Molecular Variability Within *Diaporthe/Phomopsis helianthi* from France

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ABSTRACT

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Diaporthe/Phomopsis helianthi causes brown stem canker of sunflower (*Helianthus annuus*) and is responsible for considerable yield loss. This species shows considerable variation for morphological characters, growth, and pathogenicity. Molecular variability of two sample groups was assessed with amplified fragment length polymorphism (AFLP) markers. Isolates of the first sample were collected from infected sunflower tissues from the main regions in France where the crop is grown, whereas isolates from the second sample came from stems within a single field of sunflower. A soybean strain was taken as an outgroup for AFLP analyses. Within sample one, the greatest genetic distance among isolates was 0.97, whereas it was 0.44 within sample two isolates. For the

whole of France, the average genetic distance was 0.68, whereas in the one field it was 0.12. Nei's genetic diversity indices were 0.20 and 0.06 for France and for one field, respectively. The greatest genetic distance was found between isolates from the most northern crops. The greatest genetic distance between *D. helianthi* isolates and the strain isolated from soybean was similar to that observed for *D. helianthi* isolates from different geographical areas. The problems in defining the genus *Phomopsis* are discussed. It is shown that internal transcribed spacer sequencing could be a useful criteria for *Diaporthe/Phomopsis* species determination. The considerable genetic variability of the pathogen could lead to the occurrence of new strains that could be more aggressive or more resistant to chemical control.

Additional keywords: ascomycete, Diaporthaceae, internal transcribed spacer intraspecific variability.

Stem canker, caused by *Diaporthe/Phomopsis helianthi*, is one of the most serious diseases of sunflower (*Helianthus annuus*). It appeared in 1980 in Yugoslavia (1,15) and 4 years later in France (12). A variety of symptoms has been described. Leaf and stem canker are the most common forms of attack. Cotyledon infections were first observed in 1987, and were followed by attacks on the capitulum in 1992 (19). To our knowledge, these last symptoms have only been reported in France.

Phomopsis is a cryptogrammic fungus with two different forms, the teleomorph, *D. helianthi* (Ascomycete: Diaporthaceae), and the anamorph, *P. helianthi* (Adelomycete: Sphaerioidaceae). The ascospores infect sunflower leaves in spring, and the asexual form (with α - and β -conidia) appears in late summer. The fungus survives over winter in crop debris as mycelium that is particularly resistant to cold and drought (5).

The genus *Diaporthe/Phomopsis* is characterized by wide phenotypic variability (26). Because morphological and cultural characteristics are generally insufficient for species identification, species definition in *Phomopsis* is often based on host association. However, Wehmeyer (26) concluded that this criterion was insufficient and reorganized the genus, reducing it from 650 species to only 70. In 1990, Brayford (3) demonstrated sexual compatibility between isolates from elm (*Ulmus* sp.), ash (*Fraxinus excelsior* L.), sycamore (*Acer pseudoplatanus* L.), and beech (*Fagus sylvatica* L.). *Phomopsis* internal transcribed spacer (ITS) phylogenetic analysis from various host taxa suggests that single *Phomopsis* species may infect more than one host (20).

As observed for other species of *Phomopsis*, isolates of *P. helianthi* are characterized by significant variation in cultural morphology, as well as variation in aggressiveness (25). Viguié (22) measured linear and ponderal growth (weight of mycelium after 35 days of culture at 23°C in liquid medium), morphology of the mycelium, and perithecia development in 21 strains. In addition, three artificial infection tests measuring the length of the lesions induced by these 21 isolates were used on the leaves, stems, and capitula of six sunflower hybrids to estimate the aggressiveness of *D. helianthi* strains (Viguié et al. [23]). These authors found no correlation between phenotype and aggressiveness.

The aim of the present study was to analyze molecular variability within this species. Two sample groups, one collected from different regions of France and the other from one field in the south of France, were analyzed by the amplified fragment length polymorphism (AFLP) technique. This technique has already been extensively used in molecular studies of variability in fungi (13). We also sequenced the ITS regions from seven *D. helianthi* strains and from one soybean strain and compared them with five GenBank ITS sequences from soybean (*Glycine max*).

MATERIALS AND METHODS

Isolates and culture media. Isolates were obtained from natural infections either by Institut National de la Recherche Agronomique (Clermont-Ferrand, France) or by French seed companies. Within the first sample of 52 isolates, 50 were collected from stem base, stem, petiole, leaf, and capitulum over a 5-month period from May to September 1995 from various regions in France (Table 1 provides geographical origin and identification number of these strains). In addition, one isolate (95104, Budapest) was collected from a sunflower stem in Hungary and was provided by O. Cottet (Agrosem, France). A strain of *Dia-*

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porthe sp. (96002) was collected from soybean seed (GEVES, Angers, France). This strain was considered as likely not to be *D. helianthi*.

The second sample was made up of 41 isolates, collected on a single day (31 August 1995), from sunflower stems, within one field located in the Haute-Garonne department (south of France).

Isolates of *D. helianthi* strains were obtained on solid medium containing 1% malt, 0.1% chloramphenicol, and 1.5% agar. The isolates were maintained at -20°C with a cryoprotectant mixture (15 g/liter of aqueous glycerol [RP bidistilled Normapur 99.5% minimum] and 20 mM Na₂-MOPS [sodium salt], pH 7, sterilized in an autoclave for 20 min at 120°C).

DNA extraction. The mycelium was produced over a period of 1 week at 23°C in liquid medium (1% malt) with natural light and photoperiod. Mycelium from the liquid cultures was collected and vacuum-dried through a 0.45-µm sieve. The DNA was extracted from 300 to 400 mg of mycelium according to a procedure described by Dellaporta et al. (4) and modified as follows. The mycelium was mixed with sand, 75 µl of 20% sodium dodecyl sulfate, and 750 µl of extraction buffer (100 mM Tris, pH 8, 50 mM EDTA, pH 8, and 500 mM NaCl) and was incubated at 65°C for 10 min. After centrifugation (15 min, 13,000 rpm, 4°C), 100 µl of 5 M potassium acetate was added to the supernatant. The mixture was maintained at 0°C for 20 min and centrifuged for 15 min at 13,000 rpm at 4°C. The supernatant was phenol/chloroform (1:1) extracted and ethanol precipitated. DNA was pelleted (15 min, 13,000 rpm, 4°C) and dissolved in 500 µl of TE buffer (50 mM Tris, 10 mM EDTA, pH 8). Contaminating RNA was digested by 100 ng of RNase A for 30 min at 37°C. The solution was re-extracted with phenol/chloroform (vol/vol), and the DNA was precipitated with 50 µl of 3 M sodium acetate and 1.25 ml of ethanol. The pellet was rinsed with 70% ethanol and dissolved in 100 µl of TE buffer.

AFLP procedure. The AFLP (24,28) analysis system for large genome was used according to the manufacturer's instructions (Gibco-BRL, Pontoise, France). Among the different primer combinations tested, we used A and C as selective nucleotides (*EcoRI* primer + A, *MseI* primer + C) for preamplification and *EcoRI* primer + ACA, *MseI* primer + CTA for selective amplification to analyze the two samples of strains. Experiments were carried out in triplicate.

ITS amplification and sequencing. The ITS region, which occurs between the small and large subunits of the nuclear rDNA, was amplified using ITS1 primer (5'-TCCGTAGGTGAACCTG-CGG-3') based on the 17S rDNA genes and ITS4 primer (5'-TCCTCCGCTTATTGATATGC-3') based on the 25S rDNA genes described by White et al. (27). These primers allow the amplification of ITS1, 5.8S rDNA, and ITS2 regions. Conditions of amplification were previously described by Gandeboeuf et al. (7). PCR products were cloned in pGEM-T Easy Vector System (Promega, Madison, WI). Plasmid DNA was purified according to the alkaline lysis method described by Sambrook et al. (21). About 290 ng of the plasmid DNA was used as template for cycle sequencing following the dye terminator cycle sequencing chemistry protocol (CEQ 2000; Beckman, Coulter, CA) with SP6 and T7 primers.

Data analyses. For each sample, the presence (1) or absence (0) of AFLP amplification products was scored. The 0-1 data matrix of the two samples were subjected to 50 bootstrap resamplings of markers. From these data, 50 distance matrices were calculated using the relation $D = 1 - S$, with S being Nei and Li similarity coefficient (18). For each sample, a consensus tree was drawn using the unweighted pair-group method with arithmetic average (UPGMA) procedure of the PHYLIP software (J. Felsenstein, Department of Genetics, University of Washington, Seattle). This procedure enabled us to evaluate the robustness of each node of these trees.

Comparisons of means of genetic distances were made between regions of France and field isolates and between southern and northern isolates in sample 1 using the *t* test of the Statgraphics Plus 2.1 software (Manugistics Inc., Rockville, MD). When the genetic distances were not normally distributed, their medians were compared using the Mann-Whitney W-test (Statgraphics).

Nei's genetic diversity index (17) was calculated for each sample of isolates (field and regions of France) using S-plus (Statistical Sciences, S-PLUS version 3.4; StatSci, Seattle, a division of Mathsoft Inc.).

The CLUSTAL program was used for sequence alignment of ITS sequences. The sequence from the 5.8S region was not used

TABLE 1. List of isolates of *Diaporthe/Phomopsis* sp. from different geographic origins (sample 1)^a

Isolate	Geographical origin	Infected tissue	No. of AFLP bands present	Phenotypic studies
96002 ^b	Unknown	Seed	25	—
95104 ^c	Hungary, Budapest	Stem	40	—
95004	Haute-Garonne (S)	Leaf	33	+
95006	Tarn et Garonne (S)	Leaf	37	+
95007	Tarn (S)	Stem base	59	+
95008	Tarn et Garonne (S)	Stem	25	+
95009	Tarn et Garonne (S)	Stem	37	—
95010	Gers (S)	Stem base	51	—
95013	Gers (S)	Stem base	70	—
95014	Charente (N)	Stem	61	—
95015	Puy de Dôme (N)	Leaf	69	—
95016	Haute-Garonne (S)	Leaf	42	+
95019	Haute-Garonne (S)	Leaf	40	—
95020	Haute-Garonne (S)	Stem	50	+
95022	Haute-Garonne (S)	Capitulum	46	—
95024	Haute-Garonne (S)	Leaf	50	+
95029	Tarn et Garonne (S)	Leaf	26	—
95031	Gers (S)	Leaf	31	+
95032	Tarn et Garonne (S)	Leaf	37	—
95035	Tarn et Garonne (S)	Stem	49	—
95038	Tarn et Garonne (S)	Leaf	44	—
95039	Aveyron (S)	Stem	52	—
95040	Tarn et Garonne (S)	Leaf	29	—
95044	Aude (S)	Stem	24	—
95045	Tarn et Garonne (S)	Capitulum	48	+
95047	Puy de Dôme (N)	Stem	53	—
95048	Cher (N)	Leaf	40	+
95049	Cher (N)	Petiole	111	+
95050	Cher (N)	Stem	50	+
95053	Haute-Garonne (S)	Capitulum	29	—
95056	Haute-Garonne (S)	Leaf	15	—
95057	Haute-Garonne (S)	Capitulum	12	+
95064	Haute-Garonne (S)	Stem	21	—
95068	Haute-Garonne (S)	Stem	34	—
95070	Haute-Garonne (S)	Leaf	45	—
95072	Gers (S)	Leaf	92	—
95075	Haute-Garonne (S)	Stem	103	—
95076	Haute-Garonne (S)	Capitulum	23	—
95080	Deux-Sèvres (N)	Stem base	25	+
95081	Loir et Cher (N)	Stem	4	—
95082	Loir et Cher (N)	Capitulum	60	+
95083	Dordogne (S)	Leaf	39	—
95084	Indre et Loire (N)	Stem	53	—
95089	Haute-Garonne (S)	Petiole	38	+
95095	Drôme (S)	Stem	20	—
95097	Drôme (S)	Stem	37	—
95099	Puy de Dôme (N)	Leaf	10	+
95100	Puy de Dôme (N)	Stem	16	+
95103	Cher (N)	Capitulum	11	—
95106	Allier (N)	Stem	31	—
95107	Puy de Dôme (N)	Leaf	23	—
95112	Drôme (S)	Stem	39	—

^a S or N indicates southern or northern departments of France. + or — indicates which strains have been phenotypically studied by Viguié (22) and Viguié et al. (23). The number of amplified fragment length polymorphism (AFLP) fragments ranges from 4 to 111.

^b From soybean.

^c From sunflower.

in the alignment. The dendrogram was constructed from similarity values with the UPGMA method of NTSYS-pc software (Applied Biostatistics Inc., Setauket, NY).

RESULTS

Molecular variability in different regions of France (sample 1). Fifty-two isolates were used in the AFLP analysis (Table 1). We analyzed 271 bands ranging from 60 bp to 1 kb. The number of fragments obtained varied greatly between strains (Table 1), ranging from 4 to 111, with an average of 40. The Nei genetic diversity index was 0.20.

The consensus tree, constructed after bootstrap resampling of markers, showed no geographic clustering (Fig. 1). The isolate from Eastern Europe (95104) did not differ from the others. The genetic distances varied from 0.067 to 0.97. The greatest genetic distance between the soybean strain (96002) and the sunflower isolates was 0.96. The average genetic distance between the

Phomopsis strains isolated from sunflowers was 0.68, with a standard deviation of 0.12. The average genetic distance between the isolates from southern regions of France (35 strains from Haute-Garonne, Tarn, Tarn et Garonne, Gers, Aveyron, Aude, Dordogne, and Drôme) was 0.65 (standard deviation: 0.11), whereas it was 0.73 (standard deviation = 0.12) between the north isolates (15 strains from the Puy de Dôme, Allier, Charente, Cher, Deux-Sèvres, Loir et Cher, and Indre et Loire). The *t* test showed a significant difference between the means of the two groups of genetic distances, with a 95% confidence interval of 0.08.

Molecular variability within one field (sample 2). We analyzed 101 bands ranging from 60 bp to 1 kb. The number of AFLP fragments ranged from 19 to 42, with an average of 27. The Nei genetic diversity index was 0.06.

The consensus tree is shown in Figure 2. None of the branch points was significant. The Nei and Li distances ranged from 0 to 0.44. The average distance was 0.12, with a standard deviation of 0.08.

Fig. 1. Consensus tree showing genetic distances among *Diaporthe helianthi* sample 1 strains (different regions of France), based on amplified fragment length polymorphism patterns, and constructed by unweighted pair-group method with arithmetic average clustering method (PHYLIP package), after 50 bootstrap resamplings of markers. S or N after the identification number indicates southern or northern departments of France. The numbers at the forks indicates how many times this cluster occurred among the 50 trees (numbers under 5 are not mentioned). Table 1 provides isolates characteristics.

Fig. 2. Consensus tree showing genetic distances among *Diaporthe helianthi* sample 2 strains (in one field), based on amplified fragment length polymorphism patterns and constructed by unweighted pair-group method with arithmetic average clustering method (PHYLIP package), after 50 bootstrap resamplings of markers. The numbers at the forks indicates how many times this cluster occurs among the 50 trees (numbers under 5 are not mentioned).

Comparison of genetic distances between the regions and the field isolates (samples 1 and 2) was performed with the Mann-Whitney W-test and showed that the two samples of strains were significantly different ($P < 0.05$).

ITS sequencing. The wide genetic variability revealed by AFLP prompted us to check that all the strains were from the same species, by sequencing the ITS regions from seven *D. helianthi* isolates (95004, 95016, 95045, 95048, 95049, 95057, and 95099) and from the soybean strain (96002), all of which differ considerably by their geographical origin, aggressiveness, and number of AFLP fragments. The amplified ITS fragments were approximately 540 bp. The sequences were deposited as GenBank Accession Nos. AF358435 to AF358442. The ITS1 and ITS2 regions were 180 to 186 bp and 194 to 197 bp, respectively. The 5.8S sequences of sunflower and soybean *Phomopsis* were identical (158 bp) and therefore were not used in the alignment. Five GenBank ITS sequences from *Diaporthe/Phomopsis* strains isolated from soybean (AF000564 = *D. meridionalis*, AF000567 = *D. phaseolorum*, AF132796 = *P. longicolla*, U11357 + U11411 = *Phomopsis* sp. 600, and U11362 + U11416 = *Phomopsis* sp. 642) were aligned with our sequences. The dendrogram (Fig. 3) showed that sunflower isolates (cluster 1) clustered separately from soybean isolates (clusters 2 and 3). The greatest genetic distance among sunflower *Diaporthe/P. helianthi* strains was 0.035. The distance between clusters 1 and 2 was 0.11. Ds96002 soybean strain was very closely related to *P. longicolla*, *Phomopsis* sp. 600, and *Phomopsis* sp. 642 strains. The distance between clusters 1 and 3 (*D. meridionalis* and *D. phaseolorum*) was 0.12.

DISCUSSION

We examined two samples of *Phomopsis* sunflower isolates, one from different regions of France and the other from a single field. Our aim was to characterize the molecular variability of this pathogen locally and over the entire country because its phenotypic diversity was extremely wide (22). The phenotypic characters of 18 of these isolates had been extensively studied previously by Vigi (22). Vigi found no correlation between phenotype

and aggressiveness. Table 2 shows the aggressiveness and the capacity to form perithecia of these isolates. There was no relationship between molecular variability and aggressiveness or perithecia formation. The most aggressive isolates (95004S, 95007S, 95008S, 95031S, 95089S, 95099N, and 95100N) did not clustered together (Fig. 1), nor did those that could not form perithecia (95004S, 95006S, 95020S, 95024S, 95045S, 95080N, and 95100N). Robustness of the nodes, indicated by numbers at the forks, showed that few terminal clusters were significant (values of >40). We considered a cluster significant when it was found at least 8 times out of 10.

Over the entire country, the greatest genetic distance between *D. helianthi* strains was 0.97. This highlights the extreme molecular variability among strains. It was also surprising to observe that genetic distances among *D. helianthi* strains could be as large as between *D. helianthi* strains and the soybean strain 96002 (0.97 and 0.96, respectively). Nevertheless, bootstrap resampling clearly clustered soybean strain 96002 separately from *D. helianthi* strains (Fig. 1). Studying random amplified polymorphism DNA variability between *P. helianthi* and strains of *Phomopsis* from soybean, Miric et al. (16) found that similarity between *P. sojae* and some *P. helianthi* strains was 30%, whereas 20% similarity was found between *P. helianthi* strains. Brayford showed sexual compatibility between *Phomopsis* strains from different hosts (3). These features led us to question the species definition in the genus *Diaporthe/Phomopsis*. In the literature, evolutionary relationships of species belonging to the same genus have often been investigated by rDNA spacers sequencing, mainly the noncoding ITS, because they usually vary between species within a genus but show little intraspecific variation. We have shown that ITS sequencing clustered separately the sunflower *Diaporthe* strains and the soybean ones. We found 3.5% variability between ITS1 and ITS2 region sequences of *D. helianthi* strains, which is a little higher than the intraspecific level of variability described in other species (2,8,10), whereas 11 and 12% variability were observed between sunflower *Diaporthe* ITS (Fig. 3, cluster 1) and the two soybean *Diaporthe* ITS groups (clusters 2 and 3, respectively). In addition, strains known as *D. meridionalis* and *D. phaseolorum*

Fig. 3. Dendrogram showing genetic distances after alignment of internal transcribed spacer sequences by Clustal W, between seven *Diaporthe helianthi* strains and six *Diaporthe/Phomopsis* isolates from soybean (*Glycine max*). GenBank Accession Nos.: Dh950106 = AF358435, Dh95048 = AF358436, Dh95057 = AF358437, Dh95004 = AF358438, Dh95045 = AF358439, Dh95049 = AF358440, Dh95099 = AF358441, Ds96002 = AF358442, *D. meridionalis* = AF000564, *D. phaseolorum* = AF000567, *P. longicolla* = AF132796, *Phomopsis* sp. 600 = U11357 + U11411, and *Phomopsis* sp. 642 = U11362 + U11416. The dendrogram was constructed by unweighted pair-group method with arithmetic average clustering method of the NTSYS-pc software. Number 1 shows the cluster of sunflower *Diaporthe*. Numbers 2 and 3 show the clusters of soybean *Diaporthe*.

were very different from *P. longicolla* cluster strains (Fig. 3), although they were all isolated from soybean. ITS analysis confirms here to be a powerful tool to delimit species in the genus *Diaporthe/Phomopsis* and could help to revise the taxonomy of this fungus in which host specificity does not exist.

Our data show that the average genetic distance among northern strains was significantly greater than among southern strains. Similar observations were made by Viguié (22) who showed more phenotypic variability among northern isolates than among southern ones. These findings must be discussed together with the observations that sunflower brown stem canker appeared only recently in the north (1993) but much earlier in the southern regions (1984) (12). It is possible that since only the more resistant sunflower varieties were grown for a decade in the south, the variability of the southern *Phomopsis* population decreased.

Among isolates of sample 2 (field), no significant clustering was observed (Fig. 2). Further studies, at a lower spatial scale, could bring some highlights on the distribution of *Phomopsis* genetic variation. For example, McDonald and Martinez (14) showed that a high level of genetic variability was distributed on a fine scale within a single wheat field of *Septoria tritici*. They found that deletion and insertion events were common in *S. tritici* nuclear DNA, even among isolates from the same leaf and the same lesion.

For *Diaporthe/P. helianthi*, the mean of the genetic distances was significantly lower at the local scale than at the country scale. Genetic diversity values were also lower at the local scale (field Nei index = 0.06 and regions Nei index = 0.20). These results are within the same range compared with those found for the sunflower parasitic plant *Orobancha cumana* (6), following an AFLP analysis of isolates from three fields and from different regions of Spain (mean of fields genetic diversity = 0.076, total genetic diversity = 0.12). It is interesting to note that the genus *Orobancha*, like *Phomopsis*, presents problems of species delimitation (9,11).

The present study shows that extensive genetic differences can occur between *D. helianthi* strains from different geographical origins or strains collected in one field. This should be considered when genetically representative isolates of *D. helianthi* are required in pathology and breeding research programs. Up to now,

D. helianthi damage has been limited by the use of resistant cultivars and chemical control. However, effective control of the disease causes higher selection pressure on pathogens, potentially leading to the appearance of fungicide-resistant clones or resistance gene bypass.

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TABLE 2. Phenotypic characteristics of 18 *Diaporthe helianthi* strains^a

Strain	Origin in France	Aggressiveness	Perithecia formation
95049	North	+	+
95050	North	++	+
95082	North	+	+
95089	South	+++	+
95080	North	+	—
95006	South	++	—
95008	South	+++	+
95016	South	++	+
95020	South	++	—
95024	South	+	—
95031	South	+++	+
95045	South	++	—
95048	North	+	+
95007	South	+++	+
95057	South	++	+
95004	South	+++	—
95099	North	+++	+
95100	North	+++	—

^a *Diaporthe helianthi* strains studied here and by Viguié (22) and Viguié et al. (23). Strains are listed in the table according to their position in the consensus tree (Fig. 1). The southern or northern geographical origin in France is mentioned in Table 1. Three levels of aggressiveness are noted from the least (+) to the more aggressive (+++). Aggressiveness was estimated by measuring the length of lesion after artificial infection on leaves and stem. Some strains could form perithecia (+), whereas others could not produce them (—) in the same experimental conditions according to Viguié (22) and Viguié et al. (23).

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