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Molecular and phenotypic characterisation of *Phaeomoniella chlamydospora* isolates from the demarcated wine region of Dão (Portugal)

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Summary. Sixty-eight isolates of *Phaeomoniella chlamydospora* obtained from symptomatic esca and Petri-diseased grapevines, and mostly from within the Portuguese Dão appellation, were investigated for phenotypic and molecular diversity, in order to determine intraspecific variability and population structure. *In vitro* growth features, including colony texture, colour, growing margin zonation, hyphal morphology and colony growth rate, were evaluated. Molecular characterization was performed through the sequencing of the total ITS region, and molecular analyses were used to infer phylogenetic relationships, using the Maximum Likelihood approach. Isolates were separated in two groups, supported by phenotypic and molecular analyses, but no clear correspondence was found between the two approaches. Nevertheless, both analyses revealed strong homogeneity among all isolates, despite their diverse geographical origin, year of isolation and scion/rootstock combination, supporting the clonal reproduction strategy described for this species.

Key words: esca, Petri disease, *Vitis vinifera*, genetic diversity, ITS sequences.

Introduction

The Portuguese Dão wine region or appellation has been afflicted by Grapevine Trunk Diseases (GTD), as have other wine-growing areas around the world. In previous studies (Tomáz *et al*., 1989; Sofia *et al*., 2006, 2013) esca was considered the most damaging GTD in this region. Esca is a GTD with complex etiology (Mugnai *et al*., 1999; Surico *et al*., 2006; Bruez *et al*., 2013). Over the last two decades the International Council on Grapevine Trunk Diseases (ICGTD) has promoted a wide forum gathering research on GTDs in general, and esca in particular, from which *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingfield & Mugnai) Crous & W. Gams) and *Phaeoacremonium* spp. (mostly *Phaeoacremonium aleophilum* W. Gams, Crous, M.J. Wingf. & Mugnai, now *Togninia minima* (Tul. & C. Tul.) Berlese), emerged as two of the most important pathogens related to the esca complex and Petri disease, a manifestation of esca in young plants (Crous and Gams, 2000). However *P. chlamydospora* is the most frequently isolated species (Mugnai, 1998; Clearwa-

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ter *et al*., 2000; Pascoe and Cottral, 2000; Whiteman *et al*., 2002), being considered the most important fungal organism associated with Petri disease (Ridgway *et al*., 2005; Laveau *et al*., 2009; Pouzoulet *et al*., 2013).

Originally described as *Phaeoacremonium chlamydosporum, P. chlamydospora* is an anamorphic ascomycete that is morphologically and phylogenetically different from the species included in the genus *Phaeoacremonium* (Crous and Gams, 2000). Among other characteristics, it is distinguished by a partly yeast-like growth in culture, prominently green/brown conidiophores, light green to hyaline conidiogenous cells, chlamydospore-like structures and sclerotia produced in culture. *Phaeoacremonium chlamydospora* conidia also differ from the ones produced by *Phaeoacremonium* spp. for their straight oblong-ellipsoidal to obovate form and pale brown colouration, in contrast to the dimorphic and hyaline conidia that are typical of other *Phaeoacremonium* spp. (Crous and Gams, 2000).

A comprehensive paper on the phenotypical characterisation of 57 Spanish isolates (Tello *et al.,* 2010) revealed that there was consistent phenotypic homogeneity among all *P. chlamydospora* isolates, despite diverse geographic origins, year of isolation and scion/rootstock combination isolate source.

The combination of tradition, geographical isolation and agricultural policies has made the Dão appellation a singularity amid Portuguese wine regions (Falcão, 2012). Dão's wine-growing practice is characterized by the preference for local grapevine cultivars, mainly produced by local nurseries, leading to weak penetration of alien cultivars and of foreign propagation material in general. Although previous studies on esca in this region have consistently yielded *P. chlamydospora* from symptomatic plants (Sofia *et al*., 2006, 2013), the level of diversity of this species within this appellation is still not completely known, considering the highly prevalent use of locally produced plant propagation material.

The Internal Transcribed Spacer (ITS) region of the rDNA is the most extensively sequenced DNA region in fungi (Peay *et al*., 2008), and has been proposed as the primary fungal barcode marker, due to high accuracy in fungal identification and the strongly defined barcode gap between inter- and intraspecific variation (Xu, 2006; Korabecna, 2007; Bellemain *et al*., 2010; Schoch *et al*., 2012). Furthermore, the utility of the ITS region has already been demonstrated for the correct taxonomic classification of *P. chlamydospora* (Crous and Gams, 2000).

No *P. chlamydospora* teleomorph has been described to date, and it is accepted that the reproduction of this pathogen is strictly clonal. The clonal reproduction strategy is supported by fairly high degrees of genetic homogeneity found among different populations, collected in Spain (Cobos and Martín, 2008; Tello *et al*., 2010), France (Borie *et al*., 2002; Smetham *et al*., 2010), Italy (Tegli *et al*., 2000a, 2000b), South Africa (Mostert *et al*., 2006), and New Zealand (Pottinger *et al*., 2002; Smetham *et al*., 2010).

Tegli *et al*. (2000a), using random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) analyses, described a high degree of genetic homogeneity among 15 fungal isolates from eight distinct Italian regions. Borie *et al*. (2002) found similar levels of diversity in two French regions, using RAPD analysis to study 72 isolates from one region and 34 isolates from a second; and within a French vineyard, using 47 isolates. Moreover, using RAPD, ISSR, amplified fragment length polymorphism (AFLP) and universally primed polymerase chain reaction (UP-PCR), Pottinger *et al*. (2002) verified that only slight levels of genetic variability occurred among 39 New Zealand and 6 Italian isolates, and suggested that multiple introductions of foreign fungal populations have occurred in New Zealand vineyards. Mostert *et al*. (2006), using AFLPs, concluded that 63 isolates from South Africa and 25 from Australia, France, Iran, Italy, New Zealand, Slovenia and the USA, presented low genetic diversity, and noted intra-vine, intra- and inter-vineyard variations. Also, genetic variability among different production areas was not significant, concluding that infections occurred via different inoculum sources. Cobos and Martín (2008) and Tello *et al*. (2010) observed low genetic diversity between, respectively, 35 and 57 isolates from Spain, using ISSR, RAPD and ITS region, the β-tubulin gene and the elongation factor 1-α gene. More recently, Smetham *et al*. (2010) used 60 isolates from Southern Australia and 67 from Southern France to study 18 microsatellite loci, concluding that limited genetic recombination and essentially clonal structure were present in these populations.

The aim of the present study was to evaluate the intra-specific morphological and molecular variability within a collection of Portuguese isolates. These isolates included 47 from the Dão appellation, and 21 from other Portuguese wine-producing regions.

Materials and methods

Isolate collection

The 68 isolates of *P. chlamydospora* were from different Portuguese provenances mostly in the wine-producing region of Dão (Table 1). For fungal isolation, transverse sections of wood tissues were removed from the trunks of plants that presented symptoms of esca and Petri disease, and were screened for the presence of characteristic dark lesions/spots commonly associated with the presence of this pathogen (Larignon and Dubos, 1997). These lesions were carefully separated from surrounding wood tissue using a scalpel. The obtained tissues were surface-disinfected for 1 min in 8% NaOCl solution, rinsed with sterile distilled water, dried on sterile filter paper and then placed in Petri dishes containing 2% malt agar (MA, Difco, Beckton, Dickinson and Co.), amended with 250 mg L^{-1} chloramphenicol (BioChemica, AppliChem). Plates were then incubated in the dark at 25°C to allow mycelial growth. After 8 d, colonies morphologically identical to those of *P. chlamydospora* were transferred to MA in order to get pure cultures.

Morphological characterisation

For phenotypic colony characterization (texture, colour, growing margin zonation and hyphal morphology), all obtained isolates were grown in triplicate on 2% MA, in the dark, at 25°C. Phenotypic features were described according to Crous and Gams (2000) and González and Tello (2011). In order to obtain daily growth and mean colony diameters after 30 d, for each isolate, diameters were assessed by measuring two perpendicular diameters per colony and calculating the mean. For the evaluation of the numbers of conidia, a 5 mm mycelial plug from each of the three replicate cultures was extracted from the colony growing margin, placed in a 2 mL vial containing 1 mL of sterile distilled water and vortexed for 5 s (Whiting *et al*., 2001; Tello *et al*., 2010). Numbers of conidia were counted using microscope and an improved Neubauer microscope slide cell counting chamber. Values obtained were compared referring to a two sample *t*-test.

DNA extraction, amplification and sequencing of ITS-rDNA

For DNA extraction, mycelium plugs (each of 5 mm diam.) from each of the isolates were individually plunged into 250 mL flasks containing potato dextrose broth (PDB, Difco), where mycelia were allowed to grow at 22°C. All flasks were placed on reciprocal shakers at 90 rpm min^{-1} in complete darkness. After 3 weeks, suspensions were each filtered using a paper filter disk and medium was discarded. For each isolate, 200 mg of the obtained mycelial mass was scraped into a 1.5 mL vial containing 200 µL of NucPrep™ solution (Applied Biosystems). Vials were placed on ice and each homogenized with a pestle, after which, another 600 µL of NucPrep™ solution were added to each vial, and then stored in the freezer at 5°C for 24 h. DNA was obtained using an ABI Prism™ 6100 Nucleic Acid PrepStation (Applied Biosystems), according to the manufacturer's instructions. The obtained genomic DNA was subjected to amplification of the ITS-rDNA region by PCR, using primers ITS1-F and ITS4 (White *et al*., 1990; Gardes and Bruns, 1993).

PCR reactions consisting of a final amplification volume of 25 µL, with 12.5 µL of Jump Start Taq DNA

Table 1. Details of *Phaeomoniella chlamydospora* isolates used in this study.

Phaeomoniella chlamydospora isolate	Genbank ID	Reference	Year of isolation	Geographic origin		Cultivar/rootstock	Host age (years)
				Wine appelation	County		
1	KP886950	CEVD1	2013	Dão	Mangualde	Tinta Carvalha/-	>50
2	KP886951	CEVD2	2013	Dão	Mangualde	Rufete/-	>50
3	KP886952	CEVD3	2013	Dão	Mangualde	-/-	>25

(Continued)

Table 1. (Continued).

Phaeomoniella chlamydospora isolate	Genbank ID	Reference	Year of isolation	Geographic origin		Cultivar/rootstock	Host age (years)
				Wine appelation	County		
4	KP886953	CEVD4	2013	Dão	Mangualde	Fernão Pires/-	>50
5	KP886954	CEVD5	2013	Dão	Mangualde	Português Azul/-	>50
6	KP886955	CEVD6	2013	Dão	Viseu	Baga/-	>80
7	KP886956	CEVD7	2013	Dão	Viseu	Dona Branca/-	>80
8	KP886957	CEVD8	2013	Bairrada	Pombal	Fernão Pires/-	>20
9	KP886958	CEVD9	2013	Bairrada	Pombal	Fernão Pires/-	>20
10	KP886959	CEVD10	2013	Dão	Viseu	Aragonês/-	>5
11	KP886960	CEVD11	2013	Dão	Viseu	Jaen/-	>30
12	KP886961	CEVD12	2013	Dão	Viseu	Jaen/-	>30
13	KP886962	CEVD13	2013	Dão	Viseu	Moscatel Hamb./-	10
14	KP886963	CEVD14	2013	Dão	Viseu	Bical/-	25
15	KP886964	CEVD15	2013	Dão	Viseu	Encruzado/-	25
16	KP886965	CEVD16	2013	Dão	Viseu	Arinto Gordo/-	10
17	KP886966	CEVD17	2013	Dão	Viseu	Malvasia Rei/-	25
18	KP886967	CEVD18	2013	Dão	Tondela	Touriga Nacional/-	>50
19	KP886968	CEVD19	2013	Dão	Tondela	Touriga Nacional/-	>50
20	KP886969	CEVD20	2013	Dão	Tondela	Arinto/-	>50
21	KP886970	CEVD21	2013	Dão	Tondela	Aragonês/-	25
23	KP886971	CEVD23	2013	Bairrada	Pombal	Baga/-	20
24	KP886972	CEVD24	2013	Dão	Viseu	Arinto/-	20
25	KP886973	CEVD25	2013	Bairrada	Anadia	Fernão Pires/-	25
26	KP886974	CEVD26	2012	Bairrada	Anadia	Sauvignon Blanc/-	25
27	KP886975	CEVD27	2013	Dão	P. do Castelo	Touriga Nacional/-	1
28	KP886976	CEVD28	2013	Dão	P. do Castelo	Aragonês/-	10
29	KP886977	CEVD29	2013	Açores	Pico	Aragonês/-	10
30	KP886978	CEVD30	2013	Dão	O. Hospital	Aragonês/-	>25
31	KP886979	CEVD31	2013	Dão	Tondela	Jaen/-	>25
32	KP886980	CEVD32	2013	Açores	Pico	Terrantez do Pico/-	20
33	KP886981	CEVD33	2012	Dão	Nelas	Cabernet Sauvignon/-	15
34	KP886982	CEVD34	2013	Dão	Gouveia	Syrah/-	10
35	KP886983	CEVD35	2013	Dão	Tábua	Sauvignon Blanc/-	15
36	KP886984	Ph9	2000	P. de Setúbal	Grândola	Periquita/99R	-

(Continued)

Table 1. (Continued).

Phaeomoniella chlamydospora isolate	Genbank ID	Reference	Year of isolation	Geographic origin		Cultivar/rootstock	Host age (years)
				Wine appelation	County		
37	KP886985	Ph13	2000	Bucelas	Loures	Arinto/-	-
38	KP886986	Ph14	2007	Alentejo	Monforte	Viognier/1103P	-
39	KP886987	Ph15	2007	Alentejo	Monforte	Arinto/1103P	-
40	KP886988	Ph16	2008	Alentejo	Vidigueira	Cabernet Sauvignon/169VO	2
41	KP886989	Ph17	2008	Alentejo	Vidigueira	Cabernet Sauvignon/337MM	2
42	KP886990	Ph18	2008	Alentejo	Vidigueira	Petit Verdot/400MM	2
43	KP886991	Ph19	2008	Alentejo	Vidigueira	Petit Verdot/400VO	2
44	KP886992	Ph20	2008	Alentejo	Vidigueira	Chardonnay/76PB	2
45	KP886993	Ph21	2011	Algarve	Lagoa	Arinto/1103P	-
46	KP886994	Ph22	2011	Algarve	Lagoa	Arinto/1103P	-
47	KP886995	Ph23	2011	Algarve	Lagoa	Alicante Bouschet/110R	-
48	KP886996	Ph24	2011	Arruda	A. dos Vinhos	Touriga Nacional/-	-
49	KP886997	Ph26	2012	Dão	Lousã	Cerceal da Bairrada /-	15
50	KP886998	Ph28	2012	Dão	Mangualde	Jaen/-	20
51	KP886999	Ph29	2012	Dão	Mangualde	Touriga Nacional/-	20
52	KP887000	Ph30	2012	Dão	Nelas	Jaen/SO4	15
53	KP887001	Ph31	2012	Dão	Nelas	Aragonês/SO4	15
54	KP887002	Ph32	2012	Dão	Nelas	Alfrocheiro/1103P	20
55	KP887003	Ph33	2012	Dão	Seia	Jaen/-	>50
56	KP887004	Ph34	2012	Dão	Tondela	Aragonês/-	20
57	KP887005	Ph35	2012	Dão	Mangualde	Touriga Nacional/-	25
58	KP887006	Ph36	2012	Dão	Mangualde	Encruzado/-	>50
59	KP887007	Ph37	2012	Dão	Gouveia	Gouveio/-	>50
60	KP887008	Ph38	2012	Dão	Nelas	Touriga Nacional/-	15
61	KP887009	Ph39	2012	Dão	Gouveia	Jaen/-	15
62	KP887010	Ph40	2012	Dão	Arganil	Baga/-	>80
63	KP887011	Ph42	2012	Dão	Mangualde	Jaen/-	25
64	KP887012	CEVD36	2012	Dão	Arganil	Rufete/-	>50
65	KP887013	CEVD37	2012	Dão	Tábua	TourigaNacional/1103P	15
66	KP887014	CEVD38	2013	Dão	C. do Sal	Touriga Nacional/1103P	1
67	KP887015	CEVD39	2013	Dão	C. do Sal	Touriga Nacional/1103P	1
68	KP887016	CEVD40	2013	Dão	C. do Sal	Touriga Nacional/1103P	1
69	KP887017	CEVD41	2013	Vinhos Verdes	A. de Valdevez	Loureiro/-	>25

Polymerase master mix with $MgCl_2$ and DNTP's (Sigma D9307), 0.5 μL of each primer (10 mM), 10.5 μL of ultra-pure water and 1 μL of template DNA, were performed using an ABI GeneAmp™ 9700 PCR System (Applied Biosystems), with the following conditions: initial denaturation at 95ºC for 2 min, followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 53ºC for 1 min, and extension at 72ºC for 1 min, with a final extension at 72ºC for 5 min. Each run included a negative control reaction without template DNA.

Visual confirmation of the overall amplification of the ITS region was performed using agarose gel (1.2%) electrophoresis, stained with Gel Red (Biotium) and photographed under a UV light transilluminator (Bio-Rad Gel Doc XR+). ITS region fragments were purified and sequenced using an ABI 3730 genetic analyzer, using the Big Dye v.3 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Molecular identification and phylogenetic analyses

Obtained DNA sequences were deposited in GenBank (with accession numbers: KP886950–KP887017) and compared with the sequences from the National Center of Biotechnology Information nucleotide databases using NCBI's Basic Local Alignment Search Tool (BLAST), with the option Standard nucleotide BLAST of BLASTN 2,6 (Altschul *et al.*, 1997). Molecular identification was performed following Landeweert *et al.* (2003), with similarity and taxon separations as follows: sequence similarity of 99/100%, identification to the species level; sequence similarity between 95–99%, identification to genus level; and sequence similarity equal or below 95%, identification to family or ordinal level.

Sequence alignment was performed with ClustalW 2.0 (Larkin *et al.*, 2007) and the resulting alignments were checked and manually adjusted using Geneious 7.0.6 software (www.geneious.com). Phylogenetic relationships were inferred with Maximum Likelihood (ML) using Kimura's two-parameter substitution model (Kimura, 1980). The bootstrap analysis involved 1000 replicates (MLB) to verify branches. All ML phylogenetic analyses were conducted using MEGA6 bioinformatics software (Tamura *et al.*, 2013). Phylogenetic trees were viewed and edited using FigTree 1.4.2 and a text editor. *Eutypa lata* (Genbank: KF453558.1) was used as the outgroup for the phylogenetic analyses.

Results

Phenotypic characterization

All 68 *P. chlamydospora* isolates produced typical colonies, after an incubation period of 30 d. Variability in the morphology of the colonies allowed the establishment of two distinct groups (Table 2): group I with 45 isolates and group II with 23 isolates. Group I morphology was characterised by olive-grey colour, uniform colony growing margins and filamentous somatic hyphae, while isolates included in group II developed colonies with central olive-grey colour surrounded by white growing margins. In addition, group II isolates also produced filamentous aerial mycelia on 2% MA.

All *P. chlamydospora* isolates produced the typical conidia and chlamydospore-like structures, displaying a wide range of sporulation rates, from 0.2×10^6 to 10×10^6 conidia mL^{-1}. Daily colony growth rates ranging from 0.48 mm to 0.98 mm were not significantly different, neither among colonies inside each group, nor between the two groups. Mean colony diameters after 30 d of growth ranged from 14.3 to 29.3 mm (Table 3).

Molecular identification

PCR reactions using the universal primers ITS1-F and ITS4 produced a single DNA fragment of ca. 570–600 bp for all *P. chlamydospora* isolates tested. The studied isolates were identified in comparison with reference *P. chlamydospora* ITS sequences deposited in the NCBI GenBank database. The similarity values for all sequences were 99%, with exception of isolate 61 which presented 100% similarity. These similarity values are adequate for the molecular identification of isolates (Landeweert *et al.*, 2003).

Phylogenetic characterization

The complete ITS sequences of each isolate were analysed to infer the phylogenetic relationship by using the ML approach (Figure 1). The results show separation between two distinct groups; one clustering 33 isolates with a bootstrap value of 65% and the second clustering 35 isolates separated from group 1 with a bootstrap value of 80%. The separation of these two groups occurred due to the simple nucleotide polymorphisms (SNPs), in the positions 429 (T/A) and 497 (T/C). In addition a SNP in position

Table 2. Distribution of the 68 *Phaeomoniella chlamydospora* isolates between the two morphological groups, according to colony phenotypic characteristics (after 30 d at 25°C, in 2% MA)

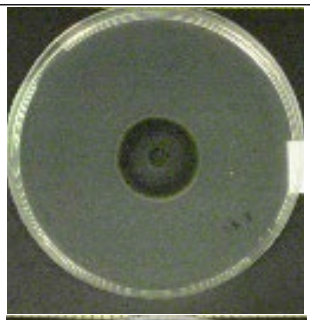
Group	*Phaeomoniella chlamydospora* isolates	Phenotype in MA culture	Texture	Colour	Growing margin	Zonation	Hyphal morphology
I	1, 2, 3, 4, 5, 9, 13, 14, 15, 16, 17, 18, 21, 23, 24, 26, 27, 28, 30, 33, 35, 39, 40, 42, 43, 44, 45, 47, 48, 49, 50, 51, 52, 53, 55, 56, 58, 59, 60, 61, 62, 64, 65 68, 69		Felty	Olive-grey	even	absent	Filamentous somatic hyphae predominant, aerial mycelium scanty
II	6, 7, 8, 10, 11, 12, 19, 20, 25, 29, 31, 32, 34, 36, 37, 38, 41, 46, 54, 57, 63, 66, 67		Felty	Olive-grey to white towards the edge	even	absent	Filamentous somatic hyphae predominant, aerial mycelium scanty

Table 3. Mean, maximum and minimum values (± standard deviations) of the colony phenotypic characters in *Phaeomoniella chlamydospora* isolates.

Phenotypic variable	Group I mean value ± SD	Group II mean value ± SD	Maximum values		Minimum values	
			Group I	Group II	Group I	Group II
Daily growth rate (mm) at 25°C [a]	0.68 ± 0.13 [ns]	0.68 ± 0.11 [ns]	0.98	0.93	0.48	0.50
Growth (mm) at 25°C, after 30 d [a]	20.54 ± 3.90 [ns]	20.52 ± 3.50 [ns]	29.3	27.87	14.31	14.83

[ns] Non-significant differences according to *t*-test at $\alpha = 0.05$
[a] Colony diameter

534 (C/A) separated isolate 37 from the rest of its group (bootstrap value of 100%).

Discussion

Several studies have examined *P. chlamydospora* isolates in Portugal (Chicau *et al*., 2000; Rego *et al*., 2000; Santos *et al*., 2006; Sofia *et al*., 2013). However, there was little available information about phenotypical and molecular variability of the species. In this study, a larger collection of *P. chlamydospora* isolates, 47 from Dão appellation and 21 from other Portuguese wine regions was characterized.

Studies concerning morphological features of *P. chlamydospora* isolates, conducted in different countries, have shown a low degree of phenotypic variation (Dupont *et al*., 1998; Whiting *et al*., 2001, 2005; Santos *et al*., 2006; Tello *et al*., 2010). Moreover, in

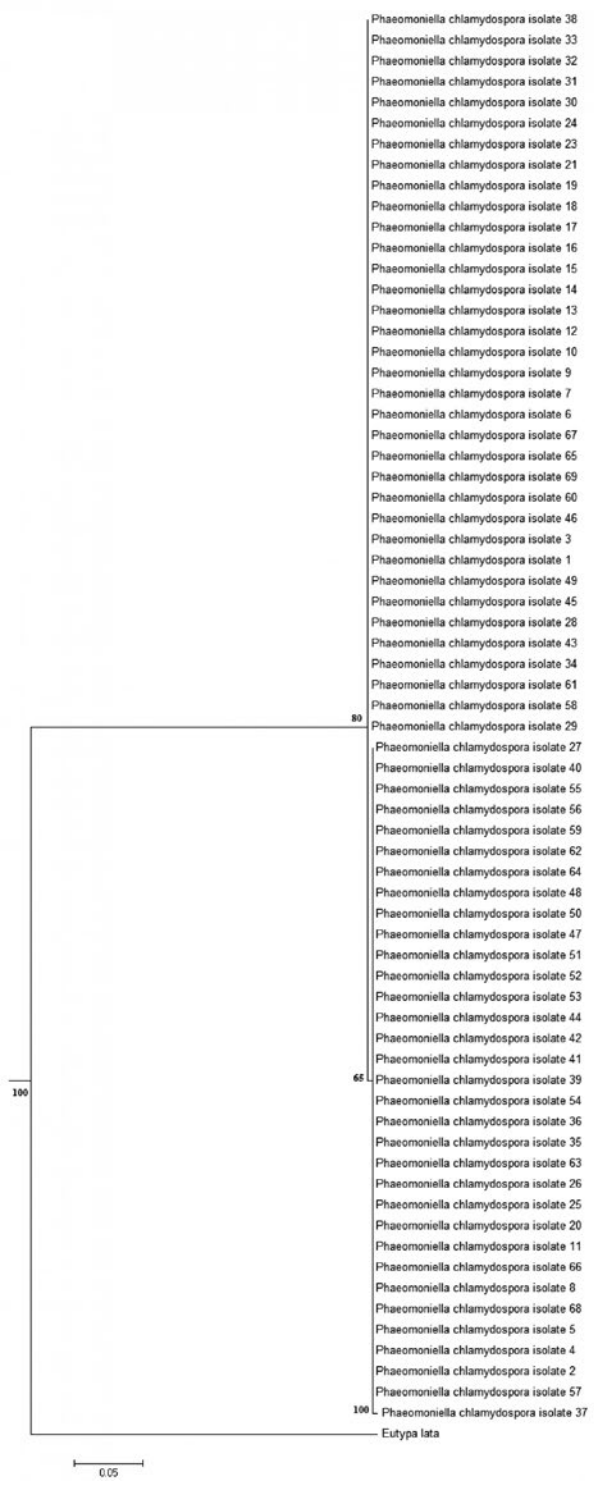


Figure 1. Maximum Likelihood tree inferred from a character alignment of the 68 rDNA- ITS sequences of *Phaeomoniella chlamydospora* obtained in this study, with *Eutypa lata* (KF453558) as an outgroup. Numbers above branches identify the statistical bootstrap percentages (Maximum likelihood bootstraps from 1000 iterations). Scale represents substitutions per site.

France, a country with an ancient and ubiquitous viticulture, similar to the Portuguese Dão appellation's viticulture, Comont *et al*. (2010) reported the coexistence of two predominant clonal lineages.

In the present research, phenotypic characteristics displayed little variation among the 68 *P. chlamydospora* isolates from Dão and other Portuguese wine-producing regions. Similar results of low morphological variation were reported in previous studies in which homogeneity was also observed (Dupont *et al*., 1998; Whiting *et al*., 2005; Tello *et al*., 2010). Nevertheless, the morphological features analysed here appeared to divide the 68 isolates into two morphotypes according to the macromorphological appearance of the cultures. Isolates from Dão were separated into the two groups together with isolates from other Portuguese regions. No clear relationships with the source rootstock/scion combination, year of isolation or geographical origin were recorded among the isolates.

Tello *et al*. (2010), for Spanish isolates, and Sofia *et al*. (2013), for Portuguese isolates, registered higher levels of sporulation and daily growth rates than recorded in the present study. These differences are probably mainly due to our usage of malt agar instead of potato dextrose agar, which is a richer growth medium likely to give greater sporulation than malt agar.

The low phenotypic variability observed in the Portuguese populations of *P. chlamydospora*, probably a consequence of predominant clonal reproduction, indicates that different criteria are needed to differentiate the population structure of a large set of isolates.

Multiple alignments of the ITS sequences clustered in two distinct groups, due to changes in the nucleotides from positions 429 (T/A) and 497 (T/C). Similar results have been observed for Spanish populations by Cobos and Martín (2008), with differences occurring in positions 369 (T/A) and 438 (T/C). In addition, isolate 37 was separated from the last group by a SNP in position 534 (C/A).

Since three different clonal lineages of *P. chlamydospora* were detected, results suggest that different sources of inoculum may have been introduced through propagation material, such as mother-plants, rootstock, grafted cuttings and/or scions (Retief *et al*., 2006; Whiteman *et al*., 2007).

Our results are in agreement with previous studies conducted in New Zealand, Spain, Australia and France (Tegli *et al*., 2000a, 2000b; Borie *et al*., 2002; Pottinger *et al*., 2002; Mostert *et al*., 2006; Cobos and Martín, 2008; Smetham *et al*. 2010; Tello *et al*., 2010). There is no clear correlation between the morphological groups and the genetic clusters.

Clonal reproduction has been described for this species (Tegli *et al*., 2000a, 2000b; Borie *et al*., 2002; Pottinger *et al*., 2002; Mostert *et al*., 2006; Cobos and Martín, 2008; Smetham *et al*., 2010; Tello *et al*., 2010). Asexual reproduction is predominant. Nonetheless, as suggested by Borie *et al*. (2002), recombinations via a parasexual cycle may be involved, explaining the slight genetic variability found.

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