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Dual labelled probe assays for differentiation of *Botryosphaeria dothidea*, *Neofusicoccum mediterraneum* and *Neofusicoccum parvum*, based on polymorphisms in the MAT1-2-1 gene

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Summary. Botryosphaeriaceous fungi are widespread, and cause serious diseases in many economically important crops. *Botryosphaeria dothidea*, *Neofusicoccum mediterraneum* and *N. parvum* are the most important members of this family in the Mediterranean region. These fungi are frequently isolated from the same host, which together with their extensive and increasing host range necessitates development of rapid and reliable diagnostic tools. Species boundaries within the *Botryosphaeriaceae* have been defined based on phylogenetic analyses of multiple gene sequences, including those of mating type genes. The MAT1-2-1 gene displayed high sequence variability between Botryosphaeriaceous species, so was selected as the target for development of a definitive diagnostic tool. This paper outlines a new and robust molecular tool, composed of three TaqMan assays based on polymorphisms located in the MAT1-2-1 gene of *B. dothidea*, *N. mediterraneum* and *N. parvum*. Each assay differentiated the target species from other *Botryosphaeriaceae*, and from non-target fungi.

Keywords. Diagnosis, mating type genes, molecular marker, real time PCR.

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Fungal pathogens in the *Botryosphaeriaceae* have been reported as the causal agents of severe losses in a wide range of hosts, including agricultural, horticultural and forest plants (Slippers *et al.*, 2007; Vakalounakis *et al.*, 2019; Aiello *et al.*, 2020; Guarnaccia *et al.*, 2020; Batista *et al.*, 2021; Luna *et al.*, 2022; Guarnaccia *et al.*, 2023). Symptoms caused by these pathogens include cankers and dieback in twigs and branches, leaf necroses, blight, and fruit rots (Chen et al., 2014; Marsberg et al., 2017). Important hosts of these pathogens are pistachio (*Pistacia vera* L.), grapevine (*Vitis vinifera*) and citrus. The main species causing high yield losses in Greece and other Mediterranean countries, where pistachio and grapevine are cultivated, are *Botryosphaeria dothidea*, *Diplodia seriata, Neofusicoccum vitifusiforme*, *N. mediterraneum* and *N. parvum* (Lazzizera *et al.*, 2008; Chen *et al.*, 2015; Stempien *et al.*, 2017; Bezerra *et al.*, 2021).

Members of the *Botryosphaeriaceae* are known to reproduce sexually, through ascospores formed in perithecia, and asexually from conidia produced in pycnidia. *Botryosphaeria dothidea* and *N. parvum* are homothallic, bearing the MAT1-1-1 and MAT1-2-1 genes in single strains, whereas *N. vitifusiforme*, and *N. mediterraneum* are heterothallic. For *N. vitifusiforme*, only MAT1-1-1 strains have been reported, whereas for *N. mediterraneum*, strains bearing MAT1-1-1 or MAT1-2-1 genes have been described (Lopes *et al*., 2017; Marsberg *et al*., 2017).

Species differentiation within the *Botryosphaeriaceae* has been mainly based on phylogenetic analyses of multiple gene sequences, such as the internal transcribed spacer region (ITS), the elongation factor 1-alpha, (TEF1-a), the beta tubulin, and the RNA polymerase II subunit (RPB2) (Pavlic *et al*., 2009; Chen *et al*., 2015). Although an RFLP analysis tool based on the ITS region could distinguish the main Botryosphaeriaceous species, this could not differentiate five species, including *N. parvum* (Slippers *et al*., 2007). More rapid molecular tools, such as PCR primers, have been developed for detection of Botryosphaeriaceous species only at the genus level, or for differentiation of *Botryosphaeria dothidea* from *Neofusicoccum* spp. (Ridgway *et al*., 2011; Palavouzis *et al*., 2022). Pathogen phylogenies based on mating type genes were comparable to multigene analyses for species differentiation. Notably, the MAT1-2-1 gene displayed high sequence variability between Botryosphaeriaceous species (Lopes *et al*., 2017).

With many species causing disease in an increasing range of hosts (Vakalounakis *et al*., 2019; Batista *et al*., 2020), rapid and accurate detection at the species level is important for the effective management of these pathogens, including quarantine measures and epidemiological studies. The present study reports development of robust and dual labelled probe molecular tools ("TaqMan" technology), targeting the MAT1-2-1 gene, for differentiation among *B. dothidea*, *N. mediterraneum* (MAT1-2-1 strains) and *N. parvum*, from fungal cultures and *in planta*.

Initially, gene sequences of MAT1-2-1 from *N. mediterraneum*, *N. parvum* and *B. dothidea* retrieved from the NCBI database, and sequences for each species derived from isolates of our own collection (deposited in GenBank under the Accession Numbers OQ632937 for *B. dothidea*, OQ632936 for *N. parvum* and OQ596433 for *N. mediterraneum*), were aligned using Clustal Omega (Madeira *et al*., 2019). Differences between sequences of the three species were detected using the MEGA software (Kumar *et al*., 2018). Based on sequence polymorphisms, dual labelled probes and primers were designed for MAT1-2-1 genes of *B. dothidea* (homothallic), *N. mediterraneum* MAT1-2-1 strain and *N. parvum* (homothallic). Primers were designed so that nucleotide polymorphisms specific for the target sequence were positioned at the 3' end of each primer, and PCR amplicons were smaller than 200 bp. Dual labelled probes were designed to have a Tm 8–10°C higher than the primers with a sequence length of 15–30 bp, avoiding a G nucleotide at the 5' end of the probe so that quenching of the 5' fluorophore was prevented. Nucleotide polymorphisms conferring probe specificity were placed close to the middle of the probe sequence. Primers and probes were screened for self-dimers, heterodimers and hairpins, using primer 3 plus software. To check target specificity, blast search against non-target *Diplodia seriata* MAT 1-2-1 sequences showed low homology with designed primers and probes. Primers and probes were then synthesized by eurofins genomics, labelling the 5' end of all probes (for *B. dothidea*, *N. mediterraneum* and *N. parvum*) with fluorescein (FAM) and the 3' end with the Black Hole Quencher-1 (BHQ1) (Table 1).

Table 1. List of primers and probe sequences for TaqMan qPCR assays, developed for detection and quantification of *Botryosphaeria dothidea, Neofusicoccum mediterraneum* and *Neofusicoccum parvum*, based on polymorphisms in the corresponding MAT1-2-1 genes. Polymorphisms of primers and probes between the different species are indicated with nucleotides letters in bold font.

Primer/probe name	Sequence (5'->3')	Target Gene	Product size (bp)
Botdo2-1_295F	TCGCATCCTCTTCCCTCC**TG**	Botryosphaeria dothidea MAT1-2-1	86
Botdo2-1_380R	AGGCCAAGACCTGCTGA**AGT**		
Tqm-Botdo2-1_316pb	**ATA**C**GTC**GC**ACCC**G**CTCC**CAAC		
Neomed2-1_260F	GTCCGCGCTCCAGTCA**TC**	Neofusicoccum mediterraneum MAT1-2-1	114
Neomed2-1_373R	AGGCTGAGGAGTGGAA**ACC**		
Tqm-Neomed2-1_324pb	CGA**CCC**TCC**AT**GC**TGA**CGG**CG**		
Neopar2-1_292F	CTGACCTTGTCCAGCA**CG**	Neofusicoccum parvum MAT1-2-1	133
Neopar2-1_424F	GCTGAGAAGCCGAA**GGTG**		
Tqm-Neopar2-1_377pb	CGA**ACT**TCC**GC**GC**CA**A**T**GG**TA**TCAAC		

Table 2. Mean Ct values (and standard deviations) for different fungi in three dual labelled probe assays targeting the MAT1-2-1 gene for diagnosis of *Botryosphaeria dothidea, Neofusicoccum mediterraneum* (MAT 1-2-1 strains) and *Neofusicoccum parvum*.

MAT1-2-1 gene target	Template	Mean Ct	St. Dev. (3 reps)
B. dothidea	*B. dothidea* 8 ng μL^{-1}	20.6	0.2
	B. dothidea 2 ng μL^{-1}	21.1	0.2
	B. dothidea 200 pg μL^{-1}	24.6	0.3
	B. dothidea 200 pg μL^{-1} & *Pistacia vera* 8 ng μL^{-1}	23.5	0.1
	B. dothidea 20 pg μL^{-1}	28.1	0.3
	B. dothidea 2 pg μL^{-1}	31.3	0.3
	B. dothidea 200 fg μL^{-1}	35.7	0.3
	N. parvum 8 ng μL^{-1}	35.6	1.1
	N. mediterraneum "58" (MAT1-2-1) 8 ng μL^{-1}	undetermined	
	N. mediterraneum "28" (MAT1-1-1) 8 ng μL^{-1}	undetermined	
	P. chlamydospora 8 ng μL^{-1}	undetermined	
	Diplodia seriata 8 ng μL^{-1}	undetermined	
	Pistacia vera 8 ng μL^{-1}	undetermined / 35.5	NA
N. mediterraneum	*N. mediterraneum* "58" (MAT1-2-1) 8 ng μL^{-1}	21.7	0.1
	N. mediterraneum "28" (MAT1-1-1) 8 ng μL^{-1}	undetermined	
	N. mediterraneum 2 ng μL^{-1}	24.7	0.0
	N. mediterraneum 200 pg μL^{-1}	28.3	0.3
	N. mediterraneum 200 pg μL^{-1} & *Pistacia vera* 8 ng μL^{-1}	26.0	0.3
	N. mediterraneum 20 pg μL^{-1}	31.9	0.3
	N. mediterraneum 2 pg μL^{-1}	35.2	1.2
	B. dothidea 8 ng μL^{-1}	36.5	0.4
	N. parvum 8 ng μL^{-1}	35.8	0.7
	P. chlamydospora 8 ng μL^{-1}	undetermined	
	Diplodia seriata 8 n μL^{-1}	undetermined	
	Pistacia vera 8 ng μL^{-1}	37.5	1.2
N. parvum	*N. parvum* 8 ng μL^{-1}	22.4	0.3
	N. parvum 2 ng μL^{-1}	24.2	0.1
	N. parvum 200 pg μL^{-1}	27.9	0.1
	N. parvum 200 pg μL^{-1} & *Pistacia vera* 8 ng/ μL^{-1}	27.7	1.0
	N. parvum 20 pg μL^{-1}	31.3	0.4
	N. parvum 2 pg μL^{-1}	34.5	0.7
	B. dothidea 8 ng μL^{-1}	36.3	0.5
	N. mediterraneum "58" (MAT1-2-1) 8 ng μL^{-1}	undetermined	
	N. mediterraneum "28" (MAT1-1-1) 8 ng μL^{-1}	undetermined	
	P. chlamydospora 8 ng μL^{-1}	undetermined	
	Diplodia seriata 8 n μL^{-1}	undetermined	
	Pistacia vera 8 ng μL^{-1}	36.0	0.1

NA: not applicable - the target was detected in one replicate.

For the three dual labelled probe assays, the PCR mixture contained 1× KAPA Probe Fast Universal qPCR kit (KK4701) with fungal DNA of different concentrations as template, 300 nM of each of the forward and

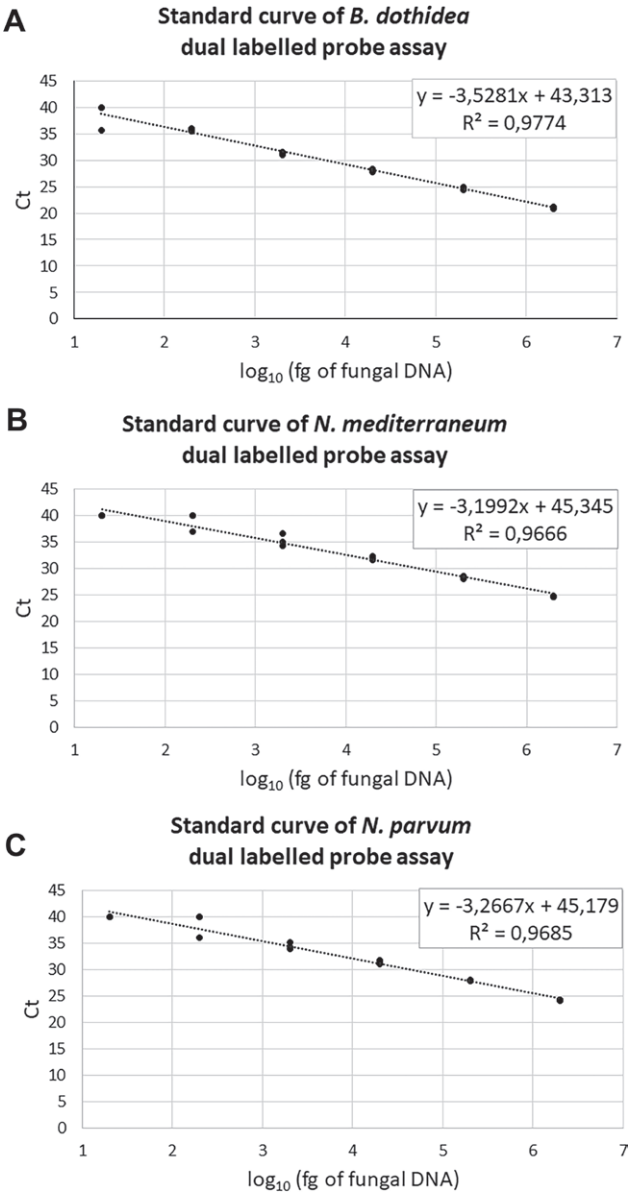

Figure 1. Standard curves for *Botryosphaeria dothidea* (A), *Neofusicoccum mediterraneum* (B) and *N. parvum* (C) specific dual labelled probe (TaqMan) assays. Mean cycle threshold (Ct) values of DNA dilution series for each pathogen were plotted against the log of the DNA concentration (three replicates per concentration of fungal DNA tested). Reactions of undetermined values were set at 40.

reverse primers and probe, and 1× Rox dye. The qPCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 40 cycles each of 3 sec at 95°C and 30 sec at 60°C, without a final extension period. All reactions were performed in a Step One Plus (Applied Biosystems) real time PCR machine cycler.

As a first step, the quality of all genomic DNA samples was assessed using universal primers ITS4 and ITS5. Amplification was performed with KAPA Taq polymerase using the manufacturer's instructions. PCR conditions consisted of initial denaturation at 95°C for 3 min, followed by 35 cycles each of 30 s at 95°C, 30 s at 55°C, and 60 s at 72°C, and a final extension period of 10 min at 72°C. DNA from the following species was used: *B. dothidea*, *N. parvum, N. mediterraneum* MAT1-2-1 strain, while *Diplodia seriata*, *P. chlamydospora* and *N. mediterraneum* MAT1-1-1 strain were included as non-target species.

All three TaqMan assays differentiated the target species from the other two species under study, with no cross-reactions. In particular, the *B. dothidea* dual labelled probe assay reliably detected target DNA up to 2 pg μL^{-1}, at mean Ct 31.3. The *N. mediterraneum* MAT1-2-1 specific assay detected target DNA up to 20 pg μL^{-1}, at mean Ct 31.9. Non-target DNA at 8 ng μL^{-1} from *Diplodia seriata, P. chlamydospora* and *N. mediterraneum* MAT1-1-1 strain was not detected, while *N. parvum* and *B. dothidea* DNA occasionally produced amplification signal at Ct above 35. The *N. parvum* specific assay detected target DNA up to 2 pg μL^{-1} at mean Ct 34.5. Non-target DNA at 8 ng μL^{-1} from *Diplodia seriata*, *P. chlamydospora* or *N. mediterraneum* was not detected, while *B. dothidea* produced a non-specific signal at Ct 36 (Table 2). For all assays, linear relationships were observed between log of DNA concentrations (serial dilution) and Ct values (Figure 1). Target DNA at 200 pg μL^{-1} for all three assays was detected at similar Ct, even if spiked with 8 ng μL^{-1} DNA from pistachio (average Ct = 24 for *B. dothidea* and 27 for *N. mediterraneum* and *N. parvum* assays). Pistachio DNA at 8 ng μL^{-1} was detected over Ct 35 for all assays.

Due to the high number of species in the *Botryosphaeriaceae* causing similar symptoms, molecular tools that enable rapid and accurate detection at the pathogen species level are considered to be important for disease management. The present study developed a diagnostic tool to differentiate among *B. dothidea*, *N. mediterraneum* and *N. parvum*. For detection of *N. mediterraneum*, the assay described here allowed detection only of MAT1-2-1 strains. Another diagnostic tool targeting the MAT1-1-1 gene has yet to be designed. The present study focused on detection of these pathogens because they prevail among species isolated from pistachio, grapevine, citrus (Rumbos and Rumbou, 2001; Vakalounakis *et al.*, 2019; Gusella *et al.*, 2021), and other hosts (including olive, pomegranate, and white willow) (personal communications, Dr Tsopelas; Dr E.J. Paplomatas).

The present study developed three TaqMan assays based on sequence variation of the MAT1-2-1 gene (Lopes *et al.*, 2017) that enabled differentiation between

Table 3. List of MAT1.2.1 sequences (Accession number, fungal strain information, host, origin and reference) from species of *Botryosphaeriaceae*, that were aligned using Clustal Omega, in order to detect polymorphisms unique for each of *Botryosphaeria dothidea, Neofusicoccum mediterraneum* (MAT1-2-1 strains) and *Neofusicoccum parvum* to design species-specific dual labelled probe qPCR assays.

	Fungal strain information	Accession Number	Host	Origin	Reference
1	*Botryosphaeria dothidea*	Genome database*			
2	*Botryosphaeria dothidea* MAT1-2-1	OQ632937	*Pistacia vera*	Greece	This study
3	*Neofusicoccum mediterraneum* MAT1-2-1	OQ596433	*Pistacia vera*	Greece	This study
4	*Neofusicoccum parvum* MAT1-2-1	OQ632936	*Pistacia vera*	Greece	This study
5	*Neofusicoccum mediterraneum* strain CAA001 MAT1-2-1	KX505884.1	*Pistacia vera*	Portugal	Lopes *et al.*, 2017
6	*Neofusicoccum parvum* strain CMW14085 MAT1-2-1	KX766044.1	N.A.	South Africa	Nagel *et al.*, 2018
7	*Neofusicoccum parvum* strain CMW9080 MAT1-2-1	KY612508.1	N.A.	South Africa	Nagel *et al.*, 2018
8	*Neofusicoccum parvum* strain CMW9081 MAT1-2-1	KX505872.1	*Populus nigra*	South Africa	Lopes *et al.*, 2017
9	*Neofusicoccum parvum* strain CBS 110301 MAT1-2-1	KX505873.1	*Populus nigra*	The Netherlands	Lopes *et al.*, 2017
10	*Neofusicoccum eucalyptorum* strain CAA511 MAT1-2-1	KX505881.1	*Eucalyptus globulus*	Portugal	Lopes *et al.*, 2017
11	*Neofusicoccum mangiferae* strain CBS 118531 MAT1-2-1	KX505889.1	*Mangifera indica*	The Netherlands	Lopes *et al.*, 2017
12	*Neofusicoccum luteum* strain CMW9076 MAT1-2-1	KY775143.1	N.A.	South Africa	Nagel *et al.*, 2018
13	*Neofusicoccum algeriense* strain CBS 137504 MAT1-2-1	KX505876.1	*Vitis vinifera*	The Netherlands	Lopes *et al.*, 2017
14	*Neofusicoccum algeriense* strain CAA322 MAT1-2-1	KX505877.1	*Eucalyptus globulus*	Portugal	Nagel *et al.*, 2018
15	*Neofusicoccum cordaticola* strain CMW14124 MAT1-2-1	KX766043.1	N.A.	South Africa	Nagel *et al.*, 2018
16	*Neofusicoccum cordaticola* strain CMW13992 MAT1-2-1	KY612506.1	N.A.	South Africa	Nagel *et al.*, 2018
17	*Neofusicoccum kwambonambiens*e strain CAA755 MAT1-2-1	KX505878.1	*Eucalyptus globulus*	Portugal	Lopes *et al.*, 2017
18	*Neofusicoccum kwambonambiens*e strain CMW14155 MAT1	KX766045.1	N.A.	South Africa	Nagel *et al.*, 2018
19	*Neofusicoccum kwambonambiens*e strain CMW14023 MAT1	KY612507.1	N.A.	South Africa	Nagel *et al.*, 2018
20	*Neofusicoccum ribis* strain CMW7054 MAT1-2-1	KX766041.1	N.A.	South Africa	Nagel *et al.*, 2018
21	*Neofusicoccum ribis* strain CMW7772 MAT1-2-1	KY612509.1	N.A.	South Africa	Nagel *et al.*, 2018
22	*Neofusicoccum ribis strain* CBS 115475 MAT1-2-1	KX505879.1	*Ribes* sp.	The Netherlands	Lopes *et al.*, 2017
23	*Neofusicoccum umdonicola st*rain CMW14106 MAT1-2-1	KX766042.1	N.A.	South Africa	Nagel *et al.*, 2018
24	*Neofusicoccum umdonicola st*rain MAT1-2-1	KY612510.1	N.A.	South Africa	Nagel *et al.*, 2018

N.A.: not applicable.

B. dothidea, *N. mediterraneum* (MAT1-2-1 strains), and *N. parvum*. The developed diagnostic tool is superior to other differentiation methods for *Botryosphaeriaceae*, as it requires no time-consuming steps such as RLFP-PCR (Slippers *et al.*, 2007) or polyacrylamide electrophoresis for SSCP analyses (Ridgway *et al.*, 2011). Furthermore, its practical application will be important, as it is possible to quantify species within infected plant tissues, potentially contributing to studies of pathogen prevalence and species interactions, and epidemiology of the diseases they cause.

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AVAILABILITY OF DATA AND MATERIAL

Sequence data that support part of the findings of this study are available at the GenBank database under the accession numbers listed in Table 3.

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