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SHORT COMMUNICATION

Differentiation between *Botryosphaeria dothidea* and *Neofusicoccum* spp. based on a single nucleotide polymorphism in the ITS region

S. Palavouzis, A. Triantafyllopoulou, A.K. Tzima and E.J. Paplomatas*

Summary Fungi belonging to the Botryosphaeriaceae family are widespread pathogens of many angiosperms, causing disease on various high value crops. The most important members of the family for the Greek region and other Mediterranean countries are *Botryosphaeria dothidea*, *Neofusicoccum hellenicum*, *Neofusicoccum mediterraneum* and *Neofusicoccum parvum*. The frequently concurrent isolation of Botryosphaeriaceae species from the same host, as well as the extensive host range of *B. dothidea*, necessitate the development of rapid and reliable detection methods. This study presents a new and robust molecular diagnostic tool, in the form of a PCR method based on primers designed on an SNP (single nucleotide polymorphism) located in the ITS region (Internal Transcribed Region) of *B. dothidea* and *Neofusicoccum* species. SNP primers constructed with or without added mismatch nucleotides were combined with the same upstream universal primer to generate distinct amplicons. When evaluated in PCR assays, mismatched primers were found to have the highest differentiation capability. The potential for further development of SNP assays in order to differentiate between species is being evaluated.

Additional keywords: diagnosis, internal transcribed spacer region, molecular marker, *Neofusicoccum*, SNPs

Botryosphaeriaceae is a family of economically important phytopathogens, with worldwide distribution (Slippers *et al*., 2007). Members of the family are known to infect a wide range of woody plants, affecting buds, panicles, petioles, rachises, fruit, the mid rib of leaflets, shoots and branches. The pathogens cause fruit rot, dieback, cankers, panicle and shoot blight (Chen *et al*., 2014).

Pistachio (*Pistacia vera* L.) is one of the most important hosts for the species in the Botryosphaeriaceae family. In Greece and other Mediterranean countries, where pistachio cultivation is widespread, the main species responsible for high yield loses are *Botryosphaeria dothidea*, *Diplodia seriata, Neofusicoccum hellenicum*, *Neofusicoccum mediterraneum* and *Neofusicoccum parvum* (Chen *et al*., 2015; Lazzizera *et al*., 2008).

To date, species differentiation within the Botryosphaeriaceae family was based on RFLPs (Slippers *et al*., 2007) or phylogenetic analysis of fingerprinting data (Abdollahzadeh and Zolfaghari, 2014) and gene sequences (Pavlic *et al*., 2009**;** Lopes *et al*., 2017). Only at the genus level, primers have been developed for detection of Botryosphaeriaceous species (Ridgway *et al*., 2011). The ITS region has been a target of choice for the design of species specific primers due to the high sensitivity achieved in single and nested PCR (Flowers *et al*., 2003).

With so many plant pathogenic species causing disease, the ability to rapidly and correctly delimit species and/ or genera is of vital importance for the successful management of these pathogens. At the same time, since some Botryosphaeriaceous species are homothallic, an impeding danger arises regarding genetic recombination that could

Laboratory of Phytopathology, Department of Crop Science, School of Plant Sciences, Agricultural University of Athens, Iera Odos 75, GR-118 55 Athens, Greece.
* Corresponding author: epaplom@aua.gr

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lead to host jumps (Batista *et al*., 2021) or the rapid development of resistance of these fungi to various fungicides. Along these lines, our study presents a robust and quick PCR method for the differentiation between *B. dothidea* and *Neofusicoccum* spp. targeting the ITS region.

Initially, sequences of *N. hellenicum*, *N. mediterraneum*, *N. parvum* and *B. dothidea* were collected from the NCBI database ("National Center for Biotechnology Information (NCBI)[Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information," 1988). Additionally, sequences for each species, derived from isolates of our own collection, were deposited on NCBI and utilized in the study. ITS sequences of *B. dothidea* and *N. hellenicum*, *N. mediterraneum*, *N. parvum* were aligned using Clustal Omega (Madeira *et al*., 2019). Differences between sequences of the species under study were detected using the MEGA software (Kumar *et al*., 2018). Based on sequence similarities, designing of SNP primers for *B. dothidea* and *Neofusicoccum* spp. with the SNP base located at the 3′ end and combined with a common upstream primer, was selected as the preferred strategy.

Downstream SNP primers were designed with a common upstream ITS5 primer. SNP base was detected on the 3′ end (A on *B. dothidea* sequence and T on *Neofusicoccum* sequence) and matching primers were developed (T on BotdoITS_384R primer and A on NeofusITS_384R primer, respectively). However, reproducible and consistent differentiation was proved difficult using these primers. Thus, new primers were designed based on the initial SNP primers. This time ITS3, which is positioned at the 5.8s rRNA, was used as the upstream primer (Table 1).

Based on the data from Gaudet *et al*. (2009), the newly designed downstream SNP primers included a destabilizing mismatch within the 3′ end of the primers. The mismatch was different for each of the downstream SNP primers. In addition, a tail of different length between the two primers was added on the 5′ end, taking care to keep the melting temperature of the primers similar (Fig. 1). Primers were checked for their specificity and characteristics using the software Primer 3 (Untergasser *et al*., 2012) and IDT DNA oligo analyzer (Owczarzy *et al*., 2008).

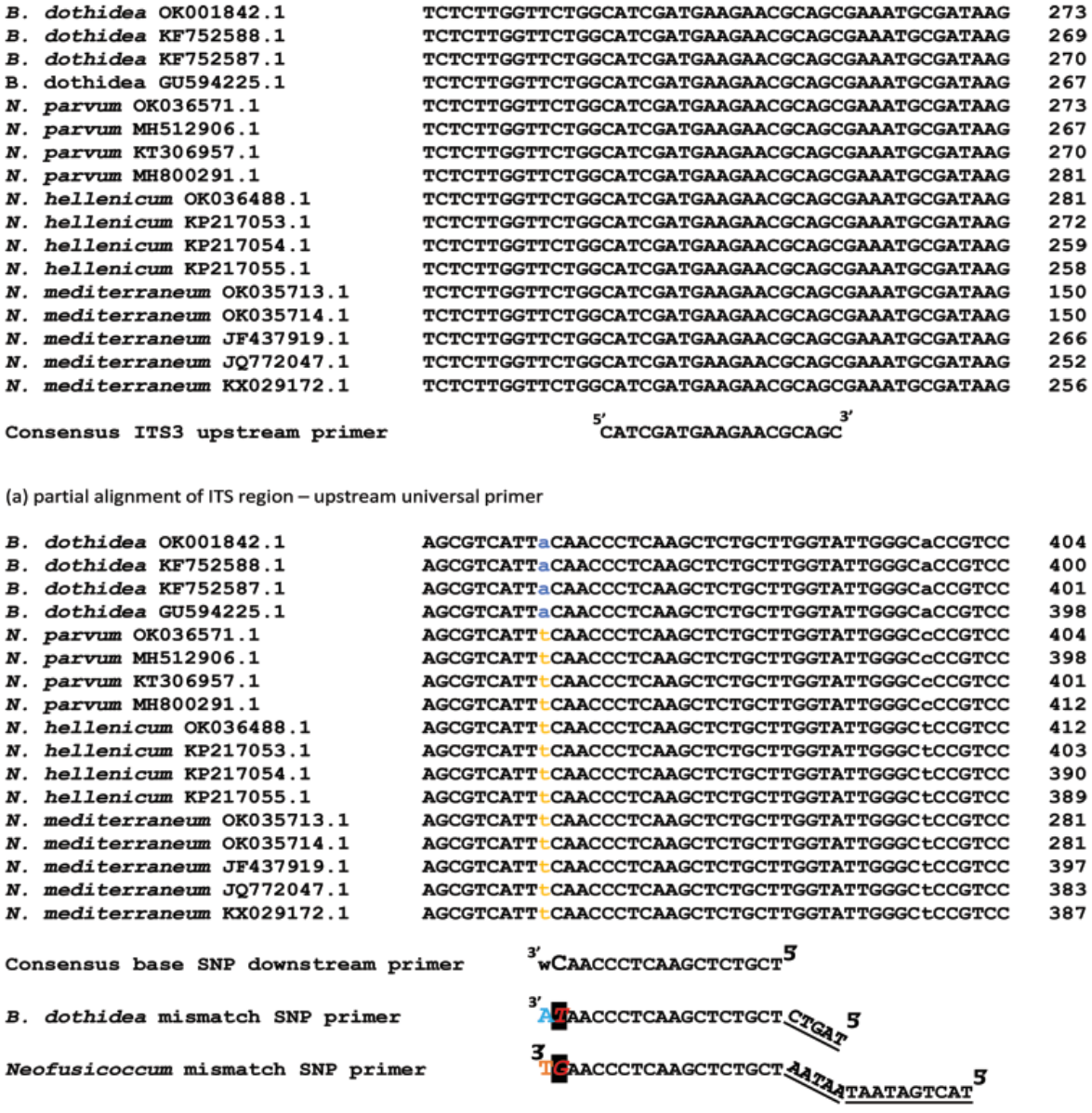
The SNP on the downstream primers selected between the species, was T for *B.*

Table 1. Primer pair sequences utilized in the present study for discrimination of *Botryosphaeria dothidea* and *Neofusicoccum* spp. For primers designed in the present study in the ITS region, the 3′ SNP base is indicated in **bold**, the mismatch base is shown in *italics & underlined* and the 5′ tail sequence in *italics*.

Primer name	Sequence (5′ > 3′)	Description	Reference
ITS4	TCCTCCGCTTATTGATATGC	Universal Downstream primer	White *et al.* (1990)
ITS5	GGAGTAAAAGTCGTAACAAGG	Universal Upstream primer	White *et al.* (1990)
BotdoITS_384R	CAGAGCTTGAGGGTTG**T**	SNP Downstream *B. dothidea* primer	This study
NeofusITS_384R	CAGAGCTTGAGGGTTG**A**	SNP Downstream *Neofusicoccum* primer	This study
ITS3	GCATCGATGAAGAACGCAGC	Universal Upstream primer	White *et al.* (1990)
TL-MS-BotdoITS_386R	*ATCAG*AGCAGAGCTTGAGGGTT*A***T**	SNP + mismatch Downstream *B. dothidea* primer	This study
TL-MS-NeofusITS_386R	*ATGACTATTATTATT*AGCAGAGCTTGAGGGTT*C***A**	SNP + mismatch Downstream *Neofusicoccum* primer	This study

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dothidea primer and A for *Neofusicoccum* spp. primer. In order to improve the specificity of the PCR, a mismatch was added next to the 3′ end. That was A for the *B. dothidea* primer and C for the *Neofusicoccum* primer. The bases used for mismatch were chosen to ensure differentiation efficiency and secondary structure of the primers. To facilitate visual gel separation, a 5 bp tail was added to the 5′ end of *B. dothidea* primer and a 15 tail on the 5′ end of the *Neofusicoccum* spp. primer (Fig. 1). Although the prim-



(a) partial alignment of ITS region – upstream universal primer

Figure 1. Illustrating the design of downstream primers TL-MS-BotdoITS_386R and TL-MS-NeofusITS_386R for selective amplification of *Botryosphaeria dothidea* and *Neofusicoccum* spp., respectively when paired with universal primer ITS3. Isolates from our own collection are indicated with an asterisk. Primer position is indicated on partial alignment of 46 bp of the ITS region of selected *B. dothidea* and *Neofusicoccum* spp. sequences to demonstrate sequence homology and SNPs between *B. dothidea, N. parvum, N. hellenicum* and *N. mediterraneum.* Next to each sequence, the name and genbank accession number is indicated. For downstream primers TL-MS-BotdoITS_386R and TL-MS-NeofusITS_386R, the SNP is indicated in bold letters, the mismatch with red letter in black background, while the 5′ tail added to each primer is shown with underlined letters.

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er pairs in the SNP primer tool (ITS3 / TL-MS-BotdoITS_386R / TL-MS-NeofusITS_386R) have low delta-G primer-dimer values, making their combination in a single reaction theoretically possible, the small difference in the length of generated PCR products limits their use as a differentiating tool in a multiplex reaction.

As a first step, the quality of all genomic DNA samples was assessed using universal primers ITS4 and ITS5. The conditions for the final amplification were as follows: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, 60 s at 72°C, and a final extension period of 10 min at 72°C. The 20 μL PCR mixture contained 1x KAPA PCR buffer with 1.5 mM $MgCl_2$ (Sigma-Aldrich), 200 μM of each dNTP, 500 μM of each primer, 1 U of KAPA Taq polymerase and 25 ng DNA as template.

For the primer pairs ITS5 (5'-GGAGTAAAAGTCGTAACAAGG-3')/BotdoITS_384R (5'-CAGAGCTTGAGGGTTGT-3') and ITS5 / NeofusITS_384R (5'-CAGAGCTTGAGGGTTGA -3') the conditions for the final amplification were as follows: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of 30 s at 95°C, 30 s at 58°C, 40 s at 72°C, and a final extension period of 10 min at 72°C. The 20 μL PCR mixture contained 1x KAPA PCR buffer with 1.5 mM $MgCl_2$ (Sigma-Aldrich), 200 μM of each dNTP, 500 μM of each primer, 1 U of KAPA Taq polymerase and 25 ng DNA as template.

For the primer pairs ITS3 (5'- GCATCGATGAAGAACGCAGC-3')/TL-MS-BotdoITS_386R (5'-ATCAGAGCAGAGCTTGAGGGTTAT-3') and ITS3 / TL-MS-NeofusITS_386R (5'-ATGACTATTATTATTAGCAGAGCTTGAGGGTTCA - 3') the conditions for the final amplification were as follows: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of 30 s at 95°C, 30 s at 64°C, 20 s at 72°C, and a final extension period of 10 min at 72°C. Lower annealing temperatures 61, 62, 63°C were also tested, but 64°C was selected due to higher stringency. The 20 μL PCR mixture contained 1x KAPA PCR buffer with 1.5 mM $MgCl_2$ Sigma-Aldrich, 200 μM of each dNTP, 500 μM of each primer, 1 U of KAPA Taq polymerase (Sigma-Aldrich) and 25 ng DNA as template.

For the SNP primers assays, DNA from the following species was used: *B. dothidea*, *N. parvum, N. mediterraneum*, *N. hellenicum,* while *Diplodia seriata* was included as an outgroup. DNA from *Colletotrichum acutatum* and *Verticillium dahliae* were utilized as negative controls.

All reactions were performed in a Veriti (Applied Biosystems) PCR machine cycler. After amplification 10 μL aliquots from each PCR product were separated by gel electrophoresis in 1% agarose for ITS5/ BotdoITS_384R and ITS5 / NeofusITS_384R PCR amplicons and in 2.5% agarose for ITS3 / TL-MS-BotdoITS_386R and ITS3/ TL-MS-NeofusITS_386R PCR amplicons in 1x TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA, pH 8) at 5 V / cm for 100 min. Gels were stained with Ethidium Bromide (1 μg / ml) for 20 min and visualized on a gel documentation system (Lumibis, DNR bio imaging systems).

For the ITS5 / BotdoITS_384R and ITS5 / NeofusITS_384R primer pairs, which have only one 3' SNP base as discriminating factor, annealing temperatures 58-61°C were chosen. However, at 58°C there was cross-species amplification and above 58°C only primer dimers or lack of product. Also, the results from each PCR were not easily reproducible. PCR with primers *B. dothidea* ITS5/ BotdoITS_384R amplified the expected fragment in *Botryosphaeria. dothidea* but also in *Neofusicoccum* spp. and *Diplodia. seriata* (Fig. 2a). In addition, PCR with *Neofusicoccum* spp. primers ITS5/ NeofusITS_384R amplified the expected fragment in all species, including non – target fungi, *C. acutatum*, *V. dahliae* (Fig. 2b). In conclusion, the single base 3' SNP primers did not differentiate efficiently and repeatedly *B. dothidea* from *Neofusicoccum* species.

For the ITS3/ TL-MS-BotdoITS_386R and ITS3/ TL-MS-NeofusITS_386R primer pairs, annealing temperatures 61 - 64°C were chosen. In all temperatures that were tested, the primers successfully differentiated between *B. dothidea* and *Neofusicoccum* spp., with *B. dothidea* SNP primers amplifying only *B.*

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dothidea DNA (Fig. 3a) and *Neofusicoccum* spp. SNP primers amplifying *Neofusicoccum* species only (Fig. 3b). An exception was DNA from *D. seriata,* which showed amplification using primers for *B. dothidea*. That can be explained by *B. dothidea* and *D. seriata* being homologous on the base sequences of the SNP primer. However, this confusion can easily be resolved, based on morphological differences of *D. seriata* conidia size, shape and color from *B. dothidea*. Therefore, the PCR differentiation will be needed only if *B. dothidea* is involved in the analysis. Based on the different length of the 5′ tail in SNP mismatch primers, the product for primer pair ITS3/ TL-MS-BotdoITS_386R was 150 bp while for primer pair ITS3/ TL-MS_NeofusITS 160 bp (Fig. 3).

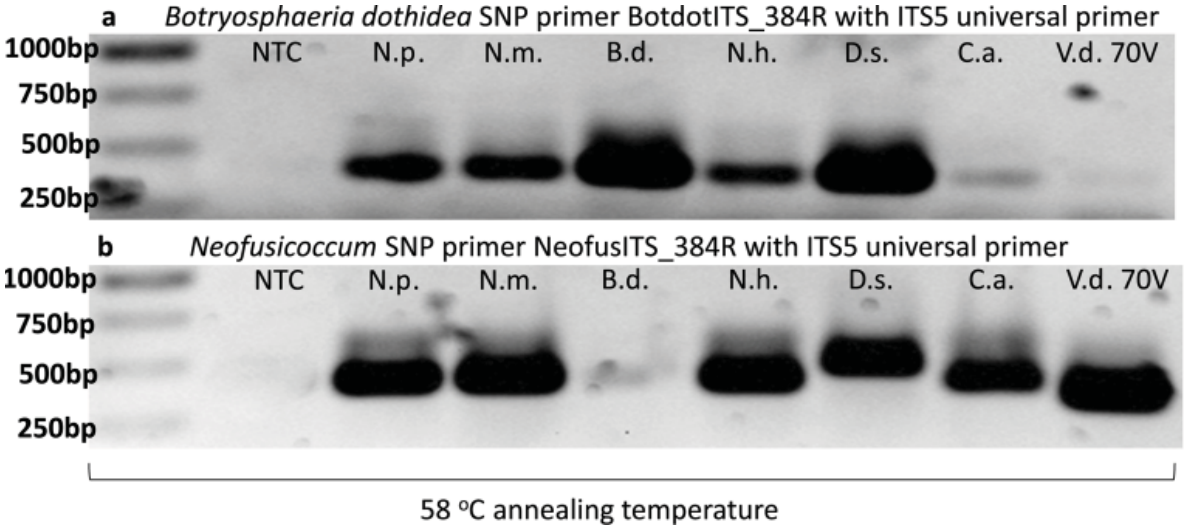


Figure 2. PCR with downstream primers bearing a single 3′ SNP paired with the ITS5 primer and annealing temperature 58°C did not discriminate between *Botryosphaeria dothidea* and *Neofusicoccum* spp. a) PCR with primers ITS5/ BotdoITS_384R amplified the expected fragment in *B. dothidea* (B.d), but also in *Neofusicoccum* spp. (N.p, N.m. N.h.) and D. seriata (D.s.). b) PCR with primers ITS5/ NeofusITS_384R amplified the expected fragment in all species, including non-target fungi. Abbreviations: N.p.=*Neofusicoccum parvum*, N.m.=*N. mediterraneum*, B.d.=*Botryosphaeria dothidea*, N.h.=*N. hellenicum*, D.s.=*Diplodia seriata*, C.a.=*Colletotrichum acutatum*, V.d. 70V=*Verticillium dahliae* strain 70V. Electrophoresis of PCR products was performed in 1% agarose gel.

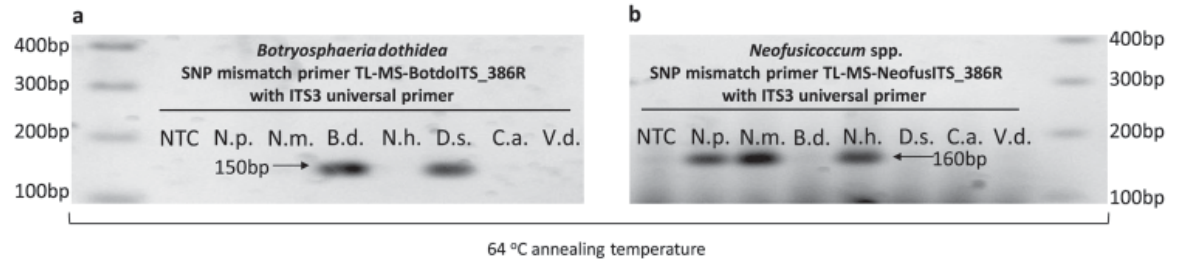


Figure 3. Successful discrimination between *B. dothidea* and *Neofusicoccum* spp. using downstream primers bearing a 3′ SNP plus mismatch and with optimization of the annealing temperature at 64°C. a) PCR with primer pair ITS3/ TL-MS-BotdoITS_386R showed amplification of the expected fragment only for *B. dothidea* (B.d) and *D. seriata* (D.s.), b) PCR with primer pair ITS3/ TL-MS_NeofusITS_386R amplified the expected fragment specifically in *Neofusicoccum* spp. (N.p., N.m., N.h.). Abbreviations: N.p.=*Neofusicoccum parvum*, N.m.=*N. mediterraneum*, B.d.=*Botryosphaeria dothidea*, N.h.=*N. hellenicum*, D.s.=*Diplodia seriata*, C.a.=*Colletotrichum acutatum*, V.d. =*Verticillium dahliae* strain 70V. Electrophoresis was performed on 2.5% agarose gel.

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Based on the plethora of species in the Botryosphaeriaceae family contributing to the same disease complex, molecular tools that enable rapid and accurate detection and differentiation, are deemed critically important. In this study, a novel, robust diagnostic tool was developed, to detect and differentiate between species of *B. dothidea* and three species of the genus *Neofusicoccum* (*N. mediterraneum*, *N. hellenicum*, *N. parvum*). These pathogens were selected as the focal point of our research because they prevail among species isolated from pistachio and other hosts (olive, pomegranate, white willow etc.) in Greece (personal communication with Dr. Tsopelas; E.J. Paplomatas, data not shown.). Furthermore, *B. dothidea* by itself is deemed critically important, since it emerges as the most widespread pathogen of the Botryosphaeriaceae family, with a documented presence in more than 24 genera of host plants, many of them of economic significance, such as pistachio and olive in the Mediterranean basin (Batista *et al*., 2021; Marsberg *et al*., 2016). As such, the ability to rapidly and accurately delimit this species, while bypassing time-consuming traditional taxonomic tools, is of major importance.

As the initial results showed, primers designed with only a 3'SNP base as discriminating factor were not successful in classifying our isolates. On the other hand, SNPs with mismatch next to the 3' end primers were efficient at differentiating these important species. In accordance with previous studies, SNP primers with mismatch base have been found to be more efficient in differentiating species or alleles compared to 3' SNP base only. Their mode of action involves inhibiting or significantly delaying, amplification of the targeted sequence (Carvalho *et al*., 2021). In the present study, the incorporation of the mismatch in the SNP primer, improved specificity and enabled the targeting of the ITS region, which is known to display high sensitivity due to the high copy numbers in genome. The developed one step PCR based protocol for differentiation of Botryosphaeriaceae species is superior to other methods, as it requires no additional steps such as RLFP- PCR (Slippers *et al*., 2007) or polyacrylamide electrophoresis for SSCP analysis (Ridgway *et al*., 2011). Furthermore, its practical application will be of outmost importance, especially in the case of perennial plant crops, which constitute a long-term investment on the producers' side.

Based on the methods evaluated, PCR with SNP primers including a mismatch is a robust and fast track protocol to differentiate between important species in the Botryosphaeriaceae. Further research, into the usage of SNP polymorphisms in the ITS region, or/and other genetic loci, for the differentiation of species in the Botryosphaeriaceae family is required.

Declarations

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Conflicts of interest/Competing interests
The authors declare no conflict of interest.

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: OK001842, OK035713, OK035714, OK036488, OK036571

Code availability
Not applicable

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Διαφοροποίηση μεταξύ *Botryosphaeria dothidea* και ειδών του γένους *Neofusicoccum* με βάση πολυμορφισμό ενός νουκλεοτιδίου στην περιοχή ITS

Σ. Παλαβούζης, Α. Τριανταφυλλοπούλου, Α.Κ. Τζίμα και Ε.Ι. Παπλωματάς

Περίληψη Οι μύκητες που ανήκουν στην οικογένεια Botryosphaeriaceae είναι ευρέως διαδεδομένα παθογόνα πολλών αγγειόσπερμων, προκαλώντας ασθένειες σε διάφορες οικονομικά σημαντικές καλλιέργειες. Τα σημαντικότερα μέλη της οικογένειας για την Ελλάδα και άλλες μεσογειακές χώρες είναι τα *Botryosphaeria dothidea, Neofusicoccum hellenicum, Neofusicoccum mediterraneum* και *Neofusicoccum parvum*. Η μεγάλη συχνότητα ταυτόχρονης απομόνωσης διαφόρων ειδών της οικογένειας Botryosphaeriaceae από τον ίδιο ξενιστή, καθώς και το εκτεταμένο εύρος ξενιστών του *B. dothidea*, καθιστούν αναγκαία την ανάπτυξη ταχέων και αξιόπιστων μεθόδων ανίχνευσης. Η παρούσα μελέτη παρουσιάζει ένα νέο και ισχυρό μοριακό διαγνωστικό εργαλείο που βασίζεται στη μέθοδο της PCR με τη χρήση εκκινητών σχεδιασμένων σε σημεία με πολυμορφισμό απλού νουκλεοτιδίου (SNP) στην περιοχή ITS (εσωτερική μεταγραφόμενη περιοχή) των ειδών *B. dothidea* και *Neofusicoccum* spp. Οι εκκινητές SNP που κατασκευάστηκαν με ή χωρίς πρόσθετα νουκλεοτίδια χωρίς ομολογία συνδυάστηκαν με τον ίδιο ανοδικό γενικό εκκινητή για να ενισχύσουν διακριτά τμήματα. Όταν αξιολογήθηκαν σε αναλύσεις PCR, οι εκκινητές με πρόσθετα νουκλεοτίδια χωρίς ομολογία βρέθηκαν να έχουν την υψηλότερη ικανότητα διαφοροποίησης. Αξιολογείται περαιτέρω η δυνατότητα ανάπτυξης δοκιμών SNP προκειμένου να γίνει διαφοροποίηση μεταξύ των ειδών Neofusicoccum.

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