Phytopathologia Mediterranea (2015) 54, 2, 335–344
DOI: 10.14601/Phytopathol_Mediterr-15441

RESEARCH PAPERS - 9TH SPECIAL ISSUE ON GRAPEVINE TRUNK DISEASES

Haplotype analysis and genetic variability of *Togninia minima* from different geographic sources

EDUARDO ABREO

Laboratorio de Bioproducción INIA Las Brujas, Ruta 48 Km 10, CP 90001, Canelones, Uruguay

Summary. *Togninia minima* (anamorph *Phaeoacremonium aleophilum*) is one of the main fungi responsible for trunk diseases of grapevines and other woody hosts worldwide. Sequences of protein-coding genes of isolates from countries in different continents have been published, presenting a useful resource for examination of the diversity and spatial distribution of *T. minima* genotypes. Single nucleotide polymorphisms (SNPs) detected in public sequences of the *actin* and partial *β-tubulin* genes were used to assess the genetic variability and to determine haplotypes of isolates of this species from different sources. The Italian sample showed the greatest allele number and the largest number of haplotypes. Most haplotypes were present in more than one country, except for haplotype 11010 which was found only in Italy and 10111 found only in Canada. Haplotype 11111 was the most conspicuous and cosmopolitan, being present in six countries and on three host plant species. One observed polymorphism in the non-coding region of the *β-tubulin* gene could be targeted with allele-specific primers to detect this particular haplotype.

Key words: *Phaeoacremonium aleophilum*, SNP, *Olea*, *Prunus*, *Vitis*, *Phaeoacremonium minimum*.

Introduction

Togninia minima (anamorph *Phaeoacremonium aleophilum*) is a pathogenic fungus responsible for the development of wood diseases mostly in *Vitis* spp. but also in fruit trees and other woody hosts (Mostert *et al*., 2006a; Cloete *et al*., 2011; Carlucci *et al*., 2013). Presence of this pathogen in *Vitis* spp. has been recorded in several countries, including Algeria (Berraf-Tebbal *et al*., 2011), Argentina (Dupont *et al*., 2002), Australia (Edwards *et al*., 2001), Canada (Úrbez-Torres *et al*., 2014), Chile (Auger *et al*., 2005), Hungary (Essakhi *et al*., 2008), Iran (Mohammadi *et al*., 2013), Israel (Essakhi *et al*., 2008), Italy (Mugnai *et al*., 1999), France (Larignon and Dubos, 1997), South Africa (Groenewald *et al*., 2001), Spain (Armengol *et al*., 2001), Turkey (Ari, 2000), Uruguay (Abreo *et al*., 2011), USA (Scheck *et al*., 1998), and former Yugoslavia (Crous *et al*., 1996).

Corresponding author: E. Abreo
E-mail: eabreo@inia.org.uy

Although the anamorph of *T. minima* is most frequently found in grapevines, the presence of perithecia resulting from the mating of isolates with opposite mating types has been reported *in vitro* (Mostert *et al*., 2003; Rooney Latham *et al*., 2005a; Gramaje *et al*., 2013), on wood pieces in moist chambers (Pascoe *et al*., 2004) and in vineyards in California (Rooney-Latham *et al*., 2005b). The genetic variability of *T. minima* has been studied in Italy (Tegli *et al*., 2000), France (Borie *et al*., 2002), Algeria (Berraf-Tebbal *et al*., 2011), and Spain (Martín and Martín, 2013; Gramaje *et al*., 2013; Martín *et al*., 2014) at national and local levels. While the Spanish and Italian studies concluded that low levels of linkage disequilibrium or large numbers of haplotypes at local or plant scales could be explained in part by the occurrence of some degree of sexual reproduction, the low genetic variability found in the French populations of *T. minima* was explained by a lower level of recombination or insufficient number of genetic markers. These studies were based on the random amplification of the fungal genome by means of RAPDs, ISSR, MSP-PCR,

ISSN (print): 0031-9465
ISSN (online): 1593-2095

UP-PCR and AFLP, with the exception of Martín and Martín (2013) who performed a multi-gene sequence analysis of Spanish isolates of *T. minima.*

In spite of these efforts, it has been acknowledged that different molecular tools are needed to obtain information on introduction frequencies, inoculum dispersal and geographical spread of *T. minima* in different regions of the world (Gramaje *et al*., 2013). Whereas the actual DNA or original specimens from throughout the world may not be readily available, there is a growing database of DNA sequences that could be used to assess the genetic diversity of geographically diverse strains of the fungus. In addition, as *T. minima* is a haploid ascomycete, SNPs and haplotypes can be identified directly from DNA sequences of the fungus, and these could be used to evaluate genetic variability.

The objective of the research outlined here was to explore the use of SNPs detected on publicly available sequences of two protein-coding nuclear genes to evaluate the genetic variability of *T. minima* representing the populations of Italy, Spain, Algeria, South Africa, Canada, and Uruguay.

Materials and methods

A search was conducted in the nucleotide database of GenBank (www.ncbi.org, last accessed 11 January, 2015) and sequences of the *actin* and partial *β-tubulin* genes of *T. minima* from different countries and hosts were retrieved. Only the sequences from strains whose identity as *T. minima* had been confirmed by means of phylogenetic analysis based on these two genes were used in subsequent analyses. Original sequences, submitters and information on related publications are available at GenBank and referenced by the corresponding accessions in Table 1.

Sequences of *actin* and partial *β-tubulin* genes were concatenated in MEGA6 (Tamura *et al*., 2013), aligned and trimmed at the irregular ends to a final span of 740 characters including gaps (TreeBase code: S17038). SNPs were visually identified. Point mutations and insertions/deletions (indels) were considered as SNPs if present in at least 10% of the retrieved sequences. This value was considered safe to avoid recent point mutations and errors at the sequencing stage.

Alleles were named for the corresponding gene abbreviation followed by the nucleotide position of the SNP on each gene sequence (referenced to strain FI 2096, accessions KJ569197 and HQ159856) and by the first letter of the alternative nucleotides to distinguish between both alleles: Act89-A/Act89-C, Act110-G/Act110-A, Act166-A/Act166-G, βT459-C/βT459-G or 0 in the case of absence of the indel βT360-AT/ βT360-0.

A matrix was constructed in which strains were characterized by the presence of the alternative SNP forms indicated by 1 or 0 (Table 1). Numbers of alleles, allelic frequencies and Nei's gene diversity were obtained using PopGene 1.31 software (Yeh *et al*., 1997) to describe country samples with at least six representative isolate sequences (a total of 63 isolates from Italy, Spain, Algeria, South Africa, Canada and Uruguay).

Haplotypes were defined as strains sharing 100% of the alleles. A cluster analysis (Euclidean distance, paired group option) of all 66 retrieved strains from nine countries in Table 1 was carried out with software PAST (Hammer *et al*., 2001) to analyze the genotypic similarity among haplotypes. A principal components analysis (variance – covariance option) was applied on single copies of the different haplotypes with software PAST (Hammer *et al*., 2001) to confirm the groups obtained in the cluster analysis. Numbers of haplotypes were calculated for the six countries with at least six representative isolates.

Results

Genetic variability analysis

Five SNPs were identified in the non-coding regions of the *actin* and *β-tubulin* genes. Four loci were biallelic and one locus was the result of a two-nucleotide indel in positions 360 and 361 in the *β-tubulin* partial sequence. SNP frequency was 0.7%. The mean observed number of alleles was 2.0 for Italy, 1.8 for Spain, South Africa and Uruguay and 1.6 for Algeria and Canada.

Allele distribution varied between country samples. SNPs in the *actin* gene were mostly unbalanced, except for Act110 in Spain and Act166 in Algeria that exhibited an even presence of both alleles. Missing alleles were only observed in this gene. Allele Act166-G was absent from Canada, Uruguay, Spain and South Africa and present only in Hungary, Italy and in higher frequency in Algeria (Table 1, 2). Samples from Algeria and Canada comprised fewer alleles since Act89-C was absent from both countries, Act110-A was absent from Algeria and Act166-G was absent from Canada.

Table 1. Strains of *Togninia minima*, source, GenBank information of sequences of the *actin* and partial *β-tubulin* genes used for single nucleotide polymorphism detection, and nucleotides at each polymorphic position.

Strain	Country	Host	GenBank accession			SNP[a]				
			Actin	β-tubulin	Author	Act89-A/C	Act110-G/A	Act166-A/G	βT360-AT/0	βT459-C/G
FI 2096	Uruguay	*Vitis* sp.	KJ569197	HQ159856	Abreo E. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
FI 2103	Uruguay	*Vitis* sp.	HQ159871	HQ159862	Abreo E. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
FI 2105	Uruguay	*Vitis* sp.	HQ159872	HQ159864	Abreo E. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
FI 2106	Uruguay	*Vitis* sp.	HQ159873	HQ159865	Abreo E. *et al.*	C (0)	A (0)	A (1)	- - (0)	G (0)
FI 2108	Uruguay	*Vitis* sp.	KJ569204	HQ159867	Abreo E. *et al.*	C (0)	A (0)	A (1)	- - (0)	G (0)
FI 2086	Uruguay	*Vitis* sp.	KJ569201	HQ159846	Abreo E. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
FI 2094	Uruguay	*Vitis* sp.	KJ569202	HQ159854	Abreo E. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
FI 2093	Uruguay	*Vitis* sp.	KJ569203	HQ159853	Abreo E. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)
Y108022z (SI)	Spain	*Vitis* sp.	JF275893	JF275879	Martin L. and Martin M.T.	A (1)	G (1)	A (1)	AT (1)	C (1)
Y235041w (SII)	Spain	*Vitis* sp.	JF275897	JF275878	Martin L. and Martin M.T.	C (0)	A (0)	A (1)	- - (0)	G (0)
Y086151x (SII)	Spain	*Vitis* sp.	JF275895	JF275876	Martin L. and Martin M.T.	A (1)	A (0)	A (1)	- - (0)	G (0)
Y168142a (SIII)	Spain	*Vitis* sp.	JF275896	JF275877	Martin L. and Martin M.T.	A (1)	G (1)	A (1)	- - (0)	G (0)
Y038053z (SIV)	Spain	*Vitis* sp.	JF275892	JF275874	Martin L. and Martin M.T.	A (1)	G (1)	A (1)	- - (0)	G (0)
Y086151a (SIV)	Spain	*Vitis* sp.	JF275894	JF275875	Martin L. and Martin M.T.	C (0)	A (0)	A (1)	- - (0)	G (0)
4ss2Pal	Italy	*Vitis* sp.	EU863496	EU863464	Essakhi S. *et al.*	A (1)	A (0)	A (1)	- - (0)	G (0)
81Pal	Italy	*Vitis* sp.	EU863497	EU863465	Essakhi S. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)
168 Pal	Italy	*Vitis* sp.	EU863498	EU863466	Essakhi S. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
138ss1Pal	Italy	*Vitis* sp.	EU863500	EU863465	Essakhi S. *et al.*	A (1)	G (1)	G (0)	AT (1)	G (0)
156Pal	Italy	*Vitis* sp.	EU863499	EU863467	Essakhi S. *et al.*	A (1)	G (1)	G (0)	AT (1)	G (0)
98Pal	Hungary	*Vitis* sp.	EU863501	EU863469	Essakhi S. *et al.*	A (1)	G (1)	G (0)	- - (0)	G (0)
21Pal	Italy	*Vitis* sp.	EU863502	EU863470	Essakhi S. *et al.*	C (0)	A (0)	A (1)	- - (0)	G (0)
144Pal	Italy	*Vitis* sp.	EU863503	EU863471	Essakhi S. *et al.*	A (1)	A (0)	A (1)	- - (0)	G (0)
CBS 100397	Italy	*Vitis* sp.	AY735498	AF246806	Mostert L. *et al.*	A (1)	G (1)	G (0)	- - (0)	G (0)
Pm37	Italy	*Vitis* sp.	KJ534035	KJ534063	Raimondo M.L. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)

(continued)

Table 1. (Continued).

Strain	Country	Host	GenBank accession			SNP[a]				
			Actin	β-tubulin	Author	Act89-A/C	Act110-G/A	Act166-A/G	βT360-AT/0	βT459-C/G
Pm36	Italy	*Vitis* sp.	KJ534034	KJ534062	Raimondo M.L. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)
Pm9	Italy	*Vitis* sp.	KJ534033	KJ534061	Raimondo M.L. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)
Pm7	Italy	*Vitis* sp.	KJ534032	KJ534060	Raimondo M.L. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)
Pm4	Italy	*Vitis* sp.	KJ534031	KJ534059	Raimondo M.L. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)
Pm2	Italy	*Vitis* sp.	KJ534030	KJ534058	Raimondo M.L. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)
Pm6	Italy	*Vitis* sp.	KJ534029	KJ534057	Raimondo M.L. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
Pm5	Italy	*Vitis* sp.	KJ534028	KJ534056	Raimondo M.L. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
Pm3	Italy	*Vitis* sp.	KJ534027	KJ534055	Raimondo M.L. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
Pm1	Italy	*Vitis* sp.	KJ534026	KJ534054	Raimondo M.L. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
Pm330	Italy	*Olea* sp	KM201189	KM201219	Carlucci A. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
Pm255	Italy	*Olea* sp.	KM201188	KM201218	Carlucci A. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
Pm115	Italy	*Olea* sp.	KM201187	KM201217	Carlucci A. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
Pm50	Italy	*Olea* sp.	KM201186	KM201216	Carlucci A. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
132Pal	Israel	*Vitis* sp.	EU863504	EU863472	Essakhi S. *et al.*	A (1)	A (0)	A (1)	- - (0)	G (0)
P12	Algeria	*Vitis* sp.	HQ605002	HQ605013	Berraf-Tebbal A. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
P14	Algeria	*Vitis* sp.	HQ605003	HQ605014	Berraf-Tebbal A. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
P16	Algeria	*Vitis* sp.	HQ605006	HQ605015	Berraf-Tebbal A. *et al.*	A (1)	G (1)	G (0)	- - (0)	G (0)
P22	Algeria	*Vitis* sp.	HQ605007	HQ605016	Berraf-Tebbal A. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
P28	Algeria	*Vitis* sp.	HQ605004	HQ605017	Berraf-Tebbal A. *et al.*	A (1)	G (1)	G (0)	- - (0)	G (0)
P29	Algeria	*Vitis* sp.	HQ605008	HQ605018	Berraf-Tebbal A. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
P49	Algeria	*Vitis* sp.	HQ605005	HQ605024	Berraf-Tebbal A. *et al.*	A (1)	G (1)	G (0)	- - (0)	G (0)
CBS 246.91 (T)	Yugoslavia	*Vitis* sp.	AY735497	AF192390	Mostert L. *et al.*/Dupont J. *et al.*	A (1)	A (0)	A (1)	- - (0)	G (0)
STE-U 6991	SouthAfrica	*Vitis* sp.	JQ038921	JQ038910	Mostert L. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
STE-U 6986	SouthAfrica	*Vitis* sp.	JQ038920	JQ038909	Mostert L. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
CBS 110703	SouthAfrica	*Vitis* sp.	DQ173115	DQ173094	Mostert L. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)

(continued)

Table 1. (Continued).

Strain	Country	Host	GenBank accession			SNP[a]				
			Actin	β-tubulin	Author	Act89-A/C	Act110-G/A	Act166-A/G	βT360-AT/0	βT459-C/G
L.M.483	SouthAfrica	*Prunus* sp.	DQ173116	DQ173095	Mostert L. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
STE-U 6088	SouthAfrica	*Prunus* sp.	EU128104	EU128062	Damm U. *et al.*	C (0)	A (0)	A (1)	- - (0)	G (0)
STE-U 6089	SouthAfrica	*Prunus* sp.	EU128105	EU128063	Damm U. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
STE-U 5836	SouthAfrica	*Prunus* sp.	EU128107	EU128065	Damm U. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
STE-U 5962	SouthAfrica	*Prunus* sp.	EU128108	EU128066	Damm U. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
STE-U 5964	SouthAfrica	*Prunus* sp.	EU128110	EU128068	Damm U. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)
STE-U 6090	SouthAfrica	*Prunus* sp.	EU128106	EU128064	Damm U. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
STE-U 5963	South Africa	*Prunus* sp.	EU128109	EU128067	Damm U. *et al.*	C (0)	A (0)	A (1)	- - (0)	G (0)
PARC395	Canada	*Vitis* sp.	KF764520	KF764681	Úrbez-Torres J.R. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)
PARC369	Canada	*Vitis* sp.	KF764519	KF764680	Úrbez-Torres J.R. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
PARC349	Canada	*Vitis* sp.	KF764518	KF764679	Úrbez-Torres J.R. *et al.*	A (1)	A (0)	A (1)	AT (1)	C (1)
PARC341	Canada	*Vitis* sp.	KF764517	KF764678	Úrbez-Torres J.R. *et al.*	A (1)	A (0)	A (1)	AT (1)	C (1)
PARC249	Canada	*Vitis* sp.	KF764516	KF764677	Úrbez-Torres J.R. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)
PARC220	Canada	*Vitis* sp.	KF764515	KF764676	Úrbez-Torres J.R. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)
PARC187	Canada	*Vitis* sp.	KF764514	KF764675	Úrbez-Torres J.R. *et al.*	A (1)	G (1)	A (1)	AT (1)	C (1)
PARC172	Canada	*Vitis* sp.	KF764513	KF764674	Úrbez-Torres J.R. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)
PARC158	Canada	*Vitis* sp.	KF764512	KF764673	Úrbez-Torres J.R. *et al.*	A (1)	G (1)	A (1)	- - (0)	G (0)

[a] Capital letters indicate the alternative nucleotides in each polymorphic position; numbers in parenthesis indicate the corresponding code used for haplotype determination.

Table 2. Distribution of the allele frequencies across the *Togninia minima* samples from six countries.

	Uruguay	Spain	Italy	Algeria	SouthAfrica	Canada	Average
Act89-A	0.75	0.67	0.95	1.00	0.82	1.00	0.86
Act89-C	0.25	0.33	0.05	0.00	0.18	0.00	0.14
Act110-G	0.75	0.50	0.86	1.00	0.82	0.78	0.78
Act110-A	0.25	0.50	0.14	0.00	0.18	0.22	0.22
Act166-A	1.00	1.00	0.86	0.57	1.00	1.00	0.91
Act166-G	0.00	0.00	0.14	0.43	0.00	0.00	0.09
βT360-AT	0.63	0.17	0.50	0.57	0.73	0.44	0.50
βT360-0	0.38	0.83	0.50	0.43	0.27	0.56	0.50
βT459-C	0.63	0.17	0.41	0.57	0.73	0.44	0.49
βT459-G	0.38	0.83	0.59	0.43	0.27	0.56	0.51

SNPs in the partial β-tubulin gene were mostly balanced, except for the Spanish sample that showed low frequency in alleles βT360-AT and βT459-C. No *β-tubulin* allele was missing in any population.

Nei's gene diversity within samples was 0.33 for Uruguay, 0.31 for Italy, 0.30 for Spain, 0.29 for Algeria, 0.28 for South Africa and 0.27 for Canada.

Genotype variability analysis

Seven haplotypes were identified based on the observed combination of alleles. Among the countries with at least six strains available, Italy showed six haplotypes, Spain four, Uruguay, South Africa and Canada three, and Algeria only two (Table 3).

Cluster analysis of haplotypes showed four main clusters (Figure 1). Cluster "A" was separated from the others and included haplotypes 11111 and 10111. Haplotype 11111 was the most abundant and most cosmopolitan including 29 strains from Algeria, Spain, Uruguay, Italy, South Africa and Canada (on *Vitis* spp., *Prunus* spp., and *Olea* spp.), while haplotype 10111 (two strains) was exclusively from Canada (*Vitis* spp.). Cluster "B" grouped haplotypes 11010, restricted to Italy, and 11000, found in Algeria, Hungary and Italy (*Vitis* spp.). Cluster "C" contained haplotype 11100 with sixteen strains from Italy, Uruguay, Spain, South Africa and Canada (*Vitis* spp. and *Prunus* spp.). Cluster "D" contained haplotype 10100, with strains from Spain, Italy and the

Table 3. Distribution of haplotypes of *Togninia minima* in samples from six countries.

Haplotype	Uruguay	Spain	Italy	Algeria	South Africa	Canada	Total
11111	5	1	9	4	8	2	29
10111	0	0	0	0	0	2	2
00100	2	2	1	0	2	0	7
11100	1	2	7	0	1	5	16
10100	0	1	2	0	0	0	3
11010	0	0	2	0	0	0	2
11000	0	0	1	3	0	0	4

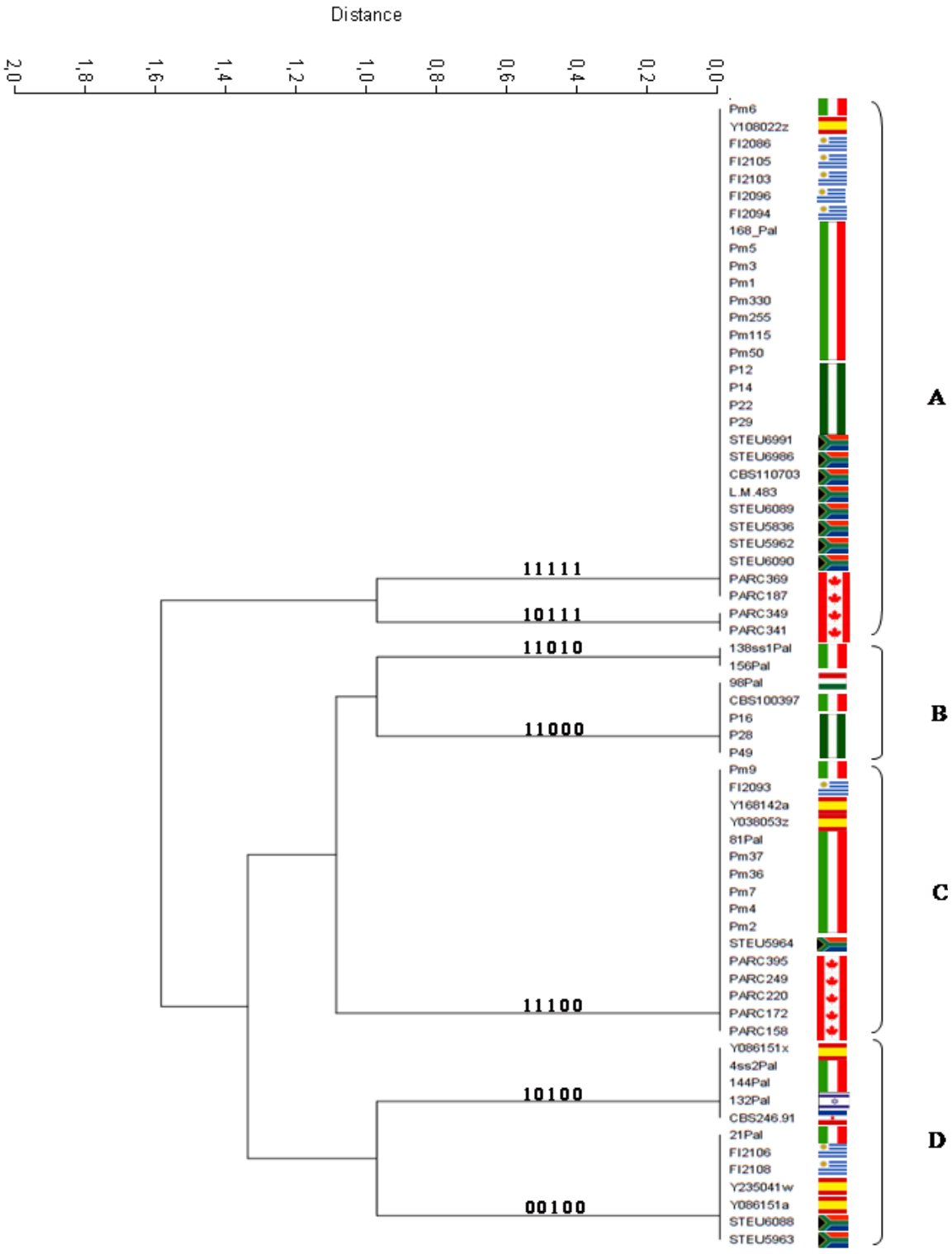

Figure 1. Cluster analysis showing haplotype grouping of isolates of *Togninia minima*. Haplotype codes are shown upon terminal branches. Country of origin of each sample is indicated by: Italy, Spain, Uruguay, South Africa, Canada, Hungary, Algeria, Israel, Yugoslavia.

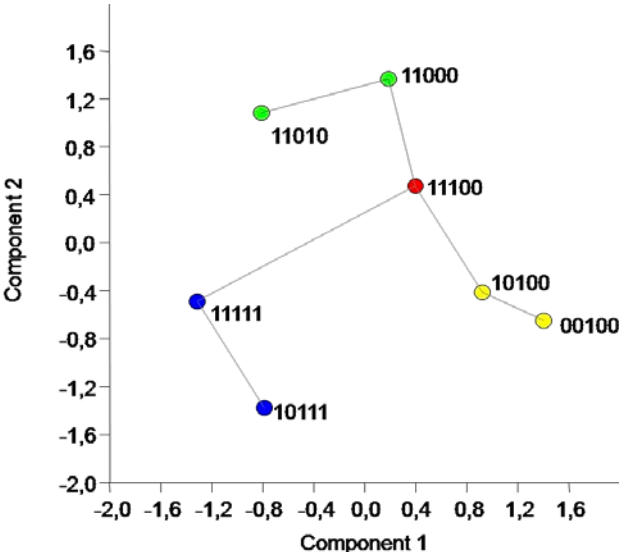

Figure 2. Principal component analysis of seven haplotypes showing a Minimal Spanning Tree to indicate the shortest possible set of connected lines linking all points. Symbols represent: ○ cluster A, ○ cluster B, ○ cluster C, and ○ cluster D in the cluster analysis.

only representatives from Israel and former Yugoslavia (*Vitis* spp. and *Prunus*), and haplotype 00100 with sequences from Spain, Italy, South Africa and Uruguay (*Vitis* spp. and *Prunus* spp.). The principal component analysis of haplotypes (Figure 2) also clearly separated haplotypes 11111 and 10111 from the others along the *x* and *y* axes representing components 1 and 2, which explained, respectively, 41% and 38% of the variance.

Haplotype 11111 and its Canadian variant haplotype 10111 could also be individualized by the presence of a C (1) in the fifth SNP, corresponding to position 459 in the fourth intron of the *β-tubulin* gene.

Discussion

Sequences of the *actin* and partial *β-tubulin* genes have been considered useful for the phylogenetic analysis of species of *Togninia* (Mostert *et al*., 2006b). At population levels, the introns of the *β-tubulin* gene have been used to analyze differences between populations of the enteric protozoan parasite *Cryptosporidium parvum* (Widmer *et al*., 1998) and the grapevine powdery mildew *Erysiphe necator* (Amrani and Corio-Costet, 2006; Brewer and Milgroom, 2010). In the present study sequences from both of these genes from *T. minima* from several countries were retrieved from GenBank and used for SNPs detection and haplotype determination. SNPs from introns of both genes were defined with a high and restrictive threshold (minor nucleotide present in at least 10% of sequences) to account for errors at sequencing and to minimize the effect of late point mutations shared only by local clonal lines.

The final SNP frequency of 0.7% was similar to that found in other fungi, such as 1% in *Candida albicans* (Jones *et al*., 2004) and 1.1–1.7% in *Tricholoma matsutake* (Xu *et al*., 2007).

Gene diversity was equal to or greater than 0.30 in Uruguay, Italy and Spain, and below this value in samples from South Africa, Algeria and Canada. Although the sample from Italy was the only one where all alleles were present, its gene diversity ranked second to samples from Uruguay as a result of three out of five SNPs being unbalanced, showing a very high frequency of one of the two alternative forms. Nevertheless, the Italian sample exhibited greater genotypic variability due to the presence of six out of seven haplotypes. The same number of haplotypes was only reached when adding together the samples from all the other countries, which suggest that the large number of haplotypes found in Italy is not just an effect of the greater number of sequences that were included from this country.

Opposite to the Italian sample, the Uruguayan sample showed the greatest gene diversity but only three haplotypes. This apparent contradiction has been observed before in populations of introduced pathogens and could be a consequence of the delay in the onset of sexual reproduction after migration, which might have attenuated the expected loss of genetic diversity due to the founder effect, as suggested by Travadon *et al*. (2012) in their study on *Eutypa lata* world populations. Brewer and Milgroom (2010) also found similar results of high gene diversity but low genotypic diversity in introduced populations of *E. necator*. In this case, one possible explanation was that the occurrence of two distinct genetic groups in the introduced populations resulted in high gene diversity because of fixed nucleotide differences between lineages, but low genotypic diversity because there was little or no variation within groups.

In contrast, the South African, Canadian and the Algerian sampled populations, with lower genetic and genotypic diversity, could be an example of the

loss of genetic diversity due to genetic drift, which may occur after many generations since the arrival of a founder population.

The sample from Spain showed intermediate gene diversity but high genotypic variability with four haplotypes identified in six sampled individuals. The greater genotypic variability of *T. minima* samples in Italy and Spain could be a consequence of both the degree of gene diversity and greater levels of sexual reproduction and recombination. In this context, Tegli (2000) found that sexual reproduction was highly feasible in Italian *T. minima* populations in view of the high genetic distance among multilocus genotypes, the low clonal fraction and the low linkage disequilibrium of the studied populations. Haplotype variability was considered greater than in France, where some regions did not show signs of recombination (Borie *et al*., 2002). In Spain, Gramaje *et al*. (2013) also found greater genetic variability than in France, although it was considered that this could be the result of the UP-PCR method used which preferentially amplifies the more variable intergenic areas of fungal genomes.

In the present study, samples of *T. minima* from Italy and Spain showed more genotypic variability than samples from two African countries, Uruguay and Canada. In the Italian, South African, Uruguayan and Algerian populations the haplotype 11111 appeared most frequently, being prevalent in three host species. Also, Martín and Martín (2013) and Martín *et al*. (2014) described a Spanish isolate whose genotype was classified as type SI, and whose *actin* and partial *β-tubulin* gene sequences, included in the present analysis, exhibited the most frequent haplotype 11111. These authors suggested that the group SI showed less genetic variability than other groups and that it was associated mostly with young plants showing Petri disease. Since young nursery plants are internationally commercialized it is possible that their international trade could have favoured the long distance dispersal of members of this group, explaining its cosmopolitan occurrence.

Haplotype 11111 and its Canadian variant haplotype 10111 were individualized by the presence of a C (1) in the fifth SNP in the *β-tubulin* gene. SNPs observed in introns of the *β-tubulin* gene have been useful for the genotyping of pathotypes of *E. necator* (Amrani and Corio-Costet, 2006) *and Cryptosporidium parvum* (Tanriverdi *et al*., 2002) with allele-specific PCR primers. It should be possible, therefore, to target this characteristic sequence polymorphism with allele-specific PCR primers to distinguish haplotypes 11111 and 10111 (cluster "A" in the haplotype distance analysis) from the others (clusters "B", "C", "D").

In conclusion, five SNPs from the non-coding regions of two protein-coding nuclear genes could be used to analyze the genotypic diversity of samples of *T. minima* from countries in Europe, Africa, North and South America. Haplotype distribution indicates that haplotype 11111 is the most frequent and cosmopolitan genotype, and is the genotype requiring further ecological, biological and phytopathological characterization.

Acknowledgments

Dr. Guillermo Pérez provided thoughtful comments on a previous manuscript version of this paper.

Literature cited

Abreo E., S. Martínez, L. Bettucci and S. Lupo, 2011. *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. in grapevines from Uruguay. *Phytopathologia Mediterranea* 50, S77–85.

Amrani L. and M.F. Corio-Costet, 2006. A single nucleotide polymorphism in the *β-tubulin* gene distinguishing two genotypes of *Erysiphe necator* expressing different symptoms on grapevine. *Plant Pathology* 55, 505–512.

Ari M.E., 2000. A general approach for esca disease in the vineyards of Turkey. *Phytopathologia Mediterranea* 39, 35–37.

Armengol J., A. Vicent, L. Torné, F. García-Figueres and J. García-Jiménez, 2001. Fungi associated with esca and grapevine declines in Spain: a three-year survey. *Phytopathologia Mediterranea* 40, S325–S329.

Auger J., I. Pérez, M. Esterio, V. Navia, W.D. Gubler and A. Eskalen, 2005. Fungi associated with grapevine wood decay and young vine decline in Chile. *Phytopathologia Mediterranea* 44, 89–90.

Berraf-Tebbal A., Z. Bouznad, J.M. Santos, M.A. Coelho, J.P. Péros and A.J.L. Phillips, 2011. *Phaeoacremonium* species associated with *Eutypa* dieback and esca of grapevines in Algeria. *Phytopathologia Mediterranea* 50, S86–S97.

Borie B., L. Jacquiot, I. Jamaux-Despréaux, P. Larignon and J.P. Péros, 2002. Genetic diversity in populations of the fungi *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* on grapevine in France. *Plant Pathology* 51, 85–96.

Brewer M.T. and M.G. Milgroom, 2010. Phylogeography and population structure of the grape powdery mildew fungus, *Erysiphe necator*, from diverse *Vitis* species. *BMC Evolutionary Biology* 10, 268; doi 10.1186/1471-2148-10-268.

Carlucci A., M.L. Raimondo, F. Cibelli, A.J.L. Phillips and F. Lops, 2013. *Pleurostomophora richardsiae*, *Neofusicoccum parvum* and *Phaeoacremonium aleophilum* associated with a decline of olives in southern Italy. *Phytopathologia Mediterranea* 52, 517–527.

Cloete M., P.H. Fourie, U. Damm, P.W. Crous and L. Mostert, 2011. Fungi associated with die-back symptoms of apple and pear trees, a possible inoculum source of grapevine trunk disease pathogens. *Phytopathologia Mediterranea* 50, S76–S190.

Crous P.W., W. Gams, M.J. Wingfield and P.S. Van Wyk, 1996. *Phaeoacremonium* gen. nov. associated with wilt and decline diseases of woody hosts and human infections. *Mycologia* 88, 786–796.

Dupont J., S. Magnin, C. Cesari and M. Gatica, 2002. ITS and β-*tubulin* markers help delineate *Phaeoacremonium* species, and the occurrence of *P. parasiticum* in grapevine disease in Argentina. *Mycological Research* 106, 1143–1150.

Edwards J., G. Marchi and I.G. Pascoe, 2001.Young esca in Australia. *Phytopathologia Mediterranea* 40, S303–S310.

Essakhi S., L. Mugnai, P.W. Crous, J.Z. Groenewald and G. Surico, 2008. Molecular and phenotypic characterisation of novel *Phaeoacremonium* species isolated from esca diseased grapevines. *Persoonia* 21, 119–134.

Gramaje D., J. Armengol and H.J. Ridgway, 2013. Genetic and virulence diversity and mating type distribution of *Togninia minima* causing grapevine trunk diseases in Spain. *European Journal of Plant Pathology* 135, 727–743.

Groenewald M., J.C. Kang, P.W. Crous and W. Gams, 2001. ITS and β-*tubulin* phylogeny of *Phaeoacremonium* and *Phaeomoniella* species. *Mycological Research* 105, 651–657.

Hammer Ø., D.A.T. Harper and P.D. Ryan, 2001. PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia electronica* 4, 1–9.

Jones T., N. Federspiel, H. Chibana, J. Dungan, S. Kalman, B. B. Magee, G. Newport, R. Thorstenson, N. Agabian *et al.* 2004. The diploid genome sequence of *Candida albicans*. *Proceedings National Academy of Science USA* 101, 7329–7334.

Larignon P. and B. Dubos, 1997. Fungi associated with esca disease in grapevine. *European Journal of Plant Pathology* 103, 147–157.

Martín L. and M.T. Martín, 2013. Multi-gene sequence analysis and phenotypic diversity of *Phaeoacremonium* species isolated from grapevines in Spain. *European Journal of Plant Pathology* 137, 343–361.

Martín L., L.E. Sáenz de Miera and M.T. Martín, 2014. AFLP and RAPD characterization of *Phaeoacremonium aleophilum* associated with *Vitis vinifera* decline in Spain. *Journal of Phytopathology* 162, 245–257.

Mohammadi H., Z. Banihashemi, D. Gramaje and J. Armengol, 2013. Fungal pathogens associated with grapevine trunk diseases in Iran. *Journal of Agricultural Science and Technology* 15, 137–150.

Mostert L., P.W. Crous, J.Z. Groenewald, W. Gams and R. Summerbell, 2003. *Togninia* (Calosphaeriales) is confirmed as teleomorph of *Phaeoacremonium* by means of morphology, sexual compatibility, and DNA phylogeny. *Mycologia* 95, 646–659.

Mostert L., F. Halleen, P. Fourie and P.W. Crous, 2006a. A review of *Phaeoacremonium* species involved in Petri disease and esca of grapevine. *Phytopathologia Mediterranea* 45, S12–S29.

Mostert L., J.Z. Groenewald, R.C. Summerbell, W. Gams and P.W. Crous, 2006b. Taxonomy and pathology of *Togninia* (Diaporthales) and its *Phaeoacremonium* anamorphs. *Studies in Mycology* 54, 1–113.

Mugnai L., A. Graniti and G. Surico, 1999. Esca (black measles) and brown wood-streaking: two old and elusive diseases of grapevines. *Plant Disease* 83, 404–416.

Pascoe I.G., J. Edwards, J.H. Cunnington and E. Cottral, 2004. Detection of the *Togninia* teleomorph of *Phaeoacremonium aleophilum* in Australia. *Phytopathologia Mediterranea* 43, 51–58.

Rooney-Latham S., A. Eskalen and W.D. Gubler, 2005a. Teleomorph formation of *Phaeoacremonium aleophilum*, cause of esca and grapevine decline in California. *Plant Disease* 89, 177–184.

Rooney-Latham S., A. Eskalen and W.D. Gubler, 2005b. Occurrence of *Togninia minima* perithecia in esca-affected vineyards in California. *Plant Disease* 89, 867–871.

Scheck H.J., S.J. Vasquez and W.D. Gubler, 1998. First report of three *Phaeoacremonium* spp. causing young grapevine decline in California. *Plant Disease* 82, 590 (abstract).

Tamura K., G. Stecher, D. Peterson, A. Filipski and S. Kumar, 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Molecular Biology and Evolution* 30, 2725–2729.

Tanriverdi S., A. Tanyeli, B. Fikri¸ F. Baslamisli, F. Koksal, Y. Kılınc, X. Feng, G. Batzer, S. Tzipori and G, Widmer, 2002. Detection and genotyping of oocysts of *Cryptosporidium parvum* by Real-Time PCR and melting curve analysis. *Journal of Clinical Microbiology* 40, 3237–3244.

Tegli S., 2000. A hypothesis about the reproductive modes of *Phaeoacremonium aleophilum* and *Phaeomoniella chlamydospora*. *Phytopathologia Mediterranea* 39, 289–298.

Tegli S., E. Santilli, E. Bertelli and G. Surico, 2000. Genetic variation within *Phaeoacremonium aleophilum* and *P. chlamydosporum* in Italy. *Phytopathologia Mediterranea* 39, 125–133.

Travadon R., K. Baumgartner, P.E. Rolshausen, W.D. Gubler, M.R. Sosnowski, P. Lecomte, F. Halleen and J.P. Péros, 2012. Genetic structure of the fungal grapevine pathogen *Eutypa lata* from four continents. *Plant Pathology* 61, 85–95.

Úrbez-Torres J.R., P. Haag, P. Bowen and D.T. O'Gorman, 2014. Grapevine trunk diseases in British Columbia: incidence and characterization of the fungal pathogens associated with esca and Petri diseases of grapevine. *Plant Disease* 98, 469–482.

Widmer G., L. Tchack, S. Tzipori and C.L. Chappell, 1998. Sequence polymorphism in the β-*tubulin* gene reveals heterogeneous and variable population structures in *Cryptosporidium parvum*. *Applied and Environmental Microbiology* 64, 4477–4481.

Xu J., H. Guo and Z-L. Yang, 2007. Single nucleotide polymorphisms in the ectomycorrhizal mushroom *Tricholoma matsutake*. *Microbiology* 153, 2002–2012.

Yeh F.C., R.C Yang, T. Boyle, Z.H. Ye and J.X. Mao, 1997. POPGENE, the user friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Center. University of Alberta, Edmonton, Canada.

Accepted for publication: March 21, 2015
Published online: September 15, 2015