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RESEARCH ARTICLE

Specific boundaries between the causal agents of the soybean stem canker

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ABSTRACT

Pathogens within the *Diaporthe* complex cause seed decay, stem blight and stem canker on soybean, representing a serious threat for this crop species. We herein utilize worldwide sequence data retrieved from Genbank in order to assess the species boundaries between the soybean stem canker causal agents, and define whether or not they should be regarded as members of the same biological species. These studies were complemented with compatibility tests, in order to validate our findings from a biological standpoint. Species delimitation assays supported the occurrence of a speciation event between *D. caulivora* and *D. phaseolourm var. meridionalis*. A speciation hypothesis between *D. aspalathi* and *D. phaseolourm* var. *meridionalis* was also supported, based on three reciprocally monophyletic substitutions at locus EF1-α. Compatibility tests further validated species delimitation assays indicating that *D. caulivora* has developed barriers to gene exchange with *D. phaseolorum* var. *meridionalis*. Clarification of the specific boundaries of the SSC pathogens and related entities will be an important asset to future research in soybean pathology, epidemiology and breeding.
Key words: *Diaporthe aspalathi*, *Diaporthe caulivora*, *Diaporthe phaseolorum* var. *meridionalis*, species delimitation.

INTRODUCTION

Diaporthe Nitschke, with over 800 specific names, constitutes the teleomorphic state of *Phomopsis* (Sacc.) Bubák, an anamorphic genus with more than 900 specific names recorded. An important number of species within this group has been reported as destructive pathogens causing cankers, diebacks, root rots, fruit rots, leaf spots, blights, decay and wilts on a wide range of plant hosts worldwide, including strategic crop species (Udayanga et al., 2011; 2012). Fungi in the *Diaporthe* species complex constitute an economically relevant threat for the soybean production chain worldwide, with five taxa traditionally recognized: *Diaporthe phaseolorum* (Cooke & Ellis) Sacc., *D. phaseolorum* var. *sojae* (Lehman) Wehm., *Phomopsis longicolla* Hobbs, *D. phaseolorum* var. *meridionalis* F. A. Fernández, *D. phaseolorum* var. *caulivora* Athow & Caldwell. The latter two have been reported as the causal agents of the soybean stem canker (SSC). Santos et al. (2011) recently described *Diaporthe novem* Santos, Vrandecic & Phillips, as a sixth soybean pathogen. Before the arrival of soybean rust to the Americas *Diaporthe* pathogens were cited as causing more economic losses in soybean production than any other single fungal pathogen, and had been a major concern in South America since 1989 (Sinclair & Backman, 1989). During the 1994/1995 growing season, yield losses due to SSC reached US$ 170 million in Brazil (Yorinori, 1996). SSC was first detected in Argentina in 1996/97, and has since then caused up to 100% yield loss in some instances (Grijalba et al., 2011).

Asexual and sexual names of fungi have recently been granted equal status in the International Code of Nomenclature for algae, fungi, and plants. Therefore, the name *Diaporthe* has been adopted for this group of fungi, regardless of the spore stage involved (Santos et al., 2011; Crous et al., 2011; Udayanga et al., 2012; Gomes et al., 2013) since *Diaporthe* (1870) predates *Phomopsis* (1905).

The taxonomic history of *Diaporthe* species as soybean pathogens starts early in the 20th century when *Diaporthe* spp. isolates were obtained from a group of unrelated hosts, including *Ipomoea batata* L., *Phaseolus lunatus* L. and *Glycine max* (L.) Merr. Following the host-specific hypothesis, those isolates were recognized as independent species and identified as *Diaporthe batatas* Harter & E. C. Field; *D. phaseolorum* (Cooke & Ellis) Sacc., and *D. sojae* Lehman, respectively (Morgan-Jones, 1985; 1989; Backman et al., 1985). In contrast, Harter & Field (1912) and Harter (1917) proposed that these three pathogens constitute a single species, reassigning them as three varieties: *D. phaseolorum* var. *phaseolorum*, *D. phaseolorum* var. *batatas*, and *D. phaseolorum* var. *sojae*. In the early 1950's, *D. phaseolorum* var. *caulivora* was

first described as the causal agent of the SSC and it was considered a perithecial variant from *D. phaseolorum* var. *batatas* (Crall, 1950), the causal agent of the dry root in sweet potato (*Ipomea batata* L.). Hobbs & Phillips (1985) proposed the differentiation of the US Northern and Southern stem cankers. Morgan Jones (1989) further split them into *formae speciales*, based on morphological and physiological differences, designating *D. phaseolorum f. sp*. *meridionalis* for the southern US teleomorphic isolates, and *D. phaseolorum f. sp*. *caulivora* for northern isolates. Fernández & Hanlin (1996), based on differences in the number and type of lesions shown by field-grown plants, readopted the concept of "variety". Since then, the accepted denomination has been *D. phaseolorum* var. *caulivora* and *D. phaseolorum* var. *meridionalis*. Based on nucleotide sequence data, cultural, phytopathological and morphological evidence, Rensburg et al. (2006) proposed that *D. phaseolorum* var. *meridionalis* should be treated at the species level along with the red bush die-back causal agent, *Diaporthe aspalthi*. Due to nomenclature reasons they renamed *D. phaseolorum* var. *meridionalis* as *Diaporthe aspalathi* Janse Rensburg, Castlebury & Crous. More recently Santos et al. (2011) raised *D. phaseolorum* var. *caulivora* to the specific level, recombining it as *Diaporthe caulivora* (Athow & Caldwell) J.M. Santos, Vrandecic & A.J.L. Phillips. This puzzling taxonomic situation has rendered the identification of biological entities from amongst the array of specific names extremely cumbersome.

Species identification in *Diaporthe* has been traditionally based on host specificity (Udayanga et al., 2011; 2012). Few morphological characters can undoubtedly differentiate among taxa (Uecker, 1988). Identification of the SSC pathogens has relied on colony appearance, growth rate, size of stromata, arrangement and morphology of perithecia, presence of α and β conida, and detection of the anamorph phase (Morgan Jones, 1985; 1989; Sinclair & Backman, 1989; Fernandez & Hanlin, 1996). The overlap shown by some of these quantitative features has led to several ambiguous identifications. Indeed, it is a well-known general fact, that morphological and phytopathological characters are affected by environmental factors and sampling, often leading to inaccurate species classification (Davis & Nixon, 1992; Padial et al., 2010; Grijalba & Ridao, 2012). In order to circumvent such limitations, the classification of *Diaporthe* species is presently being redefined to include DNA sequence data (Rehner & Uecker, 1994; Zhang et al., 1998; Mostert et al., 2001; Farr et al., 2002; Santos et al., 2010).

Nevertheless, methods for species delimitation using genealogical data typically rely upon genetic distances or gene trees (Sites & Marshall, 2003; 2004). This analytical approach requires arbitrary decisions regarding the thresholds of the species boundary (Hey, 2009). In order to circumvent this problem Yang & Rannala (2010) developed a coalescent-based approach to delimitate closely related species using DNA sequence data. This methodology includes both intra and interspecific variation. This approach to species boundary delimitation has been validated with simulated datasets (Yang & Rannala, 2010; Zhang et al., 2011) and applied to empirical datasets of rotifers, lizards (Yang & Rannala, 2010), forest geckos (Leache & Fujita, 2010), butterflies (Zhang et al., 2011) and rice (Zang et al., 2011).

The study of somatic incompatibility reactions provides a useful criterion for spatial delimitation of fungal individuals, or at least for delimitation of genetically distinct mycelia. This criterion has been applied in several fungal groups including important plant-pathogenic fungi (Pál et al., 2007). It has been proposed that the incompatibility reaction may limit the spread of harmful cytoplasmic or nuclear elements (Caten, 1972), and prevent resource plundering (Debets & Griffiths, 1998). It has also been suggested that vegetative incompatibility may promote the initiation of sexual reproduction in some species as a result of non-self recognition (Dyer et al., 1992). Individuals that share the same heterokaryon or vegetative incompatibility loci can fuse to form a heterokaryon and are then considered to belong to the same vegetative compatible group (Glass et al., 2000). In contrast, fungal isolates that differ at one or more of these loci will not fuse. Instead, programmed cell death or apoptosis occurs in the mycelial cells that are in contact with an isolate representing different vegetative compatible groups (Leslie, 1993). Previous vegetative compatibility assays have been performed for the SSC pathogens, but involving solely two Brazilian isolates (Costamilan et al., 2008).

As previously mentioned, the taxonomic rank for both SSC causal agents has been upraised to the specific level. Nevertheless, genetic and biological boundaries between them have not been addressed so far; indeed, the causal agents of tSSC are in practice still treated as part of the same biological species by soybean breeders and pathologists. As a consequence, much of the research carried out elsewhere treats these pathogens as a single biological entity. We used molecular data retrieved from many different geographic origins in order to clearly assess whether the gene pools of *D. aspalathi, D. phaseolorum* var. *meridionalis* and *D. caulivora* are in fact isolated or not. To further biologically validate the molecular evidence, we implemented vegetative compatibility assays between distinct soybean isolates of *D. phaseolorum* var. *meridionalis* and *D. caulivora*.

MATERIALS AND METHODS

Preliminary nucleotide sequence analyses and dataset assembly

Sequence data for soybean pathogen isolates originally identified as *Diaporthe phaseolorum* var. *caulivora*, *Diaporthe caulivora*, *Diaporthe phaseolroum* var. *meridionalis* and *Diaporthe aspalathi* were retrieved form Genebank. Sequence data publicly available for seven

loci was included for molecular species delimitation assays (Table 1). Multiple sequence alignment for each locus was attempted using Clustal W (Thompson et al., 1994) and Muscle (Edgar, 2004), as implemented in Mega 5.0 (Tamura et al., 2007), with different parameter settings, and slight manual modifications when necessary.

TABLE 1 - Locus name, original GenBank denomination, host, country of origin and GenBank accession numbers of the sequences included in this study.

Locus name	Original Taxon name (GenBank)	Host	Country of origin	GenBank accession number
28 S	*Diaporthe caulivora*	*Glycine max*	USA	JQ697877
		Glycine max	Serbia	JQ697878, JQ697879, JF411057, JF411058
		Glycine max	Italy	HQ445920, HQ445921 HQ445936
	Diaporthe phaseolorum var. *meridionalis*	*Glycine max*	Italy	HQ445934, HQ445935
		Glycine max	USA	JQ697875 / JF704178
CAL	*Diaporthe caulivora*	*Glycine max*	Croatia	KC343287
		Glycine soja	Canada	KC343288
	Diaporthe aspalathi	*Aspalathus linearis*	South Africa	KC343277 / KC343279
HIS 3	*Diaporthe caulivora*	*Glycine max*	Croatia	KC343529
		Glycine soja	Canada	KC343530
	Diaporthe aspalathi	*Aspalathus linearis*	South Africa	KC343519, KC343521
β-TUB	*Diaporthe caulivora*	*Glycine max*	Croatia	KC344013
		Glycine soja	Canada	KC344014
	Diaporthe aspalathi	*Aspalathus linearis*	South Africa	KC344003 / KC344005
EF1-α	*Diaporthe phaseolorum* var. *caulivora*	*Glycine max*	South Korea	HQ333508
		Glycine max	USA	AF398889, JQ697864
	Diaporthe caulivora	*Glycine max*	Croatia	KC343771, HM347687 / HM347691
		Glycine max	Italy	HQ445914
		Glycine max	Serbia	JQ697852, JF461465 / JF461467
		Glycine soja	Canada	KC343772
	Diaporthe aspalathi	*Aspalathus linearis*	South Africa	AY339353, DQ286249 / DQ286252, KC343761 / KC343763
	Diaporthe phaseolorum var. *meridionalis*	*Glycine max*	USA	AF394864, AF394865 , AF398890 / AF398893
		Glycine max	Italy	HQ445932
		Glycine max	USA	JF461479, JF461480
		Glycine max	Serbia	JQ697862 JQ697863
IGS	*Diaporthe phaseolorum* var. *caulivora*	*Glycine max*	Argentina	HM769302 / HM769322
	Diaporthe phaseolorum var. *meridionalis*	*Glycine max*	Argentina	HQ130442/ HQ130444
ITS	*Diaporthe phaseolorum* var. *caulivora*	*Glycine max*	Ex-Yugoslavia	AJ312360
		Abutilon theophrasti	Serbia	AY857867
		Glycine max	China	EF594039
		Glycine max	Argentina	EF594040, EF594041
		Glycine max	USA	EF594042, EF594043
		Glycine max	Brazil	EU622854, FJ357156 / FJ357158
		Glycine max	Argentina	HM625752 / HM625773
		Glycine max	USA	AF000212, AF000563, AF000567
		Glycine max	South Korea	HQ333503
	Diaporthe caulivora	*Glycine max*	Serbia	JF418934 / JF418937
		Dipsacus laciniatus	Croatia	HM347703, HM347704, HM347712
		Glycine max	Italy	HQ445937
		Glycine max	USA	JQ697851
		Glycine max	Croatia	KC343045
		Glycine soja	Canada	KC343046
	Diaporthe aspalathi	*Aspalathus linearis*	South Africa	AY339321, DQ286275 / DQ286278 – FJ785432, KC343035 / KC343037
	Diaporthe meridionalis	*Glycine max*	USA	AF001015, AF000564 /AF000566
		Glycine max	Italy	AJ312361
	Diaporthe phaseolorum var. *meridionalis*	*Glycine max*	China	EF594044
		Glycine max	Brazil	FJ357153 / FJ357155
		Glycine max	Argentina	HQ130438 / HQ130440
		Glycine max	USA	JF430485, JF430486
		Glycine max	Italy	JF495106
		Glycine max	Serbia	JQ697849, JQ697850
		Melastoma malabathricum	India	KF193982

28 S: 28S ribosomal RNA gene; CAL: Calmodulin gene; HIS 3: histone H3 gene; β-TUB: beta-tubulin gene; EF1-α: translation elongation factor 1 alpha gene; IGS: Intergenic Spacer of the nrDNA region; ITS: internal transcribed spacer regions of the nrDNA and intervening 5.8S nrDNA. Slashes indicate consecutive accession numbers.

Bayesian species delimitation assays

Species delimitation assays were performed using the program Bayesian Phylogenetics and Phylogeography (BPP) v. 2.0 (Rannala & Yang, 2003; Yang & Rannala, 2010). This program requires three input files, namely the sequence file (including multiple alignments for every loci under consideration), a species map file (indicating the putative species for each sequence) and a file including specific evolutionary parameters. This latter file is amenable to alternative tailoring in order to account for different evolutionary scenarios. Evolutionary parameters include a guide tree, as well as specification of prior distributions for the scaled ancestral population size (θ_0), and root age (τ_0). Priors are assigned a Gamma G (α, β) distribution, with a prior mean = α/β and prior variance = α/β^2. This information is user-provided, and constitutes the starting point (priors) for the program. Prior distributions can affect the posterior probabilities for the different speciation models (topologies). According to coalescence theory, large values for θ_0 (big population numbers) and small values for τ_0 (shallow divergence times) favor conservative models containing fewer species (Leache & Fujita 2010; Yang & Rannala, 2010). In species delimitation, the guide phylogeny is also a most important prior affecting posterior probabilities for the speciation hypotheses (Leache & Fujita, 2010; Yang & Rannala, 2010; Zang et al., 2011).

BPP v. 2.0 uses a reversible-jump Markov chain Monte Carlo (rjMCMC) algorithm to jump back and forward over different topologies and estimate the posterior distributions of species delimitation models, starting from the guide tree. Every model should be compatible with the starting priors and the sequence alignment introduced in the input files. By default, BPP assumes no admixture following a speciogenic event. The JC69 mutation model (Jukes & Cantor, 1969) is assumed to accommodate multiple hits. The sequences are supposed to be close, so that JC69 is deemed adequate. Leache & Fujita (2010) proposed posterior probability values > 0.95 as strong support for a speciation event.

The guide tree herein proposed considers *D. aspalathi*, *D. phaseolorum var. meridionalis and D. caulivora* as three separate species (completely resolved tree). Considering the huge population numbers of fungal organisms, a gamma prior distribution G(1, 10) for the root population size (θ_0) was set for every assay. Provided that no information (eg. fossil record) is available indicating species history, three different gamma priors, namely G(1, 10), G(2, 200) and G(2, 2000), were attempted for τ_0. These priors account for different divergence times from the root population. Each analysis was run at least twice, to confirm consistency between runs. Running the rjMCMC analyses for 500,000 generations (sampling interval of five) with a burn-in period of 10,000 produced consistent results across separate analyses initiated with different starting seeds. Convergence was considered as adequate only after the Estimated Sample Size (ESS) was above 300 for every node.

Somatic compatibilty tests

Vegetative compatibility was tested based on the formation of a barrage-zone. Six soybean fungal isolates were tested against each other. All isolates are housed at the Phytopathology Lab, School of Agronomy, University of Buenos Aires. *Diaporthe phaseolorum* var. *meridionalis* isolates were obtained at Asunción (Paraguay; Genbank accession number HQ130438, Dm1); Venado Tuerto (Santa Fe, Argentina; HQ130439, Dm2) and Pergamino (Buenos Aires, Argentina; HQ130440, Dm3). *Diaporthe caulivora* isolates were obtained at Trenque Lauquen (Southern Buenos Aires, Argentina, HM625758, Dc1), Urdampilleta (Western Buenos Aires, Argentina, HM625770, Dc2) and General Pirán (Buenos Aires, Argentina, HM625760, Dc7). Isolates were paired 2-3 cm apart on PDA (potato dextrose agar) in Petri dishes and incubated in darkness for a week at 20ºC and another week at 25ºC (Costamilan et al., 2008). Self-crosses were utilized as negative controls, representing no barrage formation. Each pairing was repeated twice. Hyphal interactions were recorded two weeks after the fungi were plated. The interaction zone and their boundaries were further observed under the microscope and photographed.

RESULTS

Sequence analysis

In the present study we included 162 sequences from 7 distinct nuclear loci, comprising a total of 76240 bp. ITS and EF1-α were the only genomic locations with sequences available for all taxa under study.

Bayesian species delimitation assays

Multilocus bayesian species delimitation assays, irrespective of time divergence assumptions, yielded posterior probabilities (pp) between 99 - 100% for the completely resolved tree in every evolutionary scenario (Figure 1). In comparison, the two-species (considering *D. aspalathi* and *D. ph.* var. *meridionalis* as a single species) model displayed extremely low posterior probabilities under all prior combinations (pp <0.02 in all cases).

A speciation hypothesis between *D. aspalathi* and *D. phaseolorum* var. *meridionalis* was also strongly supported (pp= 0.99 – 1.0, Figure 1) using the original three species guide tree, and under every time divergence assumption. In order to further explore this finding, a series of assays aimed at assessing the species boundaries between *D. aspalathi* and *D. phaseolorum* var. *meridionalis* were carried out, using solely those loci for which information was available for both taxa (ITS and EF1-α). A speciation hypothesis was once again favored (pp>0.99) in every evolutionary scenario. This speciation hypothesis is sustained by three reciprocally monophyletic substitutions between *D. aspalathi* and *D. phaseolorum* var. *meridionalis* at positions g.99G>T, g.161C>T and g.236 C>T of the EF1-α locus (Figure 2). The ITS region, on the other hand,

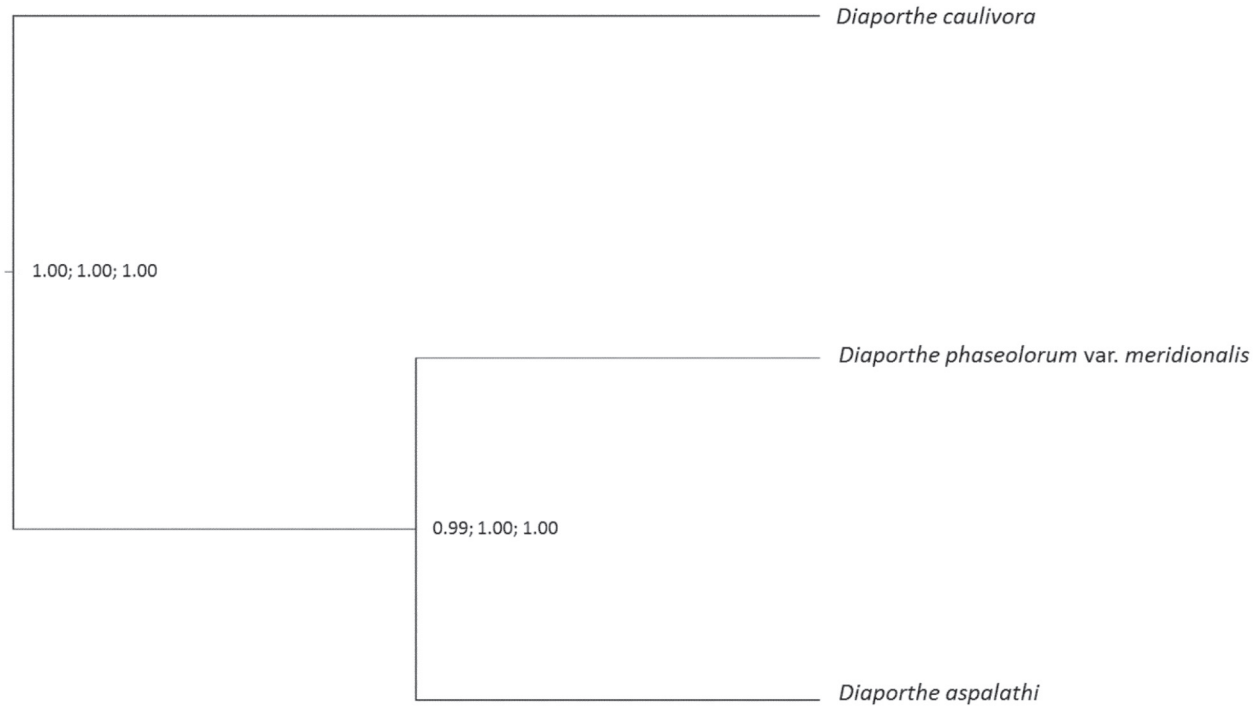

FIGURE 1 - Multilocus Bayesian species delimitation results assuming a 3-species guide tree. The speciation probabilities are provided for each node and each combination of priors. Prior mean $\theta = 0.1$ in all cases (big population numbers); this assumption results in lower speciation probabilities. Left, prior mean $\tau_0 = 0.1$; middle, prior mean $\tau_0 = 0.01$; right, prior mean $\tau_0 = 0.001$.

was identical between *D. aspalathi* and *D. phaseolorum* var. *meridionalis*, as previously stated (Rensburg et al., 2006).

Somatic compatibility tests

The vegetative compatibility tests were performed between *D. caulivora* and *D. phaseolorum* var. *meridionalis* isolates (Figure 3). The presence of a distinctive barrage, or pigmented zone and a lytic gap along the contact zone was detected in every *D. caulivora – D. phaseolorum* var. *meridionalis* confrontation assayed, seven days after contact. Microscopically, this pigmented zone comprised of a combination of compartimentalized hyphal segments, vacuolated brown hyphae and empty cells, not observed in unpaired growing mycelia. Conversely, *D. phaseolorum* var. *meridionalis – D. phaseolorum* var. *meridionalis* and *D. caulivora – D. caulivora* confrontations merged uniformly with no dark line in the contact zone.

DISCUSSION

The multilocus species delimitation test herein assayed clearly indicates that no gene exchange occurs between *D. caulivora* and the *D. aspalathi – D. phaseolorum* var. *meridionalis* cluster. Incompatibility reactions in every *D. caulivora* - *D. phaseolorum* var. *meridionalis* confrontation further strongly validate and confirm the genetic isolation between both groups. These findings supports previous results from Santos et al. (2011) who raised *D. caulivora* to the specific level using isolates from Croatia, and Grijalba et al. (2011) and Guillin et al. (2011) who reached a similar conclusion for Argentinean isolates.

Our species delimitation assays also supported a speciation hypothesis between *D. aspalathi* and *D. phaseolorum* var. *meridionalis*. This result is somehow unexpected, since it contradicts previous claims by Smit & Knox-Davies (1989a; 1989b) and Rensburg et al. (2006), who concluded that both taxa should be considered as part of the same species. These authors have mainly based their proposal on comparative morphology between the two taxa, and an ITS-based phylogenetic reconstruction including other *Diaporthe* species as well; no EF1-α sequences from *D. phaseolorum* var. *meridionalis* was available to them, and therefore they were not included in their combined ITS and EF1-α phylogenetic reconstruction. Therefore, although it is evident that these two taxa are very closely related, it is still not clear whether they are reproductively compatible. In this regard, it should be emphasized that *D. aspalathi* has solely been obtained from the red bush, *Aspalathus linearis* (Burm. f.) R. Dahlgren in South Africa, whereas isolates identified as *D. phaseolorum* var. *meridionalis* have been obtained from soybean fields worldwide. The occurrence of three reciprocally monophyletic substitutions at EF1-α suggests that both taxa have been somehow isolated for a considerable period of time, relative to the population

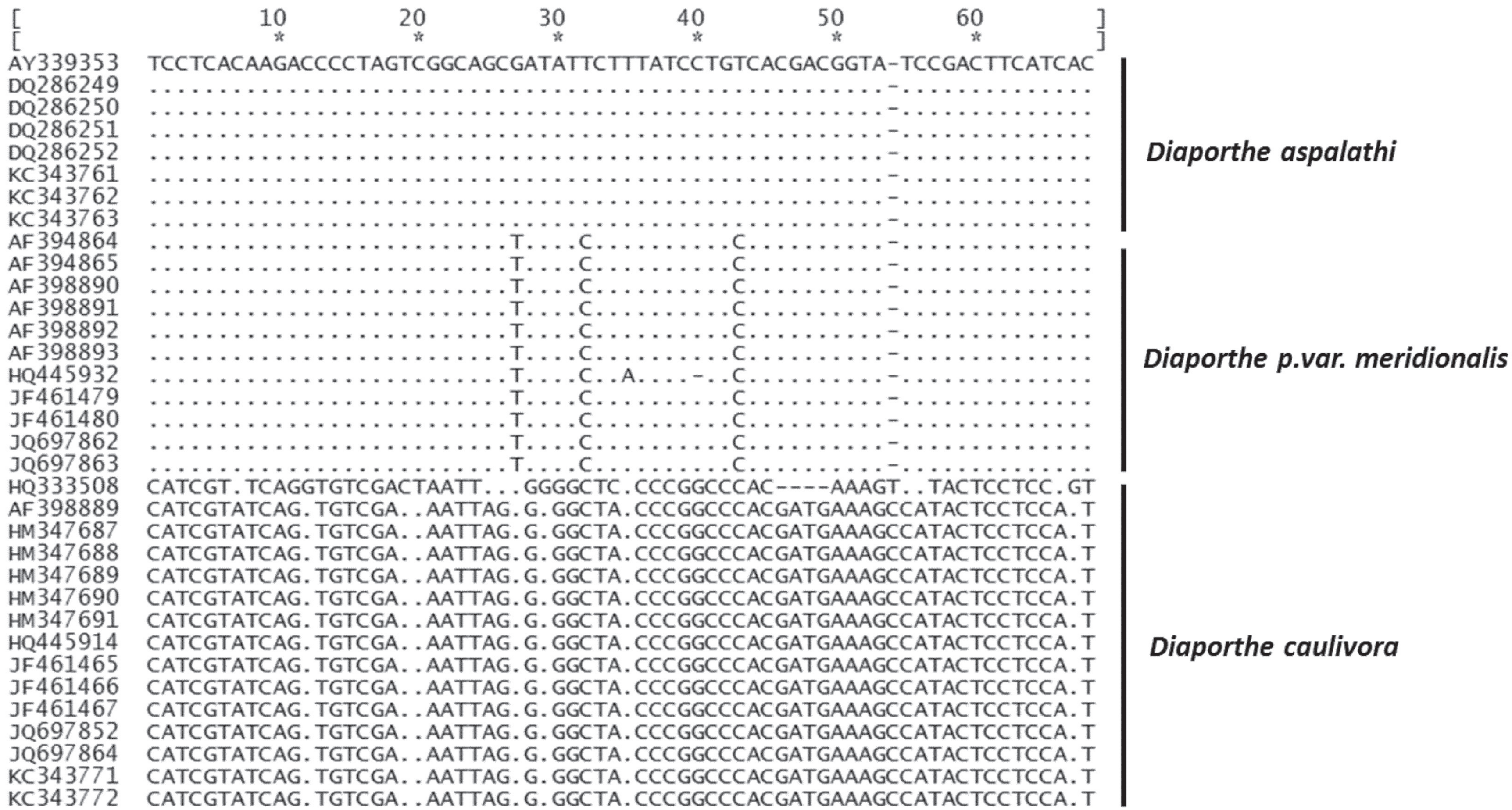

FIGURE 2 - Sequence alignment of the variable sites for the EF1-α region defining 5 haplotypes out of 34 sequences originally assigned to *Diaporthe caulivora, D. aspalathi* and *D. phaseolorum* var. *meridionalis*. Each fragment spans 306 bases. Dots indicate similarity, while hyphens indicate gaps. Numbers above indicate the nucleotide position having haplotype AY339353 as a reference sequence during alignment. Nucleotide substitutions g.99G>T, g.161C>T and g.236 C>T define *D. aspalathi* and *D. phaseolorum* var. *meridionalis* as reciprocally monophyletic groups.

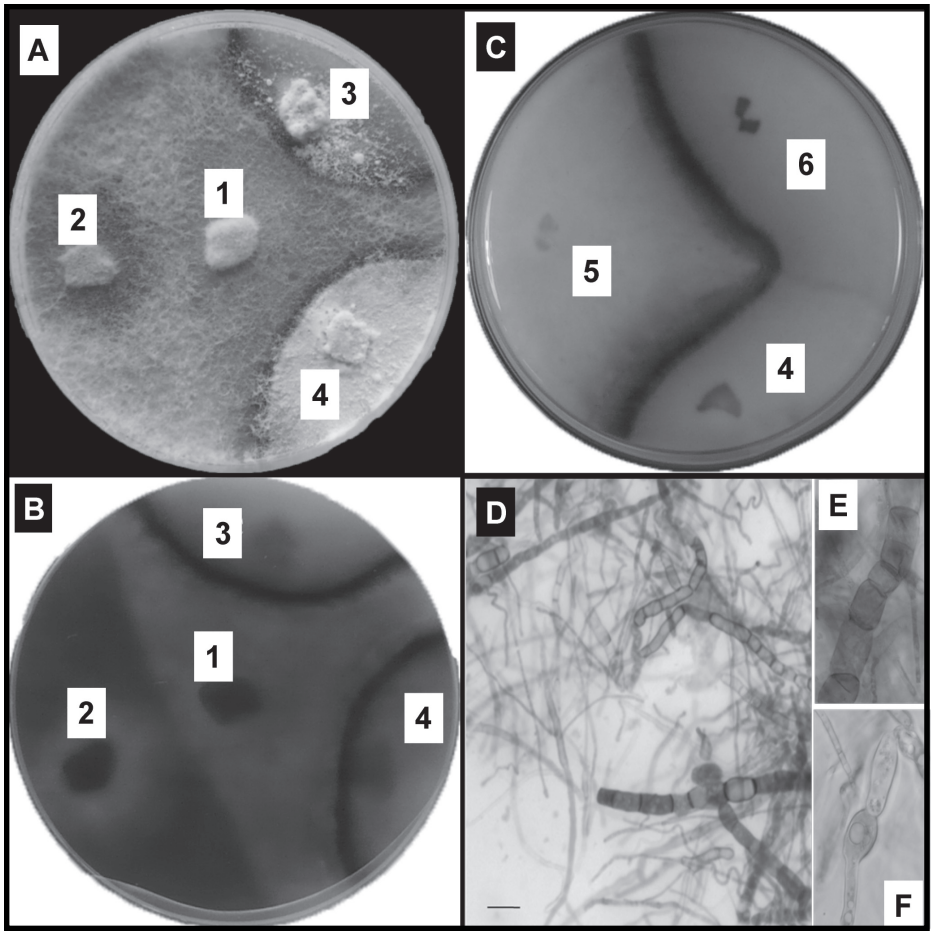

FIGURE 3 - Somatic incompatibility assays between *Diaporthe phaseolorum* var. *meridionalis* obtained from soybean fields) and *D. caulivora* isolates. **A. B**. Front and reverse of the mycelia growing in PDA: 1, *D. phaseolorum* var. *meridionalis* (HQ130439, Argentina); 2, *D. phaseolorum* var. *meridionalis* (HQ130438, Paraguay); 3, *D. caulivora* (HM625760, Argentina); 4, *D. caulivora* (HM625758, Argentina); **C**. Reverse of the mycelia growing in PDA: 5, *D. phaseolorum* var. *meridionalis* (HQ130440, Argentina); 6, *D. caulivora* (HM625770, Argentina). The darkened contact zone represents barrage formation. **D-F**. Hyphae from the barrage zone from confrontation HM625770 vs HQ130440; **D.** Thin and thickened brown hyphae; **E.** String of empty brown cells; **F.** Vacuolized cells. Bar= 10 μm.

size at the founder event (most likely a host jump from soybean to red bush). The present results suggest that genetic divergence between these two groups might be currently taking place, based on ecological grounds (host specialization). Therefore, it cannot be excluded that *D. aspalathi* and *D. phaseolorum* var. *meridionalis* constitute cryptic species at present. Further analyses with a larger number of loci are warranted, in order to assess whether a host-jump based speciation event between these two taxa has already been accomplished. No compatibility or cross infection tests involving *D. phaseolorum* var. *meridionalis* and *D. aspalathi* isolates have been attempted so far to our knowledge in order to biologically validate contrasting hypotheses. Although *D. phaseolorum* var. *meridionalis* has been formally accepted as a synonym of *D. aspalathi*, in view of the present results it is likely that this will need to be further clarified in the future.

It has been stated that traditional morphological characters no longer clarify the taxonomy of *Diaporthe* at the specific level (Brayford, 1990; Rehner & Uecker, 1994; Crous, 2005). In this regard, Udayanga et al. (2011; 2012) and Gomes (2013) proposed phylogenetic trees as platforms for future taxonomic classification within this species complex. Nevertheless, speciation is a continuous process (De Queiroz, 1998; 2007) and this implies that delimiting species using genealogical data will necessarily be accompanied by some degree of uncertainty (Leache & Fujita, 2010). This is particularly so when dealing with closely related species. Very importantly also, multiple sequence alignment for a great number of species would most likely bring about ambiguously aligned regions that could greatly skew subsequent phylogenetic analyses (Morrison, 2009), since the characters (nucleotide positions) within will most likely be homoplasic. The number of taxa included not only affects multiple alignment, but also support (or probability) values, and eventually cluster resolution within the topology. This is why multi-species phylogenetic reconstructions shall only be considered as preliminary backbones for further fine-scale analysis such as species boundary delimitations within a particular group of organisms.

Our species delimitation study for the SSC causal agents reveals the potential of the coalescent-based approach for recognizing speciation events for problematic taxa, or groups for which traditional methodologies are not clear-cut due to experimental or historical reasons. This

is, to our knowledge, the first attempt to using both infra and supra-specific data for species boundary assessment in plant pathogenic fungi. We propose that the inclusion of a coalescent-based methodology for species delimitation will greatly contribute to the resolution of *Diaporthe* species complex taxonomy. Additionally, this approach might be a great asset at establishing anamorph-teleomorph connections, an issue greatly lagging in *Diaporthe,* where *o*nly 20% of such links have been resolved so far (Udayanga et al., 2011).

Precise resolution of species boundaries will greatly contribute to optimizing downstream academic and applied studies. It is important to note that we herein adopt the traditional biological species concept (reproductive isolation amongst taxa) based on purely practical grounds: elucidation of the biological relationships amongst the SSC pathogens has implications for agricultural research. Should, for instance, *D. aspalathi* and *D. phaseolorum* var. *meridionalis* still share their gene pools, different hosts (soybean, red bush) might act as alternative sources of inocula; this should not be disregarded by producers and sanitary authorities. This could in turn contribute to the dissemination of a particular disease into new crops species and geographic areas.

Clarification of whether or not a given group of pathogens are reproductively isolated might be an indication of substantially different epidemiological conditions required by the individual taxa, as well as differential preconditions for breeding activities and strategies. In this regard, five loci have been so far described in soybean as conferring vertical resistance against *D. phaseolorum* var. *meridionalis*, whereas no major gene conferring resistance against *D. caulivora* has been described. Because of the cumbersome taxonomic history of the group, these five loci had paradoxically been named as "*Rdc*" (resistance against "*D. caulivora"*). Pioli et al. (2003) suggested that these loci should be renamed as "*Rdm*" (Resistance against "*D. phaseolorum* var. *meridionalis"*). According to the present results, *Rdm* gene stacking aimed at increasing resistance against *D. caulivora,* for instance, should not be considered as an appropriate breeding strategy, and this approach should not be favored within corporate or public breeding programs in the future.

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