0005$ ). The Rm test suggested 41 recombination events. The overall scaled recombination rate was significant ( $Rho = 29.25$ ; lower bound = 20.53, upper bound = 41.30; 95th percentile). The X-OVER protocol in RDP4 detected 15 likely unique recombination events. When those events were re-analysed on an individual basis, nine intergroup G-I  $\times$  G-II recombinant haplotypes were validated by at least six independent tests each (range of average  $p$ -value =  $10^{-19}$  to  $10^{-6}$ ; Table 1). These recombinants were likely derived from six parental haplotypes. Two G-I  $\times$  G-III recombinants were further detected but validated by four independent tests. The same two putative parental haplotypes were postulated for those recombinants. When species identification was taken into consideration, it was noted that 5/11 recombinants represented interspecific events between *C. aff. kikuchii* and *Cercospora* sp. *Q* (three cases) or *C. aff. kikuchii* and *C. alchemillicola* (two cases) (Table 1).

**Fig. 3** Neighbor-Net split graph based on uncorrected p-distance among *Cercospora* isolates from dataset 1, excluding *C. sojina* (3351 sites; 66 terminals; fit = 97.89; LSfit = 99.94). The colours in terminals indicate species identification: *C. kikuchii* and *C. aff. kikuchii* (blue); *C. cf. flagellaris* (red); *C. cf. sigesbeckiae* (purple); *Cercospora* sp. *Q* (green); *C. alchemillicola* (pink); *Cercospora* sp. *H* (orange); *Cercospora* sp. (black). The scale bar is in genetic distance units. The weight of each edge, shown in numbers, is proportional to the edge length



**Table 1** Recombination analyses for *Cercospora* isolates. Only validated events are presented

	Recombinants										
	37A3 (G-II) <sup>b</sup>	39A1 (G-II)	7A2 (G-II)	72A (G-II)	63B (G-II)	39B (G-II)	62A2 (G-II)	63A2 (G-II)	39A1bis (G-I)	2A2 (G-I)	91B (G-I)
BpP <sup>a</sup> beginning	1850	2183	1331	1851	1793	1382	1732	605	1480	2906	2635
BpP ending	137	2953	2953	385	135	3257	3114	1332	3192	3267	3241
Test average <i>p</i> -value	1.89 × 10 <sup>-10</sup>				4.05 × 10 <sup>-11</sup>			4.91 × 10 <sup>-12</sup>	7.99 × 10 <sup>-15</sup>	1.95 × 10 <sup>-10</sup>	
RDP											
GENECONV	4.82 × 10 <sup>-09</sup>				4.59 × 10 <sup>-10</sup>			4.48 × 10 <sup>-09</sup>	1.60 × 10 <sup>-13</sup>	6.74 × 10 <sup>-12</sup>	
BootScan	<i>1.76 × 10<sup>-03</sup></i>				–			<i>8.37 × 10<sup>-04</sup></i>	–	–	
MaxChi	1.49 × 10 <sup>-08</sup>				1.56 × 10 <sup>-06</sup>			1.06 × 10 <sup>-11</sup>	1.49 × 10 <sup>-07</sup>	4.15 × 10 <sup>-04</sup>	
Chimaera	7.85 × 10 <sup>-08</sup>				4.86 × 10 <sup>-07</sup>			6.45 × 10 <sup>-12</sup>	1.39 × 10 <sup>-07</sup>	1.35 × 10 <sup>-04</sup>	
SiScan	3.03 × 10 <sup>-07</sup>				2.12 × 10 <sup>-08</sup>			1.25 × 10 <sup>-13</sup>	1.95 × 10 <sup>-07</sup>	1.33 × 10 <sup>-07</sup>	
3Seq	2.43 × 10 <sup>-16</sup>				4.08 × 10 <sup>-16</sup>			1.17 × 10 <sup>-18</sup>	1.01 × 10 <sup>-19</sup>	9.48 × 10 <sup>-09</sup>	
Major parental	39A2 (G-II)				CkA110* (G-II)			5A (G-I)	91B (G-I)	87A2 (G-I)	
Minor parental	87A2 (G-I)				38A1bis (G-I)			39A2 (G-II)	39A2 (G-II)	Ck666** (G-III)	

<sup>a</sup> BpP, breakpoint position on each recombinant sequence (in base pairs)

<sup>b</sup> G-I to G-III, referring to the groups inferred from DAPC analyses. Values in *italics* are above the established acceptance threshold; empty cells indicate that no results were obtained

\**Cercospora* sp. Q; \*\**C. alchemillicola*; all others, *C. aff. kikuchii*

Network topologies, pairwise homoplasy indices and minimum recombination tests (Rm) provided evidence for data conflict in dataset 1, suggesting intra- and intergroup genetic exchange. Recombination analyses validated the occurrence of interspecific recombinants, suggesting that species boundaries between such groups are diffuse.

## Discussion

The present study surveyed the genetic puzzle of S-PSS causal agents across the major South American soybean producing countries. Particularly for Argentina, both the number of analysed *Cercospora* isolates associating S-PSS and sampled locations increased in comparison to previous studies (Soares et al. 2015; Groenewald et al. 2013). Along with the utilisation of analytical procedures that do not assume strictly bifurcated evolutionary patterns, our study showed that interspecific gene flow may play a significant role in the evolutionary dynamics of *Cercospora* species associated with S-PSS in this geographic region. The epidemiological scenario for S-PSS in South America emerges as being different from that communicated by Albu et al. (2016) for North America, where *C. cf. flagellaris* and *C. cf. sigesbeckiae* appeared as prevalent species. Our results suggest a more complex scenario in Argentina and Brazil. Isolates morphologically resembling *C. kikuchii* but also lineages that could be ascribed to five additional taxa (i.e. *C. kikuchii*, *C. alchemillicola*, *C. cf. sigesbeckiae*, and *Cercospora* spp. Q and H) were encountered. Furthermore, various isolates could only be identified as *C. aff. kikuchii* or *Cercospora* sp., as it was not clear whether these represented additional undescribed species.

Although most Argentinean and Brazilian soybean producing areas were sampled for this report, continuous studies are required to attain updated epidemiological pictures. While a strikingly high haplotypic diversity (Hd = 1.0) was encountered, our analyses also indicated that significant intra- and interlocus recombination rates may occur. Congruence analysis provided the first indication of significant conflict, while network analyses allowed the identification of non-tree-like associations. As was already reported for other species of *Cercospora* (Groenewald et al. 2006, 2008; Bolton et al. 2012; Bakhshi et al. 2015), our data suggest that S-PSS causal agents are capable of sexual reproduction and/or parasexual activity. For example, *Mycosphaerella*-like teleomorphs have been found for a few species, such as *M. fijiensis* (*C. fijiensis* = *Paracercospora fijiensis*), *M. musicola* (*C. musae* = *Pseudocercospora musae*) and *M. arachidicola* (*C. arachidicola*) (Corlett 1991; Crous et al. 2004b). We suggest, however, for the first time, that, also, interspecies recombinant events can occur in ecologically defined *Cercospora* species complexes.

Delimiting specific boundaries in the presence of recombination is daunting, open to debate and depends upon applied species concepts (De Queiroz 2007). Gene flow between *C. kikuchii* and *Cercospora* sp. *Q*, and *C. kikuchii* and *Cercospora* sp. *H* is suggested on the basis of our analyses. Based on the high number of recombination events reported here between *C. kikuchii* and *Cercospora* sp. *Q*, and assuming that they do not share a recent common ancestor, it might be proposed that they represent two diverging biological entities, whose reproductive isolation mechanisms have not been completely developed at present. Similarly, although *C. kikuchii* and *Cercospora* sp. *H* are not closely related, as evidenced by Groenewald et al. (2013) and herein, the interspecific genetic exchange detected amongst them may suggest that they have not yet evolved as independent species.

### Multiple origins of the S-PSS agents, impact on agricultural practices

*Cercospora* species associated with S-PSS appear to have diverged between 0.5–1.7 Mya (Soares et al. 2015), well before archaeological records suggest that the first cultivated soybean seeds was sown in East Asia (some 5500 years ago; Lee et al. 2011). Cultivated soybean is well known for its narrow genetic base, produced by successive bottleneck breeding during its domestication and introduction into the Western Hemisphere; modern soybean cultivars could be traced back to, at most, 16 ancestral genotypes (Hyten et al. 2006). The results from these and other studies indicate that a number of diverse cercosporoid fungi may have opportunistically colonised modern soybean cultivars, constituting a cryptic array of pathogens triggering the same defence reactions, which were collectively identified as “*C. kikuchii*”. For instance, isolates assigned here as *Cercospora* sp. *Q* and those as *Cercospora* sp. *H* produce the same symptoms that *C. kikuchii* produced on soybean seeds. Noteworthy, *Cercospora* sp. *Q* is an ubiquitous, polyphagous pathogen and *Cercospora* sp. *H* was originally isolated from Myrtaceae and Convolvulaceae (Groenewald et al. 2013).

Because no robust relationships were established for isolates V14 and V27 (from Tucumán, Argentina), Ck680 (from Mato Grosso do Sul, Brazil) and CkA119 (from Minas Gerais, Brazil), they are considered different, currently undescribed *Cercospora* species. As already shown by Soares et al. (2015) and further verified here, *C. cf. sigesbeckiae* (from Japan and South Korea) is currently known from a single gathering in South America. No *C. flagellaris*, known to occur in the USA, Near East and East Asia (Groenewald et al. 2013; Bakhshi et al. 2015; Albu et al. 2016), has been detected in South America so far. Accordingly, measures should be considered to avoid its introduction. It is worth mentioning that, even though maize/soybean crop rotation is a common practice, no *C. zeina* or *C. zea-maydis* were isolated from

soybean until now, suggesting that they are potentially host-specific. Noticeably, these two species show a distant phylogenetic relationship with all the aforementioned *Cercospora* associated with soybean (Fig. 2 and Groenewald et al. 2013).

Evidences from interspecific recombination (Table 1) and network analyses (Fig 3; Supplemental Fig. S1) suggested that soybean-associated *Cercospora* isolates from different phylogenetic lineages are capable of sharing their genetic reservoirs. Recombination events among *Cercospora* species were detected for *C. alchemillicola*, *Cercospora* sp. *Q* and *C. aff. kikuchii*. Similar events that may have affected also pathogenicity genes may have contributed to the similar pathogenic behaviour of these taxa on soybean. Interspecific genetic exchange events, which could be either sexually or asexually, may lead to more aggressive or resistant haplotypes and altered epidemiological situations. This may have implications for disease control, plant breeding, quarantine regulations and international seed trade. Not only should pathogen population structure be taken into account for risk framework development (McDonald and Linde 2002) but also analyses documenting interspecific genetic exchange events.

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