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Phenotypical characterization in *Plasmopara halstedii* (sunflower downy mildew) isolates of several races

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Abstract: Sakr, N. 2015. Phenotypical characterization in *Plasmopara halstedii* (sunflower downy mildew) isolates of several races. *Bot. Complut*. 39: 7-18.

Phenotypic variation (morphological and pathogenic characters), and genetic variability were studied in 50 isolates of seven *Plasmopara halstedii* (sunflower downy mildew) races 100, 300, 304, 314, 710, 704 and 714. There were significant morphological, aggressiveness, and genetic differences for pathogen isolates. However, there was no relationship between morphology of zoosporangia and sporangiophores and pathogenic and genetic characteristics for the races used in our study. Also, our results provided evidence that no relation between pathogenic traits and multilocus haplotypes may be established in *P. halstedii*. The hypothesis explaining the absence of relationships among phenotypic and genetic characteristics is discussed.

Key words: *Helianthus annuus*, morphology, multilocus haplotypes, obligate parasite, pathogenicity.

Resumen: Sakr, N. 2015. Caracterización fenotípica en aislados de varias razas de *Plasmopara halstedii* (mildiu del girasol). *Bot. Complut*. 39: 7-18.

Se ha estudiado la variación fenotípica (caracteres morfológicos y patógenos) y la variabilidad genética de 50 aislamientos de las razas 100, 300, 304, 314, 710, 704 y 714 de *Plasmopara halstedii* (mildiu del girasol). Se han encontrado diferencias significativas en la morfología, la agresividad y las variaciones genéticas para los aislados patógenos. Sin embargo, no se ha encontrado relación entre la morfología de los zoosporangios y esporangióforos, y las características patogénicas y genéticas entre las razas estudiadas. También nuestros resultados aportan evidencia de que no existe relación entre los patógenos estudiados y los haplotipos con múltiples loci resistentes en *P. halstedii*. Se comenta la hipótesis explicativa de la ausencia de relaciones entre las características fenotípicas y genotípicas.

Palabras clave: *Helianthus annuus*, morfología, haplotipos con multiples loci, parásito obligado, patogenicidad.

INTRODUCTION

Sunflower downy mildew is caused by *Plasmopara halstedii*. It is diploid, homothallic, and can reproduce sexually and asexually. The sexual phase is required to produce over wintering propagules, but during the sunflower growing season from spring to autumn there can be several asexual generations (Tourvieille de Labrouhe *et al.* 2000, Sakr 2014). Historically, this Oomycete was originally described by Farlow in 1882 as *Peronospora halstedii*, the name referring to *Halsted*, who first collected it on *Eupatorium purpureum*. After a revision of the genus *Peronospora*, the fungus was renamed *Plasmopara halstedii* in 1888 (Berlese et De Toni), and this name has become generally accepted and conventionally used in many parts of the world (Sackston 1981). *P. halstedii* shows physiological races (pathotypes) capable of infecting a variable range of sunflower genotypes. In the last decades, new races of *P. halstedii* were discovered worldwide in the cultivation areas of sunflower (As-Sadi *et al.* 2011, Sakr 2014).

P. halstedii has long been considered as a single species complex with a broad host range showing infectivity to > 80 genera of the *Asteroideae* and *Cichorioideae* subfamilies of the *Asteraceae* (Leppik 1966). Attempts have been made to split up the *P. halstedii* complex into various new taxa related to specific host species (Leppik 1966). However, cross infection studies for the determination of host specificity were mostly lacking in this process. As another concept, on the basis of pathological assessments and of morphological examinations, Novotelnova (1966) differentiated between species and forms according to artificial infection studies on annual and perennial *Helian-*

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thus species, giving the name *Plasmopara helianthi* to the fungus, thought to be confined to the genus *Helianthus*, with further specialization on intrageneric taxa as formae speciales: f.sp. *helianthi* (downy mildew of sunflower), f.sp. *perennis*, and f.sp. *patens*. The species name *Plasmopara helianthi* is now regarded as taxonomically invalid, because its introduction by Novotelnova (1966) did not adhere to the rules of the International Code of Botanical Nomenclature (Gulya *et al.* 1997). Novotelnova (1966) differentiation between species and forms of this fungus on the basis of minor morphological traits is not convincing when facing the great variability of biometric characters observed even among sporangiophores and sporangia of single isolates of the pathogen (Delanoe 1972). However, the distinctiveness of the given micromorphological characters and the infection specificity was doubted and the epithet *helianthi* was widely abandoned.

Characterization of new phenotypic aspects (morphological and pathogenic characters), and genetic characteristics in an obligate parasite such as *P. halstedii* based on interactions with sunflower plants is of interest for its taxonomy. *P. halstedii* is characterized by a high level of evolutionary potential expressed by high virulence, aggressiveness and a great potential in developing new races (Tourvieille de Labrouhe *et al.* 2000, 2010; Sakr 2009, 2010, 2011a,b, 2012, 2014; Sakr *et al.* 2009, 2011; As-Sadi *et al.* 2011). For morphology in *P. halstedii*, Spring & Thins (2004) found phenotypic limited tools for analyzing *P. halstedii*. Concerning genotypic diversity in *P. halstedii*, the interest of Internal Transcribed Spacer (ITS) (Spring *et al.* 2006) and Expressed Sequence Tag (EST) (Delmotte *et al.* 2008) sequences to characterize *P. halstedii* isolates has been showed, but races can still not be defined with certainty. However, As-Sadi *et al.* (2011) reported that genetic distance between four *P. halstedii* races can be detected using Single Nucleotide Polymorphisms (SNPs) discovered in CRN (a cell-death-inducing proteins that cause crinkling and necrosis phenotypes in the leaves of infected plants) effector sequences. In order to generate information about the phenotypic and genetic variability in *P. halstedii*, the aim of this study was to analyze morphological, genetic, and pathogenic characters for 50 *P. halstedii* isolates of seven races 100, 300, 304, 314, 704, 710 and 714.

MATERIALS AND METHODS

Oomycete isolates and race identification. Fifty *P. halstedii* isolates used in this study were sampled in France and collected at INRA, Clermont-Ferrand. Manipulation of this quarantined pathogen followed European regulations (No 2003/DRAF/70). Pathogen isolates were sampled from naturally infected plants in sunflower parcels at Clermont-Ferrand by the Plant Protection Service during its annual downy mildew survey. Virulence profiles of all races (Table 1) were obtained according to the method described by Tourvieille de Labrouhe *et al.* (2000) which is based on the reaction of a series of differential lines. The *P. halstedii* isolates were multiplied on the same sunflower genotype in the same conditions, such that any original *intra-isolate* variability would be maintained to the same extent for the 50 isolates. In addition, the isolates are multiplied using their zoospores, which could be a continued source of new variability, even for originally single zoosporangium isolates. All the pathogenic (virulence and aggressiveness) tests were carried out in growth chambers regulated at 18 hrs of light, 18°C ± 1 and RH of 65-90%.

Measurement of aggressiveness in isolates. To characterize aggressiveness criteria: percentage infection, latent period, sporulation density and reduction of hypocotyl length for 50 *P. halstedii* isolates (Sakr 2009, 2010, 2011a,b, 2012, 2014; Sakr *et al.* 2009, 2011), one INRA inbred line FU was used. It carried no *Pl* gene, but is known to a have high level of quantitative resistance (Tourvieille de Labrouhe *et al.* 2008). The index of aggressiveness of *P. halstedii* isolates was calculated as the ration of (percentage infection × sporulation density) / (latent period × dwarfing). The index of aggressiveness of the *P. halstedii* isolate was used to summarize all values for two criteria on sunflower inbred lines `FU` in one value to facilitate the comparison between the different *P. halstedii* isolates (Sakr 2012, 2014).

Morphological observations. After 13 days of infection of the sunflower inbred line `FU`, the zoosporangia and sporangiophores suspensions for 50 isolates were obtained by grouping all sporulated cotyledons in a small container and adding 1 ml of physiological water for each cotyledon (9g NaCl + 1L sterilized water). This slowed zoosporangia maturation to facilitate observations before liberation of zoospores (Sakr *et al.* 2007). Identification of form and measurement of size was carried out on 50 zoosporangia per treatment under a light microscope (magnification X400) with 2 replications. Zoosporangia size was calculated from an oval $\pi \times a \times b$, $a = {}^1/_2$ length, $b = {}^1/_2$ width. Furthermore, sporangiophore dimensions were observed by measuring 50 fresh sporangiophores in physiological water under a light microscope (magnification X400) with 2 replications.

DNA extraction and molecular typing. The 12 EST derived markers were sequenced by Giresse *et al.* (2007). In the current study, these markers were used because the other molecular markers were non-specific, insufficiently polymorphic within *P. halstedii*, and no genetic structure in *P. halstedii* populations was identified by using these markers (Giresse *et al.* 2007). GenBank accession numbers for 12 EST-derived genomic markers (Giresse *et al.* 2007) were presented as following: *Pha6* CB174585, *Pha39* CB174648, *Pha42* CB174650, *Pha43* CB174680, *Pha54* CB174708, *Pha56* CB174714, *Pha74*

Table 1
Virulence profile of 50 *Plasmopara haslstedii* isolates on nine sunflower differencial lines. **R**: resistant = incompatible interaction. **S**: susceptible = compatible interaction. *Data from:* Tourvieille de Labroubuhe *et al.* 2000)

Isolates of *P. halstedii*	**Differential lines**								
	D1 Ha-304 without *Pl* gene	**D2 Rha-265 *Pl2***	**D3 Rha-274 *Pl***	**D4 PMI3 *Pl(?)***	**D5 PM-17 *Pl(?)***	**D6 803-1 *Pl(?)***	**D7 HAR-4 *Pl(?)***	**D8 QHP1 *Pl(?)***	**D9 Ha-335 *Pl6***
MIL001 M2, MIL001 M3 MIL001 M4, MIL001 M5 MIL001 M6	S	R	R	R	R	R	R	R	R
DU1842 M1, DU1842 M2 DU1842 M3, DU1842 M4 DU1842 M5	S	S	R	R	R	R	R	R	R
DU1943 M1, DU1943 M2 DU1943 M3, DU1943 M4 DU1943 M5	S	S	R	S	R	R	R	R	S
DU1767 M1, DU1767 M2 DU1767 M3, DU1767 M4 DU1767 M5	S	S	R	R	R	R	R	R	S
MIL002 M1, MIL002 M2 MIL002 M3, MIL002 M4 MIL002 M5	S	S	S	S	S	S	S	S	S
DU 1552, DU 1555, DU 1564 DU 1571, DU 1635, DU 1651	S	S	S	S	R	R	R	R	R
DU 1659, DU 1670, DU 1753 DU 1776, DU 1777, DU 1782	S	S	S	S	S	S	S	S	S
DU 1839, DU 1845, DU 1865	S	S	S	S	S	S	S	S	S
DU1915 M3, DU1915 M5 DU1915 M6	S	S	S	S	R	R	R	R	S
DU1734 M1, DU1734 M2 DU1734 M3, DU1734 M7 DU1734 M8	S	S	S	R	R	R	R	R	S

CB174642, *Pha79* CB174692, *Pha82* CB174573, *Pha99* CB174703, *Pha106* CB174676, and *Pha120* CB174660. For 50 isolates tested, DNA was isolated from infected plant tissue, and then the 12 polymorphic EST-derived markers were used to genotype *P. halstedii* isolates. The polygenetic relations between the 50 isolates were obtained by building a Neighbour-Joining (NJ) tree (Jin & Chakraborty 1993) using Populations 1.2.28 Software (Librado & Rozas 2009). A Bootstrap analysis was performed on 10.000 replicates.

Statistical analyses. All statistical analyses were performed using StatBox 6.7® (GimmerSoft) software. Before statistical analysis, the percentages were transformed using the Arcsines function. A normality test showed that the transformed variables were normaly distributed, so the values obtained were submitted to a one-way analysis of variance (ANOVA). The Newman-Keuls test (Snedecor & Gochran 1989) was used to compare the means at $P=0.05$. The sample correlation coefficients (Pearson r) were calculated using overall mean values per treatment at $P=0.05$ and $P=0.01$.

RESULTS

Analysis of pathogenicity for 50 isolates. Table 2 presents aggressiveness data of 50 *P. halstedii* isolates. All isolates showed high percentage infection values and it varied between 90.6 and 100%. The latent period ranged from 7.8 days for isolates DU1842 M4, DU1842M5 and DU1943M4 to 12.5 days for isolate DU1734M7. Sporulation density varied six fold: 3.91×10^5 zoosporangia were produced by cotyledons for isolate DU1915 M2 and 19.68×10^5 for isolate MIL001 M2. Diseased plants had hypocotyls with only one third the mean lengths of *P. halstedii*-free sunflower inbred line 'FU' (30.85 ± 0.6 mm and 90.0 ± 2.3 mm respectively) whatever the isolate of *P. halstedii*. Hypocotyl length varied from 25.6 mm for isolate DU1915 M6 to 44.9 mm for isolate DU1943 M5. There were significant differences for index of aggressiveness (F-value = 44,099, *P*-value = 0.0001) in sunflower downy mildew isolates. The mean index of aggressiveness varied between five-fold: 1.5 for isolates of race 714 and 7.04 for isolates of 8.3 for isolates of race 300. Isolates of races 300 and 304 (mean index of aggressiveness = 6.01) were the most aggressiveness, followed by isolates of races 100 (mean index of aggressiveness = 4.88) and 314 (mean index of aggressiveness = 3.98), and isolates of races 714, 704 (mean index of aggressiveness = 1.92) and 710 (mean index of aggressiveness = 2.3) were the least aggressiveness.

Morphology of zoosporangia and sporangiophores. The results showed that the two forms most observed were oval and round, irrespective of the *P. halstedii* isolate (Fig. 1). The proportion of oval form varied from 37 to 94% and the zoosporangia size from 315 to 918 μm^2 (Table 3). The proportion of oval zoosporangia varied within the races; for example, for race 100, it varied from 87% to 94% and for race 710 it ranged from 37% to 92%. Mean sporangiophore length was the highest in DU1767M3 isolate (800.0 µm). The sporangiophore length ranged between 299.0 and 800 µm. Mean sporangiophore width was the largest in DU1767M1 isolate. The sporangial width varied from 4.3 µm to 16.0 µm. Zoosporangia size also varied considerably within and between races, with no relation to form. The dimensions for zoosporangia sizes were recorded as following: 11.4-62.4 µm to 11.4-100.8 µm. Moreover, the dimensions of sporangiophores ranged significantly within and between races (Table 1). Regarding sporangiophores sizes, length varied from 299.0 to 800 µm and width ranged from 4.3 to 16.0 µm. There was thus no relationship between morphology of zoosporangia and sporangiophores and virulence profiles of all races (Tables 1 and 3). All aggressiveness criteria (Table 2) were not correlated with both form or size of zoosporangia (Table 3) ($r = -0.135$ and $r = -0.123$ for percentage infection, $r = 0.053$ and $r = 0.056$ for latent period, $r = 0.179$and $r = -0.250$ for sporulation density and $r = -0.127$ and $r = 0.170$ for hypocotyl length), and length or width of sporangiophore (Table 3) ($r = -0.123$ and $r = 0.215$ for percentage infection, $r = 0.064$ and $r = -0.207$ for latent period, $r = -0.277$ and $r = 0.044$ for sporulation density and $r = 0.117$ and $r = -0.041$ for hypocotyl length).

Molecular analysis. The combination of 12-EST derived markers revealed five multilocus haplotypes (MLH) among 50 *P. halstedii* isolates (Table 4). There was no *intra-race* genetic variation for all pathotypes tested. Races 100 and 710 were different for all genomic markers excepting Pha54. Furthermore, races 100, 300, 304 had the same genetic background. The Neighbour-Joining tree showed that races 714, 704 and 314 had an intermediary genetic position between races 100 and 710 (Fig. 2). Consequently, there were five genetically-identified haplotypes among *P. halstedii* isolates tested: haplotype of isolates of races 100, 300 and 304, haplotype of isolates of race 314, haplotype of isolates of race 704, haplotype of isolates of race 714, and haplotype of isolates of race 710 (Table 4 and Fig. 2).

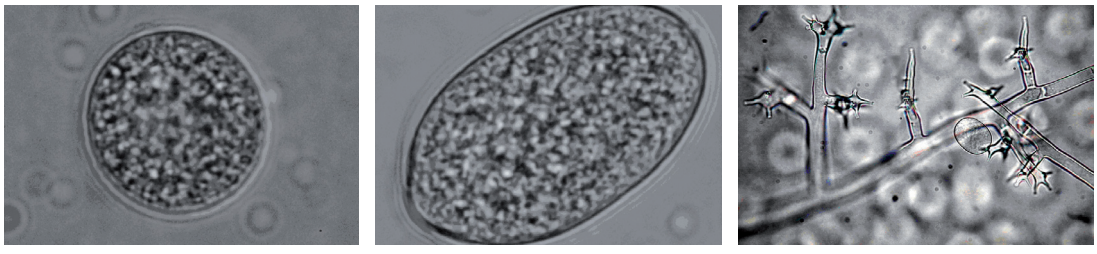

Fig. 1– *Plasmopara halstedii* zoosporangia forms and sporangiophores observed on sunflower inbred line 'FU': round (left), oval (center) and sporangiophore (right), the dimensions of zoosporangia and sporangiophores were magnified X400.

Table 2
Aggressiveness values on sunflower inbred line 'FU' for 50 isolates of *Plasmopara halstedii*. [a] 60 plants per replication. [b] 10 plants per replication. [c] 18 counts per replication. [d]10 plants per replication, index of aggressiveness = (percentage infection × sporulation density) / (latent period × dwarfing) (Sakr 2009, 2010, 2011a,b, 2012, 2014; Sakr *et al.* 2009, 2011), F-tests (**P = 0.01), least significant differences LSD (P = 0.05).

Isolates of *P. halstedii*	Race	Percentage infection[a] Mean (%)	Latent period[b] Mean (days)	Sporulation density[c] Mean (10^5 zoosporangia per cotyledon)	Hypocotyl length[d] Mean (mm)	Index of aggresiveness
MIL001 M2	100	95	9.1	19.68	32.4	6.3
MIL001 M3		100	9.3	12.70	28.7	4.8
MIL001 M4		95.6	10.2	11.97	28.3	4.0
MIL001 M5		97.2	8.9	13.16	27.9	5.2
MIL001 M6		92.1	8.6	14.11	36.8	4.1
DU1842 M1	300	98.3	7.9	16.61	29.1	7.1
DU1842 M2		98.8	8.1	17.20	28.4	7.4
DU1842 M3		98.9	8.8	18.33	29.0	7.1
DU1842 M4		99.4	7.8	14.03	33.6	5.3
DU1842 M5		100	7.8	17.42	26.8	8.3
DU1943 M1	314	100	8.6	13.25	42.1	3.7
DU1943 M2		100	8.5	12.75	40.9	3.7
DU1943 M3		99.4	8.9	11.30	35.6	3.5
DU1943 M4		98.9	8.2	18.27	39.7	5.6
DU1943 M5		98.3	7.9	12.10	44.9	3.4
DU1767 M1	304	100	7.9	13.04	35.2	4.7
DU1767 M2		98.9	8.7	13.60	27.9	5.5
DU1767 M3		100	8.0	16.26	27.6	7.4
DU1767 M4		100	8.6	15.31	26.2	6.8
DU1767 M5		100	8.0	13.32	27.4	6.1
MIL002 M1	710	96	10.4	7.44	28.3	2.4
MIL002 M2		95.6	11.8	5.45	32.8	1.3
MIL002 M3		90.6	11.1	8.45	29.6	2.3
MIL002 M4		95.6	10.3	8.25	26.1	2.9
MIL002 M5		94.4	10.5	5.56	27.4	1.8
DU 1552		100	9.7	5.6	37.8	1.5
DU 1555		98.9	9.1	7.4	39.6	2.0
DU 1564		99.4	9.3	7.4	36.9	2.1
DU 1571		98.9	9.3	7.7	39.6	2.1
DU 1635		99.3	8.6	11.9	38.7	3.6
DU 1651		100	8.8	9.5	36.9	2.9
DU 1659		100	8.7	9.7	28.8	3.9
DU 1670		99.4	8.9	11.3	40.5	3.1
DU 1753		100	8.8	5.7	32.4	2.0
DU 1776		100	8.8	8.3	37.8	2.5
DU 1777		100	8.5	7.2	37.8	2.2
DU 1782		98.9	9.1	7.1	35.1	2.2

Table 2 (cont.)

Isolates of *P. halstedii*	Race	Percentage infection[a] Mean (%)	Latent period[b] Mean (days)	Sporulation density[c] Mean (10^5 zoosporangia per cotyledon)	Hypocotyl length[d] Mean (mm)	Index of aggresiveness
DU 1839		100	9.1	6.8	37.8	2.0
DU 1845		97	9.6	5.8	36	1.6
DU 1865		97	9.4	6.9	35.1	2.0
DU1915 M1	714	95.9	10.6	6.20	28.3	2.0
DU1915 M2		95.5	11.9	3.33	28.2	1.0
DU1915 M3		95	10.2	3.91	29.6	1.2
DU1915 M5		98.3	11.5	4.07	26.7	1.3
DU1915 M6		91.1	11.2	7.62	25.6	2.4
DU1734 M1	704	95	11.0	4.37	26.6	1.4
DU1734 M2		98.3	10.9	7.72	26.5	2.6
DU1734 M3		95.6	10.5	5.84	26.8	2.0
DU1734 M7		97.8	12.5	5.58	28.3	1.5
DU1734 M8		95.6	11.6	8.07	31.0	2.1
F isolates		3.03**	55.47**	12.39**	5.46**	
LSD		25.6	0.53	357	6.12	

DISCUSSION

For *Plasmopara halstedii*, it has not yet been possible to use molecular differences to define pathogenicity characteristics (As-Sadi *et al.* 2011), and direct race identification of *P. halstedii* is not yet possible (Sakr 2014); so, it appeared useful to continue phenotypic (morphological and pathogenic characters) and genetic studies. Bearing this in mind, pathogenic, morphological and genetic characteristics were identified for 50 *P. halstedii* isolates of several races based on interactions with sunflower plants.

Differences in aggressiveness of *P. halstedii* races are indicated when pathogen isolates vary in the amount of damage that they cause in sunflower plants. High percentage infection, short latent period, high sporulation density, and significant reduction in the length of the hypocotyl represent high aggressiveness (Sakr 2009, 2011a,b, 2012, 2014; Sakr *et al.* 2009, 2011). It is possible that variability between *P. halstedii* isolates used is due to the origin of pathogen isolates used in this study (Tables 1 and 2). These isolates belong to several races and may be found to be an effect of additional virulence genes in *P. halstedii* isolates as observed for the same pathosystem (Sakr 2009, 2011a,b, 2012, 2014; Sakr *et al.* 2009) and another oomycete *P. infestans* (Montarry *et al.* 2010).

From observations of 5000 zoosporangia and sporangiophores, isolates showed different proportions of the oval form, zoosporangia size, and sporangiophores dimension (Table 3). Both the effects of host plant genotype and the different isolates used in other studies may explain the different measurements reported. For zoosporangia sizes, the dimensions recorded as following: 30-33 µm to 36-57 µm in Nishimura (1922), 14-20 µm to 18-30 µm according to Hall (1989) and 12-16 µm to 27-40 µm in Spring *et al.* (2003). Regarding sporangiophores sizes, Kulkarni *et al.* (2009) reported that sporangiophores length varied from 325 to 700 µm and sporangiophores width ranged from 5 to 10 µm.

There was no *intra-race* genetic variation (Table 4), but five genetically-identified haplotypes were detected among *P. halstedii* isolates of all races (Fig. 2). Our results observed genetic distances between two races 100 and 710 in agreement with the conclusions of Delmotte *et al.* (2008), Sakr (2011a) and As-Sadi *et al.* (2011). By using the same EST-derived markers, Delmotte *et al.* (2008) and Sakr (2011a) found that races 100, 300 and 304 had the same genetic clade as observed in our study (Fig. 2). However, As-Sadi *et al.* (2011) reported that certain SNPs might allow for clear differentiation between races 304 and 100, which has not been detected in our work (Fig. 2) and previously studies (Delmotte *et al.* 2008, Sakr 2011a).

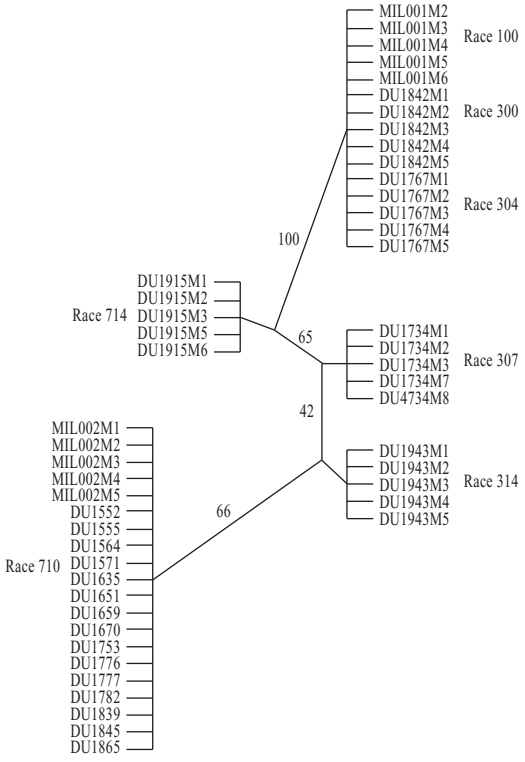

Fig. 2— Phylogenetic tree according to Neighbour-Joining analysis of 12 EST-derived markers. Figures on branches indicate bootstrap values (10.000 replicates).

Delmotte *et al.* (2008) grouped races 710, 704 and 714 together in the same genetic clade; however, this association was not identified in the present work and in previously analyses (Sakr 2011a). Either the isolates used in our study were different from the ones used by Delmotte *et al.* (2008), or the intrarace variance in the EST-derived marker may explain the different results reported.

There was no relationship between morphology of zoosporangia and sporangiophores (Table 3) and virulent (Table 1) and aggressiveness characteristics (Table 2) for the races used in our study. In accordance with our results, for another oomycete, *Phytophthora capsici,* Islam *et al.* (2004) did not find any relationship between groups of isolates characterized for their growth patterns on artificial medium and their pathogenicity. However, for the same pathosystem, Sakr (2011b) found a relationship between another phenotypic character (viability of zoosporangia) and aggressiveness in *P. halstedii*. This is in contrast with the results of De Wet *et al.* (2003) who observed morphological differences between strains of *Sphaeropsis sapinea*, which divided them into three morphotypes (A, B and C) that presented differences in pathogenicity (virulence and aggressiveness). Also, in the pathosystem *Sclerotinia sclerotiorum* / rapeseed and mustard, Ghasolia & Asha (2007) described nine groups based on morphological characters which were divided into two series according to degree of aggressiveness.

Table 3

Morphometric data of zoosporangia and sporangiophores obtained on sunflower genotype 'FU' for 50 isolates of *Plasmopara halstedii.* [a] 50 zoosporangia per replication. [b] 50 zoosporangia per replication. [c] 50 sporangiophores per replication. [d] 50 sporangiophores per replication. F-tests (***P* = 0.01), least significant differences LSD (*P* = 0.05).

Isolates of *P. halstedii*	**Race**	**% of oval zoosporangia[a]**	**Size of zoosporangia in µm² [b]**	**Sporangiophore length (µm)[c]**	**Sporangiophore width (µm)[d]**
MIL001 M2	100	87	315.8	325.9	12.3
MIL001 M3		94	434.9	550.2	15.1
MIL001 M4		88	392.9	715.9	10.9
MIL001 M5		91	418.7	660.2	8.7
MIL001 M6		90	432.2	489.3	6.9
DU1842 M1	300	88	398.0	333.8	5.1
DU1842 M2		89	511.7	568.2	4.3
DU1842 M3		68	436.4	663.6	7.7
DU1842 M4		82	315.2	785.2	14.8
DU1842 M5		89	381.4	559.3	12.3
DU1943 M1	314	93	424.8	663.5	9.7

Table 3 (cont.)

Isolates of *P. halstedii*	Race	% of oval zoosporangia[a]	Size of zoosporangia in µm² [b]	Sporangiophore length (µm)[c]	Sporangiophore width (µm)[d]
DU1943 M2		86	425.4	489.3	6.3
DU1943 M3		80	387.6	356.2	8.2
DU1943 M4		56	372.0	689.3	10.9
DU1943 M5		56	380.4	299.0	14.3
DU1767 M1	304	86	394.0	478.6	16.0
DU1767 M2		78	422.3	559.6	8.3
DU1767 M3		90	505.2	800.0	6.5
DU1767 M4		91	478.7	456.3	8.1
DU1767 M5		63	344.7	765.3	9.6
MIL002 M1	710	82	463.5	459.3	7.8
MIL002 M2		92	513.3	569.3	6.6
MIL002 M3		90	918.6	553.6	8.9
MIL002 M4		37	352.9	440.4	12.0
MIL002 M5		53	419.4	554.3	11.9
DU 1552		77	558.5	636.9	15.2
DU 1555		71	534.4	496.3	12.3
DU 1564		61	510.9	444.5	11.0
DU 1571		61	549.4	725.9	12.3
DU 1635		83	551.6	563.6	14.3
DU 1651		78	578.2	453.5	16.0
DU 1659		73	600.4	660.3	12.3
DU 1670		67	465.8	478.3	13.3
DU 1753		72	547.1	698.5	14.5
DU 1776		69	485.7	669.3	12.5
DU 1777		74	513.4	559.6	14.6
DU 1782		78	546.4	598.6	12.6
DU 1839		78	518.5	785.6	14.9
DU 1845		87	320.5	589.6	12.3
DU 1865		68	497.4	489.3	9.96
DU1915 M1	714	90	477.8	700.9	8.5
DU1915 M2		90	477.8	456.3	7.6
DU1915 M3		93	734.6	552.1	6.2
DU1915 M5		86	374.6	456.3	5.0
DU1915 M6		87	358.9	558.2	6.8
DU1734 M1	704	74	505.4	335.6	7.4
DU1734 M2		68	357.1	663.3	6.8
DU1734 M3		68	314.3	712.3	8.1
DU1734 M7		74	302.2	322.2	9.6
DU1734 M8		89	436.9	455.9	6.0
F isolates		7.52**	8.40**	7.69**	4.89**
LSD		13.2	110.3	101.3	9.63

Table 4
Multilocus haplotypes (MLH) characterized using 12 EST-derived genomic markers on 50 isolates of *Plasmopara halstedii.* Genetic markers are variants in the DNA code (known as alleles) that, alone or in combination, are associated with a specific phenotype, the values of two figures indicate to the alleles of 50 *Plasmopara halstedii* isolates for 12 EST-derived genomic markers. For each isolate, the race and the two alleles at each derived genomic marker were indicated.

Isolates of *P. halstedii*	**EST-derived markers**											
	Pha6	***Pha39***	***Pha42***	***Pha43***	***Pha54***	***Pha56***	***Pha74***	***Pha79***	***Pha82***	***Pha99***	***Pha106***	***Pha120***
MIL001 M2	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
MIL001 M3	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
MIL001 M4	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
MIL001 M5	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
MIL001 M6	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1842 M1	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1842 M2	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1842 M3	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1842 M4	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1842 M5	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1943 M1	1/1	2/2	1/1	2/2	1/1	2/2	2/2	3/3	2/2	1/1	2/2	1/1
DU1943 M2	1/1	2/2	1/1	2/2	1/1	2/2	2/2	3/3	2/2	1/1	2/2	1/1
DU1943 M3	1/1	2/2	1/1	2/2	1/1	2/2	2/2	3/3	2/2	1/1	2/2	1/1
DU1943 M4	1/1	2/2	1/1	2/2	1/1	2/2	2/2	3/3	2/2	1/1	2/2	1/1
DU1943 M5	1/1	2/2	1/1	2/2	1/1	2/2	2/2	3/3	2/2	1/1	2/2	1/1
DU1767 M1	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1767 M2	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1767 M3	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1767 M4	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
DU1767 M5	2/2	2/2	1/1	1/1	1/1	1/1	1/1	3/3	2/2	2/2	1/1	2/2
MIL002 M1	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
MIL002 M2	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
MIL002 M3	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
MIL002 M4	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
MIL002 M5	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1552	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1555	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1564	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1571	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1635	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1651	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1659	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1670	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1753	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1776	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1777	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1782	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1839	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU 1845	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1

Table 4 (cont.)

Isolates of *P. halstedii*	EST-derived markers											
	Pha6	***Pha39***	***Pha42***	***Pha43***	***Pha54***	***Pha56***	***Pha74***	***Pha79***	***Pha82***	***Pha99***	***Pha106***	***Pha120***
DU 1865	1/1	1/1	2/2	2/2	1/1	2/2	2/2	1/1	1/1	1/1	2/2	1/1
DU1915 M1	1/1	2/2	1/2	1/1	1/1	1/1	1/1	3/3	2/2	1/1	2/2	1/1
DU1915 M2	1/1	2/2	1/2	1/1	1/1	1/1	1/1	3/3	2/2	1/1	2/2	1/1
DU1915 M3	1/1	2/2	1/2	1/1	1/1	1/1	1/1	3/3	2/2	1/1	2/2	1/1
DU1915 M5	1/1	2/2	1/2	1/1	1/1	1/1	1/1	3/3	2/2	1/1	2/2	1/1
DU1915 M6	1/1	2/2	1/2	1/1	1/1	1/1	1/1	3/3	2/2	1/1	2/2	1/1
DU1734 M1	2/2	2/2	2/2	1/1	1/1	1/1	2/2	3/3	1/1	1/1	2/2	1/1
DU1734 M2	2/2	2/2	2/2	1/1	1/1	1/1	2/2	3/3	1/1	1/1	2/2	1/1
DU1734 M3	2/2	2/2	2/2	1/1	1/1	1/1	2/2	3/3	1/1	1/1	2/2	1/1
DU1734 M7	2/2	2/2	2/2	1/1	1/1	1/1	2/2	3/3	1/1	1/1	2/2	1/1
DU1734 M8	2/2	2/2	2/2	1/1	1/1	1/1	2/2	3/3	1/1	1/1	2/2	1/1

No correlation was detected between five EST haplotypes (Table 4 and Fig. 2) and morphological characteristics (Table 3) for *P. halstedii* races used in our experiment. In accordance with our results, Mahdizadeh *et al.* (2011) reported no correlation between genetic diversity based on inter simple sequence repeat (ISSR) markers and morphological characteristics for *Macrophomina phaseolina*. However, regarding *Sphaeropsis sapinea*, De Wet *et al.* (2003) found that phenotypic groups A, B and C were separated into three differential genetic clades, by using multiple gene genealogies inferred from partial sequences of six protein-coding genes and six microsatellite loci.

No correlation was detected between pathogenicity traits (Tables 1 and 2) and EST haplotypes (Table 4 and Fig. 2). Indeed, for *P. halstedii*, Sakr (2011a) found no correlation between aggressiveness traits and EST genotypes. The lack of matching between virulence and aggressiveness traits and groups based on molecular markers was not surprising. Indeed, Montarry *et al.* (2006) did not find a clear correlation between pathogenicity phenotypes and genotypes based on Amplified fragment length polymorphism (AFLP) markers for *Phytophthora infestans*. Pathogenicity is known to evolve through mutation without highly altering molecular fingerprints (Goodwin 1997). Because most molecular markers used for fingerprinting are selectively neutral, they can be used to assess evolutionary forces other than selection (such as gene flow or genetic drift). Since tools for classification of obligate parasitic *Peronosporaceae* are very limited (Spring & Thines 2004), it appears desirable to continue research for new methods. It is necessary to underline morphological, pathogenic and genetic variability on a large collection of *P. halstedii* isolates with different races from several parts of the world to provide a better insight into interactions between this obligate parasite and its host.

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