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RESEARCH PAPERS

Multiplex PCR for specific identification and determination of mating type in *Togninia minima* (anamorph *Phaeoacremonium aleophilum*), a causal agent of esca disease of grapevine

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Summary. *Togninia minima* is one of the fungi involved in esca disease of grapevine, worldwide. It has a biallelic heterothallic mating system. A multiplex PCR test was developed that can detect the species as well as the mating type. A *T. minima*-specific primer set, with expected amplicon size of 500 bp, was designed based on *β-tubulin* gene sequences. A previously designed degenerate primer set (NcHMG1 and NcHMG2) was successfully used to amplify a fragment of approximately 300 bp from the *Mat1*-2 gene of *T. minima*. The obtained sequence showed substantial homology to the *Mat1*-2 gene sequences of other related ascomycetes. A more specific primer set, with expected amplicon size of 230 bp, was designed based on the same *Mat1-2* gene sequence. The specificity of the new primer set was verified on DNA extracted from a set of *Phaeoacremonium* and other fungal species frequently occurring on grapevine. Both primer sets were combined in a multiplex PCR for the simultaneous identification and determination of mating types of *T. minima.* A 500 bp amplicon was obtained from all available *T. minima* isolates and none from the other *Phaeoacremonium* spp. A 230 bp amplicon confirmed *T. minima* isolates that have the *Mat1*-2 allele. The species-specific β-tubulin-based primer set served as an internal control to confirm that the PCR reaction with the mating type primer set had worked properly. The efficacy of the multiplex test was evaluated on 31 isolates of *T. minima* from different vineyards in the Azarshahr region (East Azerbaijan province, Iran). Isolates of both mating types were found from the sampled areas; however, Mat1-2 isolates were more frequent than Mat1-1 isolates (19:12). This multiplex PCR assay developed can facilitate rapid screening of mating types in populations of *T. minima***.**

Key words: Molecular monitoring, heterothallic, *β-tubulin* gene, mating type gene, specific primer, sexual reproduction.

Introduction

Petri disease and the whole esca complex are important diseases of grapevines, affecting young and old vines and causing significant economic damage to grapevine industries (Mugnai *et al.*, 1999; Rooney-Latham *et al.*, 2005a; Mostert *et al.*, 2006; Essakhi *et al.*, 2008). Several species of *Phaeoacremonium* (teleomorph: *Togninia* Berl., Calosphaeriales) are associated with Petri disease and esca symptoms on grapevines (Mostert *et al.*, 2006; Damm *et al.*, 2008; Essakhi *et al.*, 2008; Graham *et al.*, 2009; Gramaje *et al.*, 2009, 2012; Arzanlou *et al.*, 2013). However, the chaetothyrialean *Phaeomoniella chlamydospora* (W. Gams, Crous, M.J. Wingf. & Mugnai) Crous & W. Gams is more often associated with typical Petri disease symptoms on grapevines (Mugnai *et al.*, 1999; Edwards and Pascoe, 2004).

Species of *Phaeoacremonium* are known from a wide spectrum of substrates such as woody hosts, larvae and adults of bark beetles, humans with subcutaneous infections and occasionally from soil (Mostert *et al.*, 2005, 2006). However, the majority of species of this genus are commonly associated with decline and dieback symptoms on various woody

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hosts or as endophytes (Mostert *et al.*, 2006; Damm *et al.*, 2008; Essakhi *et al.*, 2008; Graham *et al.*, 2009; Gramaje *et al.*, 2009, 2012). *Phaeoacremonium* spp. occurring on grapevines have been extensively studied. Since the description of *Phaeoacremonium*, the number of species in this genus has increased to 36 (www.mycobank.org), of which 25 were described from grapevines with esca or Petri disease symptoms (Mostert *et al*., 2005, 2006; Essakhi *et al*., 2008; Graham *et al*., 2009; Gramaje *et al*., 2009).

Since January 2013 a pleomorphic fungus may have only one name (Art. 59 of the new ICN, McNeill *et al*., 2012). It is therefore likely that the older generic name *Togninia* will persist for the whole genus. *Togninia minima* (anamorph *Phaeoacremonium aleophilum*) is known as the dominant and most widely distributed species associated with esca and Petri disease of grapevines, followed by *T. parasitica* (Mostert *et al.*, 2006; Essakhi *et al.*, 2008).

Pathogenicity trials have shown that *T. minima* is one of the causal agents in the esca complex diseases in grapevine (Adalat *et al.*, 2000; Gubler *et al.*, 2001; Arzanlou *et al.*, 2013). Several studies have been conducted on the life cycle, epidemiology and genetic diversity of this pathogen (Mugnai *et al.*, 1999; Adalat *et al.*, 2000; Eskalen & Gubler, 2001; Eskalen *et al.*, 2001, 2005; Overton *et al.*, 2004; Mostert *et al.*, 2006). These studies were mainly focused on the spread of *Phaeoacremonium* spp., inoculum sources and portals of entry to host plants. Infected propagation material, infected soils and airborne spores (conidia or ascospores) were considered as the sources for inoculum in vineyards (Adalat *et al.*, 2000; Eskalen & Gubler, 2001; Eskalen *et al.*, 2001, 2005). The presence of airborne propagules for *T. minima* and *T. fraxinopennsylvanica* has been verified in vineyards using spore trapping techniques (Eskalen & Gubler 2001; Eskalen *et al.*, 2005). Studies on the role of ascospores as inoculum sources were controversial until the discovery of the teleomorph connection for *Pm. aleophilum* (as *T. minima*) by Mostert *et al.* (2003). High genetic diversity observed among strains of *T. minima* collected from individual vineyards in different parts of the world indicate ongoing recombination and occurrence of sexuality for this species (Péros *et al.*, 2000; Tegli *et al.*, 2000). Besides *Pm. aleophilum,* teleomorph connections have been established for eleven species in *Togninia* (Eskalen *et al*., 2005; Mostert *et al*., 2005, 2006; Hu et al., 2012). The remaining species are only known from their anamorphs.

A biallelic heterothallic mating system was proposed for *T. minima* based on *in vitro* crossing of field isolates from individual diseased vines on autoclaved cane sections (Mostert *et al.*, 2003). Formation of perithecia for *T. minima* on dead vascular tissues and surface of decayed pruning wounds under field conditions have been reported by Rooney-Latham *et al.* (2005b). The presence of perithecia in vineyards indicates that under favourable conditions ascospores, which are actively released, could be sources of inoculum.

Sexual reproduction together with mutation and natural selection are the main forces driving evolution and speciation (Milgroom, 1996; Turgeon, 1998). In plant pathogenic fungi, sexual reproduction benefits the pathogens by generating variation at the population level by means of meiotic recombination; ascospores with modified genetics will then purge the genome from deleterious mutations (Turgeon, 1998; Zhan *et al.*, 2007; Arzanlou *et al.*, 2010; Bakhshi *et al.*, 2011). Sexual reproduction in heterothallic species like *T. minima* requires the presence of two mating types, Mat1-1 and Mat1-2 (Turgeon, 1998; Waalwijk *et al.*, 2002; Bakhshi *et al.*, 2011). Rapid determination of the mating type of *T. minima* would be useful for assessing the potential of mating within a population in a vineyard and on a wider scale in regions. Currently, determination of mating type alleles in *T. minima* is based on an *in vitro* crossing protocol, which is laborious and requires much incubator space and a long waiting period for the formation of perithecia. The present study was aimed at developing a multiplex PCR test for specific identification and determination of the mating type of *T. minima.*

Materials and methods

Fungal isolation and DNA extraction

The *Phaeoacremonium* isolates and other fungal species commonly associated with grapevine trunk diseases used to test the specificity of the designed primers are listed in Table 1. Genomic DNA was extracted from 8-day-old cultures of isolates grown on 2% malt extract agar (MEA) at 25°C in darkness using the method of Möller *et al.* (1992).

Molecular diagnostics

Part of the *β-tubulin* gene was amplified from *Phaeoacremonium* isolates obtained from grapevines

Table 1. *Togninia / Phaeoacremonium* and other fungal species used to test the specificity of PCR primers.

Fungus	Collection number	Mating identiy	Country of origin	GenBank accession number	
				ITS	β-tubulin
Phaeoacremonium iranianum	CCTU[a]271	—	WA[b], Iran	KF179096	KF179086
Togninia minima	CCTU1251	Mat1-2	WA, Iran	KF179087	KF179078
T. minima	CCTU1255	Mat1-2	WA, Iran	KF179088	KF179079
T. minima	CCTU1256	Mat1-2	WA, Iran	KF179089	KF179080
T. minima	CCTU1257	Mat1-2	WA, Iran	KF179090	KF179081
T. minima	CCTU1260	Mat1-2	WA, Iran	KF179091	KF179082
T. minima	CCTU1261	Mat1-2	WA, Iran	KF179092	KF179083
T. minima	CCTU1266	Mat1-2	WA, Iran	KF179093	KF179084
T. minima	CCTU1269	Mat1-2	WA, Iran	KF179094	KF179085
T. minima	CCTU1290	Mat1-1	Malekan, Iran	—	—
T. minima	CCTU1336	Mat1-1	Shabestar, Iran	—	—
T. fraxinopennsylvanica	CCTU182	—	WA, Iran	KF179095	KF179099
T. krajdenii	CBS 109479	—	Ontario, Canada	—	—
T. parasitica	CBS 109666	—	Alabama, USA	—	—
Acremonium strictum	CCTU176	—	WA, Iran	KF179098	—
Botryosphaeria dothidea	CCTU735	—	WA, Iran	JQ663991	—
Fusarium proliferatum	CCTU101		WA, Iran	KF179097	—
Phaeomoniella chlamydospora	CCTU1294	—	WA*, Iran	—	—

[a] CCTU, Culture Collection of Tabriz University, Iran.
[b] West Azerbaijan province; isolates originated from different vineyards in West Azerbaijan province.

with esca symptoms. Amplification was performed with the T1 (O'Donnell and Cigelnik, 1997) and Bt2b (Glass and Donaldson, 1995) primer pair in a total reaction volume of 12.5 μL, which was composed of 10–15 ng genomic DNA, 1× PCR buffer (Cinnagen), 60 μL of 1 mM dNTPs, 1.5 mM $MgCl_2$, 0.2 pM of each primer, 0.5 μL DMSO and 0.5 U Taq polymerase (Cinnagen). The cycling conditions consisted of 5 min at 96°C for primary denaturation, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 60 s, with a final extension at 72°C for 7 min. The amplicons were sequenced using the BigDye Terminator v3.1 (Applied Biosystems) Cycle Sequencing Kit according to the recommendation of the vendor and analysed on an ABI Prism 3700 (Applied Biosystems). Raw sequence files were edited manually by using SeqMan™II (DNASTAR) and a consensus sequence was computed from the forward and reverse sequence. A megablast nucleotide search analysis was carried out at NCBI's GenBank nucleotide database and sequences with high similarity were downloaded from GenBank and included in alignment files. The sequences were aligned automatically using the ClustalW algorithm implemented in MEGA 5 (Tamura *et al.*, 2011). The alignments were checked by eye and a minimal number of gaps were inserted where necessary for proper alignment. Based on the alignment, a primer pair specific to *T. minima* (PmaleoF/PmaleoR) with expected amplicon size of 500 bp was designed (Table 2). The specificity of this primer set was tested on genomic DNA from the *Phaeoacremonium* and other

Table 2. Primers used to develop a multiplex PCR method for molecular identification and determination of mating type alleles in *Togninia minima*.

Primer name	Sequence (5′ → 3′)	Target	Reference
NcHMG1	CCYCGYCCYCCYAAYGCNTAYAT	Mat1-2	Arie *et al.*, 1997
NcHMG2	CGNGGRTTRTARCGRTARTNRGG	Mat1-2	Arie *et al.*, 1997
ChHMG1	AAGGCNCCNCGYCCNATGAAC	Mat1-2	Arie *et al.*, 1997
ChHMG2	CTNGGNGTGTAYTTGTAATTNGG	Mat1-2	Arie *et al.*, 1997
MAT1-1 F	CGCCCTCTKAAYGSCTTCATG	Mat1-1	Kerényi *et al.*, 2004
MAT1-1 R	GGARTARACYTTAGCAATYAGGGC	Mat1-1	Kerényi *et al.*, 2004
PmaleoF	CTCTGCGACGCGTCCCAGATTG	β-tubulin	This study
PmaleoR	TCGCGATGGCCCACTGCCTAC	β-tubulin	This study
PmaleoMat1-2F	CCTATCGTCAAGGCAGCTCATCC	Mat1-2	This study
PmaleoMat1-2R	CTTCTCGTAGTGCTTGCGCTTGC	Mat1-2	This study

fungal species listed in Table 1. The reaction mixture had a total volume of 12.5 μL and contained 1 μL of diluted gDNA (5–20 ng), 1× PCR buffer (CinnaGen), 1.5 mM $MgCl_2$, 40 μM of each dNTP, 1 pmol of each primer, and 0.7 units Taq polymerase. The PCR conditions comprised 40 cycles with annealing at 58°C (30 s). The products were separated by electrophoresis at 80 V for 45 min on a 1% (w:v) agarose gel containing 0.1 μg mL^{-1} ethidium bromide in 1× TAE buffer (0.4 M tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualized under UV-light.

Amplification of mating type genes by PCR

Three previously designed degenerate primer pairs (Arie *et al.*, 1997; Kerényi *et al.*, 2004) were used to amplify a fragment of the mating type genes from *T. minima* isolates (Table 2). The reaction mixture and PCR conditions were the same as described by Arie *et al.* (1997) and Kerényi *et al.* (2004).

Primer design for mating type genes

PCR products from the DNA of *T. minima* isolates using the Mat1-2 degenerate primer set (NcHMG1/NcHMG2), showing the expected product size were cloned into a pGEM-T vector system directly from the PCR reaction, and plasmids were then engineered in *Escherichia coli* strain JM109, following the recommendations of the supplier (Promega). White colonies, carrying the insert, were subjected to PCR amplification using the M13F and M13R universal primer set. For this purpose, ten white colonies were picked up using a sterile inoculation needle and transferred in 10 μL of Luria broth (LB). The PCR reaction was performed in total volume of 12.5 μL containing 1× PCR buffer, 1.5 mM $MgCl_2$, 48 μM dNTPs, 0.2 μM of the standard universal primers pair, 0.7 U of Taq DNA polymerase and 1–2 μL of the recombinant DNA (white colonies in 10 μL of LB). The thermal cycling conditions comprised 40 cycles with annealing at 55°C for 20 s. Amplicons from two isolates were sequenced and analysed using the procedure described above (Molecular diagnostics). Contigs obtained were then analysed using the basic local alignment search tools (BLAST) at NCBI (http://www.ncbi.nlm.nih.gov). From the resulting sequences a primer set was designed for Mat1-2 isolates with an expected amplicon size of 230 bp (Table 2). The specificity of this primer set was tested on genomic DNA from the *Phaeoacremonium* and other fungal species listed in Table 1. The reaction mixture had a total volume of 12.5 μL and contained 1 μL of diluted gDNA (5–20 ng), 1× PCR buffer, 1.5 mM $MgCl_2$, 40 μM of each dNTP, 1 pmol of each primer, and 0.7 units Taq polymerase. The PCR conditions comprised 40 cycles with annealing at 58°C (30 s).

Multiplex PCR assay

The *T. minima*-specific primer set was used in combination with the *Mat1-2* gene-specific primer set for the simultaneous identification of *T. minima* and determination of its mating type. The species-specific primer set served as the internal control for the mating type primer as well, to check whether the mating type primer had worked properly. The PCR amplification was performed under the same conditions as with the *T. minima*-specific primer set.

Validation of the multiplex PCR test

To validate the efficacy of primers developed in this study, we subsequently analysed distribution of mating type alleles among *T. minima* isolates from different vineyards in the Azarshahr region (East Azerbaijan province, Iran). A total of 31 *T. minima* isolates were recovered from 12 vineyards in this region. The identity of *T. minima* isolates as well as mating type allele were determined using the multiplex PCR test.

To further confirm the reliability of the multiplex PCR method developed for determining mating identity of *T. minima* isolates, representative Mat1-1 and Mat1-2 isolates were mated under laboratory conditions following the method described by Mostert *et al.* (2003). In brief, ten *T. minima* isolates (five Mat1-1 and five Mat1-2) were grown on 2% MEA for 12 d. Conidial suspensions were prepared by washing the surfaces of colonies in 5 mL distilled water. Aliquots (100 μL) from the conidial suspension of two opposite mating types were pipetted onto twice-autoclaved pieces of grapevine cane placed on 2% water agar (WA). Plates were incubated at 22°C under continuous white light until the formation and maturation of perithecia. For the controls, 200 μL aliquots of the single isolates were pipetted onto the canes in WA plates.

Results

Molecular diagnostics

Approximately 720 nucleotides were obtained from the *β-tubulin* gene of *Phaeoacremonium* isolates using the T1 and Bt2b primer set. Blast analysis of the sequences revealed the identity of the isolates as *T. minima*, *T. fraxinopennsylvanica* and *Pm. iranianum*. The sequence data generated in this study were deposited in GenBank (Table 1). The alignment of these sequences together with sequence data for other *Phaeoacremonium* spp. from GenBank enabled the design of a *T. minima*-specific primer set (PmaleoF/PmaleoR). This primer set specifically amplified a 500 bp fragment from all *T. minima* isolates. No PCR product was obtained from other *Phaeoacremonium* spp. and other fungal species listed in Table 1 (Figure 1).

Amplification of mating type genes by PCR

From the degenerate primer sets (NcHMG1/NcHMG2; ChHMG1/ChHMG2 and MAT1-1 F/MAT1-1 R) used for the amplification of mating type genes from the DNA of *T. minima* isolates, only the NcHMG1/NcHMG2 primer set amplified a fragment with expected product size (300 bp) from the DNA of six out of eight T. *minima* isolates, besides several other amplicons with different sizes (data not shown). The target sequence for the NcHMG1/NcHMG2 primer set is the conserved region (HMG-box) of the *Mat1-2* gene in ascomycetes. The other primer sets (ChHMG1/ChHMG2 and MAT1-1 F/ MAT1-1 R) tested in this study failed to amplify parts of the mating type region from *T. minima* isolates. The target site for the ChHMG1/ChHMG2 primer set is the conserved region of the *Mat1-2* gene (HMG-

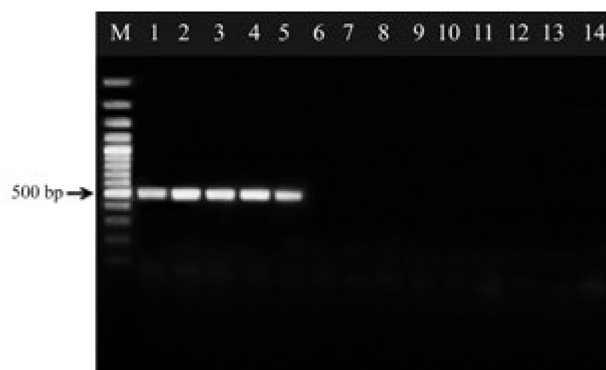

Figure 1. Identification of *Togninia minima* using a species-specific primer set (PmaleoF/PmaleoR) based on sequences of the *β-tubulin* gene. Lane M: 100 bp DNA Ladder; Lanes 1–5, different isolates of *Togninia minima*; Lanes 6, *Pm. iranianum*; 7, *T. fraxinopennsylvanica*; 8, *T. krajdenii*; 9, *T. parasitica*; 10, *Phaeomoniella chlamydospora*; 11, *Acremonium strictum*; 12, *Fusarium proliferatum*; 13, *Botryosphaeria dothidea*; 14, negative control (master mix).

box) in ascomycetes, and for the MAT1-1F/MAT1-1R primer set is the *Mat1-1* gene in *Fusarium* species. The PCR products from the white colonies with approximately 450 bp size (300 bp for the expected product of the mating type gene + 150 bp for the vector) were subsequently purified and sequenced. A directed BLASTX search revealed some homology with the Mat1-2 sequences from *Magnaporthe grisea* (T.T. Hebert) M.E. Barr, showing 78% identity at the amino-acid level (GenBank: AB080671.2). The sequences for the isolates CCTU1255 and CCTU1256 were deposited in GenBank with the accession numbers KF179076 and KF179077.

Primer design for mating type genes

The primer set PmaleoMat1-2F/PmaleoMat1-2R was designed based on the partial sequence of *Mat1-2* gene from the *T. minima* isolates. Melting temperature (Tm) of the reverse and forward primers was set as the same as for the *T. minima*-specific primer set (PmaleoR/PmaleoF), to enable the combination of both primer sets in a single reaction. This primer set successfully amplified a 230 bp fragment from six out of eight *T. minima* isolates tested in this study (Figure 2). The six *T. minima* isolates yielding 230 bp fragments were designated as Mat1-2 and two isolates with no amplicon were designated as Mat1-1. No amplicon was obtained from the other *Phaeoacremonium* species and other fungal species tested (Table 1).

Multiplex PCR

The combination of the *T. minima*-specific primer set (PmaleoR/PmaleoF) together with the PmaleoMat1-2F/PmaleoMat1-2R primers in a single reaction enabled the simultaneous identification of *T. minima* and the determination of the mating type. A 500 bp and a 230 bp sized PCR products were amplified from Mat1-2 isolates of *T. minima*, while for the Mat1-1 isolates only a 500 bp amplicon was present (Figure 3). For all *T. minima* isolates the 500 bp amplicon was always present, and the presence or absence of the 230 bp sized band determined the mating type. No amplicon was obtained from the other *Phaeoacremonium* species and other fungal species tested (Table 1).

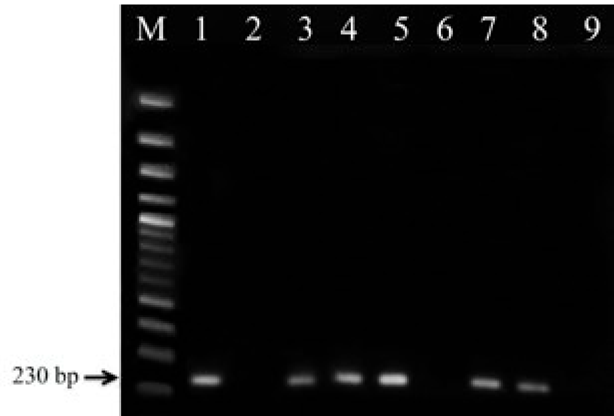

Figure 2. Amplification results with the Mat1-2-specific primer set (PmaleoMat1-2F/PmaleoMat1-2R) using template DNA from *Togninia minima* isolates. Lane M, 100 bp DNA ladder; lanes 1, 3–5, 7, 8, Mat 1–2 isolates; Lanes 2, 6, Mat 1–1 isolates; Lane 9, negative control (master mix).

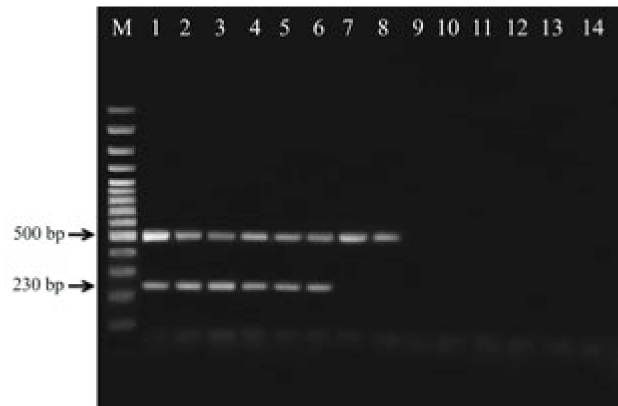

Figure 3. Multiplex PCR using the combination of the *Toninia minima*-specific primer set (PmaleoF/PmaleoR) together with PmaleoMat1-2F/PmaleoMat1-2R for the simultaneous identification of *T. minima* and determination of mating type allele. Lane M, 100 bp DNA ladder; lanes 1–8, the PmaleoF/ PmaleoR primer set amplifies a 500 bp sized band from all *T. minima* isolates; the Mat1-2 primer set amplifies a 230 bp band only from Mat1-2 isolates of *T. minima* (lanes 1–6); Lane 7-8, Mat1-1 isolates of *T. minima* (no amplicon with *Mat1*-2 primer set); Lane 9, *Pm. iranianum*; Lane 10, *T. fraxinopennsylvanica*; 11, *T. krajdenii*; Lane 12, *T. parasitica*; Lane 13, *Phaeomoniella chlamydospora*; Lane 14, negative control (master mix).

Validation of multiplex PCR test

The efficacy of the multiplex PCR assay was evaluated on a set of 31 *T. minima* from 12 different vineyards in the Azarshahr region in East Azerbaijan province, Iran (Table 3). The amplification results revealed that both mating types were present among the 31 *T. minima* tested in this study. However, the distribution of mating alleles among the isolates was uneven. The 500 bp amplicon, specific to *T. minima*, was amplified from all of the isolates; the 230 bp sized band, specific to *Mat1-2* gene of *T. minima*, was, however, amplified only from 19 out of 31 isolates (Table 3). For the remaining 12 isolates in which the 230 bp sized band was absent, the mating identity was determined as Mat1-1. The distribution of mating type alleles among the isolates tested in this study deviated from the 1:1 ratio; Mat1-2 isolates were more frequent than Mat1-1 (at a ratio of 2:1).

The reliability of the multiplex PCR method developed for determining the mating identity of *T. minima* isolates was further confirmed by crossing representatives from Mat1-1 and Mat1-2 isolates under laboratory conditions. Approximately 3–4 weeks after mating, the *Togninia* teleomorph appeared on the canes and agar surfaces between Mat1-1 and Mat1-2 isolates of *T. minima*. The control plates (self crossings) did not produce perithecia after 2 months of incubation. The morphological characteristics of the *Togninia* teleomorph were in full agreement with the description of *T. minima* provided by Mostert *et al.* (2003).

Table 3. Validation of multiplex PCR method developed for screening of mating types in *Togninia minima* on field isolates collected from 12 vineyards in the Azarshahr region (East Azerbaijan province, Iran).

Vineyard number	Number of isolates	Mat1-1 frequency	Mat1-2 frequency
1	3	1	2
2	2	0	2
3	2	1	1
4	3	2	1
5	2	0	2
6	2	0	2
7	3	1	2
8	3	2	1
9	2	2	0
10	3	0	3
11	2	1	1
12	4	2	2
Total	31	12	19

Discussion

Molecular-based identification tools developed in recent years have alleviated the problems associated with the cultural and morphology-based identification of *Phaeoacremonium* spp. (Tegli *et al.*, 2000; Dupont *et al.*, 2002; Mostert *et al.*, 2006). These problems are mainly associated with the lack of distinctive morphological features among the members of this genus. Molecular-based tools, such as PCR-RFLP and species-specific primers based on protein-coding genes, have been used for the identification of *Phaeoacremonium* spp. (Tegli *et al.*, 2000; Dupont *et al.*, 2002). Mostert *et al.* (2006) developed a multiplex PCR protocol for identification of the 22 *Phaeoacremonium* spp. known at that stage, based on sequences from the *β-tubulin* and *actin* genes. The *β-tubulin* gene has been shown to be useful in molecular diagnostics of plant pathogenic fungal species from pure cultures and even from infected plant specimens (Arzanlou *et al.*, 2007). Since the development of the multiplex PCR assay by Mostert *et al.* (2006), several additional *Phaeoacremonium* species have been described and the number of species in this genus has increased to 36. Hence the possibility of a cross reaction of these primers with newly described *Phaeoacremonium* spp. remains to be tested. There is also a need for designing new primer sets for the molecular identification of the recently described species.

We designed a new primer set (PmaleoF/PmaleoR) based on the sequence data from the *β-tubulin* gene for the specific identification of *T. minima* isolates, which we intended to combine with the *T. minima* Mat1-2-specific primer set in a multiplex assay. The previously designed primer set specific to *T. minima* (Mostert *et al.*, 2006) was not suitable for the multiplex assay. Our PmaleoF/PmaleoR primer set amplified a fragment of 500-bp size only from *T. minima* isolates (Figure 1) and none from other available *Phaeoacremonium* spp. or other fungal species known from grapevine (Table 1).

Togninia minima has a biallelic heterothallic mating system; the determination of mating type identity of individuals was based on an *in vitro* crossing protocol (Mostert *et al.*, 2003). A primer set NcHMG1/NcHMG2 was designed by Arie *et al.* (1997), based on the conserved region (HMG-box) of Mat1-2 mating type gene in *Neurospora crassa* Shear & B.O. Dodge and *Podospora anserina* (Ces. ex Rabenhorst) Niessl. This was successfully used to amplify a conserved part of *Mat1-2* gene from heterothallic pyrenomycetes, namely *Haematonectria haematococca* (Berk. & Broome) Samuels & Nirenberg and *Cryphonectria parasitica* (Murrill) M.E. Barr, as well as the homothallic pyrenomycete *Gaeumannomyces graminis* (Sacc.) Arx & D.L. Olivier (Arie *et al.*, 1997). This primer set has been used by other researchers to amplify the conserved part of the *Mat1-2* gene from *Fusarium* spp. and *Ophiosphaerella korrae* (J. Walker & A.M. Smith) R.A. Shoemaker (Hsiang *et al.*, 2003; Kerényi *et al.*, 2004). This primer set also amplified a fragment of approximately 300 bp from some of the *T. minima* isolates. The PCR products obtained with the NcHMG1/NcHMG2 primer set revealed substantial homology to the *Mat1-2* gene of other ascomycetes at the amino acid level. A more specific primer set (PmaleoMat1-2F/PmaleoMat1-2R) with the expected amplicon size of 230 bp was designed based on the *T. minima Mat1-2* sequence obtained in this study. This primer set amplified a fragment of 230 bp only from some of the *T. minima* isolates (Figure 2), which were of Mat1-2.

Amplification with the other primer sets listed in Table 2 was not successful. The ChHMG1/ChHMG2 primer is based the conserved region (HMG-box) of the *Mat1-2* mating type gene in *Cochliobolus heterostrophus* (Drechsler) Drechsler (Arie *et al.*, 1997). It was not surprising, therefore, that this primer set did not amplify the target sequence from *T. minima.* Groenewald *et al.* (2006) did also not succeed in amplifying the *Mat1-2* mating type gene from *Cercospora* spp. using ChHMG1/ChHMG2. Our efforts to amplify the mating type gene of *Phaeomoniella chlamydospora* were also not successful (M. Arzanlou unpublished data). We tried to amplify a fragment from the conserved region of the *Mat1-1* gene in *T. minima* isolates with the primer set MAT1-1F/ MAT1-1R without success. This primer set had been designed by Kerényi *et al.* (2004) to amplify the *Mat1-1* gene from *Fusarium* spp.

Over the last decade, mating type genes have been cloned and characterized for several groups of fungi using a variety of cloning strategies, including functional complementation, subtractive hybridisation, genomic library screening with heterologous probes, and PCR-based approaches (Arie *et al.*, 1997; Turgeon, 1998; Waalwijk *et al.*, 2002; Groenewald *et al.*, 2006; Arzanlou *et al.*, 2010). With the availability of partial sequences for the *Mat1-2* gene of *T. minima* it will be possible to characterize the *Mat1-2* idiomorph using the chromosome-walking strategy or other molecular techniques, and to subsequently characterize the *Mat1-1* idiomorph by designing primers based on sequences for the region flanking the *Mat1-2* idiomorph. Analysis of mating type loci in closely related species provides insights to the evolutionary plasticity of this unique region of the genome as well as evolutionary history of closely related species (Arzanlou *et al.*, 2010).

The combination of the *T. minima*-specific primer set (PmaleoR/PmaleoF) with the *T. minima* Mat1-2-specific primer set (PmaleoMat1-2F/PmaleoMat1-2R) in a multiplex PCR assay enabled the identification of *T. minima* and its mating type in one step. In the multiplex assay a 500 bp was always present for *T. minima* isolates and the presence or absence of 230-bp amplicon determined the mating identity. Because no amplicon of the *Mat1-1* gene from *T. minima* occurred, the *T. minima*-specific primer set can serve as a positive internal control to check whether PCR with the Mat1-2 primer set has worked properly. Multiplex PCR-based tests have previously been described to allow determination of mating types in plant pathogenic fungi (Waalwijk *et al.*, 2002; Bakhshi *et al.*, 2011). PCR-based tests are more reliable and faster than conventional methods which are based on time-consuming multiple pairings between wide ranges of strains and selected tester strains of the two mating types (Waalwijk *et al.*, 2002; Mostert *et al.*, 2003; Bakhshi *et al.*, 2011).

We further validated the efficacy of the multiplex method developed in this study on 31 *T. minima* isolates collected from 12 different vineyards. The results revealed that both mating types were present among the isolates, with Mat1-2 isolates being prevalent; 19 out of 31 isolates were determined as Mat1-2 and 12 as Mat1-1 (Table 3). Our results agree with the findings of other researchers who have reported co-occurrence of both mating types in individual vineyards and even on one vine (Pascoe *et al.*, 2004; Rooney-Latham *et al.*, 2005b). Perithecia of *T. minima* have been found on dead host vascular tissues and the surface of decayed

pruning wounds under field conditions (Pascoe *et al.*, 2004; Rooney-Latham *et al.*, 2005b). Although both mating types were detected among the *T. minima* isolates examined in the present study, no perithecia of *T. minima* were observed under field conditions in the areas sampled here. Indirect evidence for sexual recombination in populations of *T. minima* is based on the high genetic diversity among strains from individual vineyards, which suggests that active sexuality is occurring in this species (Tegli *et al.*, 2000; Péros *et al.*, 2000; Mostert *et al.*, 2006).

Because *T. minima* is a primary pathogen involved in esca disease of grapevines worldwide, and that multiple species of *Phaeoacremonium* can co-occur on a single vine, the primers designed in this study will facilitate concurrent identification and screening of mating type alleles in *T. minima.* This approach will be useful for population studies, in order to understand the modes of reproduction in different geographical areas and the type of inoculum sources.

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