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Characterization of *Penicillium s.s.* and *Aspergillus* sect. *nigri* causing postharvest rots of pomegranate fruit in Southern Italy

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Abstract

Most of diseases of pomegranate fruit are caused by fungal pathogens, which provoke postharvest yield and economical losses. *Aspergillus* and *Penicillium sensu lato* (*s.l.*) are the main wound pathogens of pomegranate fruit. In the present investigation, the populations of *Aspergillus* and *Penicillium s.l.* isolated from pomegranate fruit in Southern Italy were characterized. Since the morphological identification of species belonging to these genera is laborious, molecular approaches, such as PCR and High-Resolution Melting (HRM), were used. Particularly, a specific primer pair was designed to discriminate, within the *Penicillium s.l.* population, *Penicillium sensu stricto* (*s.s.*) from *Talaromyces* strains. Then, a new HRM assay for species identification within *Penicillium s.s.* according to SNPs present in a portion of the beta-tubulin gene was set up. Similarly, *Aspergillus* sect. *nigri* population was characterized arranging a HRM assay, which primer pair was designed on a portion of the calmodulin gene. According to these assays, 10% of the *Penicillium s.l.* population proved to be made up of *Talaromyces biverticillius* strains. Furthermore, six species of *Penicillium s.s.* (*P. adametzioides, P. brevicompactum, P. citrinum, P. glabrum, P. pagulum,* and *P. johnkrugii*) and four of black aspergilli (*A. tubingensis, A. welwitschiae, A. japonicus,* and *A. uvarum*) were identified; all species belonging to both genera disclosed different incidences in postharvest rotted pomegranate fruit. Moreover, since *Aspergillus* and *Penicillium* are potentially producers of mycotoxins, like ochratoxin A and fumonisins, the presence/absence of genes involved in mycotoxin biosynthetic pathways was tested. Some *Aspergillus* strains belonging to species *A. welwitschiae* proved to possess fumonisin genes. The setup of molecular tools to characterize *Penicillium s.l.* and *Aspergillus* sect. *nigri* species infecting pomegranate fruit after harvest is of paramount importance for their effective control, even more considering the ability of these fungal genera to produce mycotoxins, which are hazardous for human health and potentially present also in by-products.

Keywords: High Resolution Melting, genotyping, *Punica granatum*, blue mould, Aspergillus rot.

1. INTRODUCTION

Because of their cosmeceutical and nutraceutical properties (Diplock, 1999), the consumption and related demand of pomegranate fruit and their by-products have rapidly increased in the last twenty years (Özgüven *et al.*, 2015). Italian weather is favorable to pomegranate cultivation, so that more than 1500 ha are cropped with pomegranates in Italy (Cossio *et al.*, 2017), especially in Apulia and Sicily regions, where 'Wonderful' and 'Akko' are the most cultivated varieties. The high quality of Apulian fruit favored the economic competition with the other Mediterranean producers (Özguven *et al.*, 2015). However, since few and not updated data are available in literature and on official databases, pomegranate production and the related market are currently strongly underestimated.

Since pomegranate fruit are produced in a short period of the year, the adoption of optimal storage conditions is fundamental: for example, fruit could be placed in a micro-perforated plastic bag and organized in a single layer, preferably in cardboard boxes (Arendse, 2014). Moreover, depending on cultivars, a temperature of 7-8 °C, and a relative humidity of 90-95% should allow a storage period waving between 4 and 6 months. This last stage is critical since huge losses, caused mainly by fungal pathogens, may take place (Selcuk and Erkan, 2014). There are two main types of postharvest pathogens: "latent pathogens" and "wound pathogens": the former infect fruit in the field during blooming or fruit development (e.g., *Botrytis* spp. and *Alternaria* spp.), remaining latent until favorable conditions allow their re-activation after harvest; whereas the latter need a wound to penetrate the host, both in the field and after harvest (Palou *et al.*, 2013). *Aspergillus* spp. and *Penicillium* spp. are wound pathogens infecting fruit through pre-existing lesions, injuries, wounds, and other abiotic damages (Bardas *et al.,* 2009; Tziros *et al.*, 2008). Particularly, most of the *Aspergillus* spp. infecting pomegranate fruit belong to section *nigri* (Khokhar *et al.*, 2012; Kanetis *et al.*, 2015; Yehia, 2013). Morphological and molecular identification at the species level of these two genera is quite complicated. Indeed, several species share morphologic and metabolic features. Moreover, although the universal barcoding region ITS is considered enough to distinguish the four phylogenetic complexes, the sequencing of ß-tubulin gene is necessary to obtain the

identification of *Penicillium* at species level, and calmodulin is considered the most reliable gene to identify all the black aspergilli (Samson *et al.*, 2014; Visagie *et al.*, 2014). Moreover, within the genus *Penicillium* some further distinctions should be done. Indeed, a modern concept of *Penicillium sensu lato* (*s.l.*), derived from the pioneering monographic revisions of Thom (1930) and Rapper & Thom (1949), was established and later formalized by the recognition of four subgenera, *Aspergilloides*, *Furcatum*, *Penicillium,* and *Biverticillium* by Pitt (1979). The delineation, species composition, and taxonomic rank of this latter subgenus were modified over time culminating in a study by Malloch (1986), who, based on a consideration of morphological and ecological factors, and anamorph-teleomorph connections, was the first to speculate that this subgenus should be removed from *Penicillium* as a separate genus. Moreover, since *Penicillium* subgenus *Biverticillium* and *Talaromyces* formed a monophyletic clade distinct from the other subgenera of *Penicillium*, they were recombined under the *Talaromyces* genus definition (Samson *et al.*, 2011a). However, *Penicillium sensu stricto* (*s.s.*) species are not easy to be differentiated from those of *Talaromyces* genus according to sole morphology. Moreover, morphological identification at species level is further complicated being *Penicillium* spp. less susceptible to changes in environmental conditions. Finally, concerning the molecular approach, databases show outdated accepted species and lack in verifying deposited strains (Visagie *et al.*, 2014).

A precise identification is fundamental since some species belonging to the genera *Penicillium* and *Aspergillus* produce mycotoxins; for example, some *Penicillium* spp. can produce ochratoxins, patulin, and citrinin, while species of *Aspergillus* can produce ochratoxins, aflatoxins, and fumonisins (Sanzani *et al.*, 2016). The presence of these harmful compounds in pomegranates and their by-products is potentially hazardous for human health. Indeed, these metabolites have several negative effects (cytotoxic, neurotoxic, etc.) so that European Commission regulated their content in food and feed (Regulation 1881/2006/EC; Regulation 401/2006/EC; Directive 2002/32/EC; Recommendation 2006/576/EC). Mycotoxins presence was reported in entire pomegranate fruit (Ammar and El-Naggar, 2014), in fresh arils (Nallathambi and Umamaheswari, 2009), in dried

pomegranate seeds (Iqbal *et al.*, 2018), and in juices (Myresiotis *et al.*, 2015; Rahimi and Jeiran, 2014). Over the last few years, there has been intensive research aimed at developing molecular tools, mainly based on conventional and real-time PCR, for both the characterization and the detection of various genera and species in different plant tissues and environmental samples (Sanzani *et al.*, 2014). However, as far as we know, none of them permitted to carry out the task efficiently and rapidly. For example, a fast and specific assay for the detection and characterization of *Penicillium* spp. and *Aspergillus* spp. of pomegranate fruit would be particularly useful. At this regard, High-Resolution Melting (HRM), a technique already utilized for DNA genotyping of fungal genera and species (Garganese *et al.*, 2018; Sanzani *et al.*, 2013), would be sensitive enough to allow the detection of single nucleotide polymorphisms (SNPs) among otherwise identical nucleotide sequences. In fact, it measures the rate of double-stranded DNA dissociation to single-stranded molecules with increasing temperature. Upon the melting of the amplicon, a dissociation rate relevant to its thermodynamic properties is obtained, which triggers specific changes in fluorescence measurement, so that melting curves can be differentiated even when sharing the same Tm values. This time-efficient methodology facilitates high-throughput analyses, since many samples can be screened simultaneously, it does not require expensive equipment and reagents (e.g., labelled probes), and it is free of post-PCR manipulation or analysis such as, sequencing or fragment detection.

The main aims of this research were: i) the setup of a molecular assay to differentiate mistakable genera *Penicillium* and *Talaromyces*; ii) the identification of *Aspergillus* and *Penicillium* isolates at species level; and iii) the evaluation of the presence/absence of genes involved in the mycotoxin biosynthetic pathways.

2. MATERIALS AND METHODS

2.1 Collection of isolates and morphological identification

Between 2015 and 2017, 155 symptomatic pomegranate fruit, belonging to cultivars Akko, Mollar de Elche, Wonderful, and Wonderful One, were collected. They came from orchards, local markets, and packinghouses in Apulia and Basilicata (Southern Italy), and they were immediately processed upon arrival to the laboratory. According to rot severity and fruit integrity, symptomatic pomegranate fruit were differently surface-sterilized: whole pomegranates were 2-min dipped in a 2% sodium hypochlorite solution, then rinsed for 1 min in sterile distilled water and air dried at room temperature; damaged fruit were disinfected by spraying 70% ethanol and air-dried. Using a sterile scalpel and in aseptic conditions, marginal rotted tissue fragments were plated on semi-selective PDA (Potato Dextrose Agar) (Conda, Madrid, Spain) amended with 250 mg/L of both streptomycin and ampicillin (Sigma-Aldrich, St. Louis, USA). Finally, for seriously compromised fruit, spores arising from the epidermis were directly plated in sterile conditions on semi-selective PDA. All plates were incubated in the dark at 24±1°C for 5 days. Observing the macro- and micro-morphological characteristics of each colony according to Barnett and Hunter (1999), 90 and 25 colonies belonging to *Aspergillus* and *Penicillium sensu lato* (*Penicillium* s.*l.*) genera, respectively, were preliminarily identified; they were sub-cultured onto new PDA plates to obtain pure cultures. The monoconidial form of each isolate was deposited in the fungal collection of the Department of Soil, Plant, and Food Sciences (DISSPA), University of Bari Aldo Moro, Italy.

Moreover, for the morphological identification, *Penicillium s.l.* isolates were transferred onto MEA (Malt Extract Agar, Conda) and G25N (25% glycerol nitrate agar, Pitt, 1973) media, and incubated in the dark at 25 or 35±1 °C, respectively (Pitt, 1979). Similarly, to observe their morphological features, *Aspergillus* isolates were cultured on MEA (Samson *et al.*, 2007; Visagie *et al.*, 2014). Isolates of both genera were sub-divided into groups according to macro- and micro-morphological features and exudate production (De Vries *et al*., 2016; Samson *et al*., 2014).

2.2 Molecular identification

2.2.1 DNA extractions

Isolates were transferred to PDB (Potato Dextrose Broth, Conda), and cultures were grown at 24±1 °C and 150 rpm in the dark for 7 days. Each culture was aseptically filtered, and the mycelium dried and stored at -20 °C until use. The genomic DNA of each collected isolates was extracted from 100 mg of dried mycelium as reported by Doyle and Doyle (1990). DNA purity and quantity were assessed with a spectrophotometer (Nanodrop 2000, Thermo Scientific, Waltham, MA, USA). All PCR reactions were run in a T100 thermalcycler (Bio-Rad, Hercules, CA, USA). Amplicons were resolved in 1.7% agarose gel in TBE buffer (1×), pre-stained with GelRed® (Biotium, Landing Parkway Fremont, CA, USA), and visualized by Gel Doc™ EZ System (Bio-Rad). All primer pairs used in this study were designed by Primer3 software (http://primer3.ut.ee/) and synthetized by Macrogen Europe (Amsterdam, The Netherlands).

2.2.2 Genus *Penicillium*

2.2.2.1 Penicillium s.l. identification and sequences alignment

To confirm the morphological identification, a portion of ß-tubulin gene of two (when available) *Penicillium s.l.* isolates, randomly selected from each morphological group and potentially belonging to different species, was amplified using primers Bt2a/Bt2b (Glass and Donaldson, 1995; Table 1). PCR mixture (50 µL) contained: 50 ng of DNA, 0.2 µM of each primer, and 1×EmeraldAmp PCR Master Mix (Takara, Clontech, USA), reactions were carried out according to Sanzani *et al*. (2013). Expected bands (about 450 bp in size) were excised from the agarose gel and purified by Isolate II Genomic DNA kit (Bioline, London, UK) according to manufacturer's recommendations. Purified products were sequenced in both directions by Macrogen Europe. The accuracy of the obtained sequences was evaluated by FinchTV software (http://jblseqdat.bioc.cam.ac.uk/gnmweb/download/soft/FinchTV_1.4/doc/). The nucleotide sequences were submitted to the online BLAST search engine of the National Centre for Biotechnology Information (NCBI) and deposited in GenBank (Table 2). All sequences were aligned using the free software MULTALIN (http://npsapbil.ibcp.fr/cgi-

bin/npsa_automat.pl?page=/NPSA/npsa_multalin.html). This allowed to detect the presence of deletions and single nucleotide polymorphisms (SNPs).

2.2.2.2 *Talaromyces* specific primers design

On the basis of the sequencing results and observed variability, the primer pair TALF/TALR (Table 1) able to distinguish *Talaromyces* genus from *Penicillium sensu stricto* (*Penicillium s.s.*) was designed. PCR mixtures were arranged in 25 µL containing: 25 ng of DNA, 0.2 µM of each primer, 2 mM of $MgCl_2$, and 1× Dream Taq Hot Start Green PCR Master Mix (Thermo Fischer Scientific, Milan, Italy). Reactions were run under the following conditions: 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min, and 72 °C for 7 min. The primer pair was initially tested with DNA of sequenced *Penicillium s.s.* and *Talaromyces* strains, and then used to screen the whole *Penicillium s.l.* collection.

2.2.2.3 HRM assay for *Penicillium s.s.* species

The sequence alignment described above, highlighted the presence of enough SNPs to differentiate *Penicillium s.s.* species present in the collection, and thus suitable to design a HRM assay. To this purpose, a degenerated primer pair PPF1/PPR1 was designed (Table 1). Twenty µL reaction mixture, containing 1× SsoFast Eva Green Master Mix (Bio-Rad), 0.2 µM of each primer, and 15 ng of DNA, were run in a CFX96 Touch Real-time PCR Detection System (Bio-Rad). Cycling conditions were: 98 °C for 2 min, followed by 40 cycles of 98 °C for 5 s, 60 °C for 10 s and 72 °C for 10 s. The amount of fluorescence, measured at the end of each cycle, was analyzed via CFX-Manager Software v1.6 (Bio-Rad). Melting curves were obtained at temperatures ranging from 65 to 95 °C. Acquisition was performed every 0.2 °C increase in temperature, with a 10s-step. The Precision Melt analysis software (Bio-Rad) automatically clustered the samples according to their melting profiles. A cut-off genotype confidence percentage (GCP) value ≥95% was set for assigning isolates to an identical genotype. To further confirm the reaction specificity amplicons

were run on 1.5% agarose gel and UV visualized as above reported. Initially, two isolates per species was pre-screened to test the efficiency of the assay. The clustering profile was then confirmed by building-up a phylogenetic tree using the freely available software MEGA6 the Maximum Likelihood method using the Tamura-Nei model (Hall, 2013). Analyses were performed with 500 bootstrap replications. Then, all 90 isolates belonging to *Penicillium s.s.* sub-collection were included in HRM reactions using the specific primer pair.

2.2.2.4 Mycotoxin gene evaluation

To detect the putative ability of collected *Penicillium s.s.* isolates to produce ochratoxin A (OTA), the presence of a biosynthetic gene coding a non-ribosomal peptide synthetase (*nps*) was evaluated. Sequences available in GenBank of *Penicillium carneum* CBS 468.95 (JN097803), *Penicillium aurantiogriseum* CECT 2264 (JN097802), *Penicillium melanoconidium* CBS 109605 (JN097799), *Penicillium verrucosum* CBS 323.92 (JN097798), and *Penicillium nordicum* CBS 110769 (JN097797), reported to produce OTA (Cabañes *et al.*, 2010; Lund and Frisvad, 2003), were aligned and a specific primer pair for this region P-OTAF/P-OTAR was designed. PCR reaction mixture (25 μL) was prepared with 25 ng of DNA, 0.2 μM of each primer, 2 mM of $MgCl_2$, and 1× Dream Taq Hot Start (Thermo Fischer Scientific), and run according to the following conditions: 95 °C for 3 min, 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min. To estimate the presence/absence of this OTA gene in the *Penicillium s.s.* collection, an aliquot of one representative strain per HRM cluster was run on 1.5% agarose gel and UV visualized. To ensure the reliability of the assay, an OTA-producer *P. nordicum* strain (PN1, GenBank Accesion no. MK895710) of the DISSPA collection was used as a positive control.

2.2.3 Genus *Aspergillus*

2.2.3.1 Isolate identification and sequence alignment

The morphological identification of collected *Aspergillus* sect. *nigri* isolates was confirmed amplifying for two random selected isolates of each morphological group, a portion of calmodulin gene (Samson *et al.*, 2014), using primer pair CMD5/CMD6 (Hong *et al.*, 2005; Table 3). PCR mixtures (50 µL) were prepared using Phusion Green High-Fidelity DNA Polymerase (Thermo Fisher Scientific), according to manufacturer's recommendations, 0.2 µM of each primer and 100 ng of DNA and run according to the conditions reported by the authors (Hong *et al.*, 2005). PCR products were resolved, visualized, excised, purified, sequenced, and analyzed as reported above for *Penicillium s.l.* and the nucleotide sequences were deposited in GenBank (Table 4). The alignment of calmodulin sequences, using MULTALIN software, allowed the detection of SNPs and deletions useful for designing HRM primers.

2.2.3.2 HRM primer design

Because of the high number of SNPs and deletions among species recorded for calmodulin gene, it was chosen to design the HRM primer pair HRM-CMDF/HRM-CMDR (Table 3). The HRM reaction mixture and amplification conditions were the same reported above for *Penicillium s.s.* A cut-off GCP value ≥95% was set for assigning isolates to an identical genotype. To further confirm the reaction specificity, amplicons were run on 1.5% agarose gel and UV visualized. Initially, one isolate per species was pre-screened to test the efficiency of the assay. The clustering profile was then confirmed by building-up a phylogenetic tree as reported above. Then, all the 25 *Aspergillus* isolates were included in HRM reactions using the specific primer pair.

2.2.3.3 Mycotoxin gene evaluation

The putative ability of a representative *Aspergillus* strain per each HRM cluster to produce OTA and fumonisins was analyzed arranging PCRs for partial regions of *ota1*, *ota3*, *fum8*, and *fum15* genes (Susca *et al.*, 2016). PCR mixture were arranged in 25 µL containing 25 ng of DNA, 0.2 µM of each primer, 2 mM of $MgCl_2$, and 1× Dream Taq Hot Start (Thermo Fischer Scientific), and

reactions were carried-out according to the authors' conditions (Table 3; Susca *et al.*, 2014, 2016). To estimate the presence/absence of these genes, an amplicon aliquot of all the tested strains was run on 1.5% agarose gel and UV visualized.

3. RESULTS

3.1 *Penicillium s.l.* isolates

Twenty-six percent of fruit collected in this study showed decay symptoms putatively caused by *Penicillium s.l.* They were either just-harvested or from cold storage or shelf life according to standard procedures. Isolations allowed the build-up of a *Penicillium s.l.* collection using monoconidial isolates, which were lately grouped according to their micro- and macro-morphological characteristics. Despite the use of specific media and growth conditions, the discriminating features were not enough to assign each of them with certainty to a particular species. Therefore, a molecular characterization was performed by sequencing a portion of ß-tubulin gene for two representative isolate per group, which were identified as strains of the genera: *Penicillium s.s.,* in particular of the species *P. adametzioides* Abe (ex. Smith), *P. brevicompactum* (Dierckx), *P. citrinum* (Sopp), *P. glabrum* (Wehmer) Westling, *P. pagulum* (Visagie & K. Jacobs), and *P. johnkrugii* (Rivera, Houbraken & Seifert); and *Talaromyces,* in particular of the species *Talaromyces albobiverticillius* (H.M. Hsieh, Y.M. Ju & S.Y. Hsieh) Samson, Yilmaz, Frisvad & Seifert (Table 2). This latter isolate group had 3-8 acerose phialides. The slender aculeate or lanceolate shape of phialides highlighted the symmetrical, biverticillate conidiophores. Conidia were pinkish-white, from globose to sub-globose; in addition, the production of soluble pigments shading from yellowish-orange to purplish-red was observed as a characteristic feature (Yilmaz *et al.*, 2014).

3.1.1 Talaromyces discrimination

Since sequence alignment evidenced a conservation of ß-tubulin gene portion among *Talaromyces* strains, but a difference from *Penicillium s.s.* sufficient to discriminate them, the specific primer pair TALF/TALR was designed (Fig. 1A). A preliminary test including sequenced *Penicillium s.s.* and *Talaromyces* strains confirmed the efficiency of the primer pair in discriminating the two genera in the pomegranate collection (Fig. 1B). According to this assay, 10% of the total *Penicillium s.l.* collection proved to be made up of *T. albobiverticillius* strains, and thus 2.6% of postharvest infections recorded on pomegranate fruit in this study were caused by this fungal pathogen.

3.1.2 *Penicillium s.s.* characterization by HRM

The presence of SNPs (transversions and transitions) and deletions among ß-tubulin sequences of *Penicillium s.s.* species (Fig. 2A) allowed to set up a HRM assay (Table 1), which was employed to characterize all the isolates of the new *Penicillium s.s.* collection, corresponding to 90% of the *Penicillium s.l.* collection. The assay proved to be specific as confirmed by derivative melt plots and gel electrophoresis observation of a single distinct band of about 130 bp (data not shown). The HRM assay was initially tested on sequenced strains (Fig. 2B) and further validated by a phylogenetic tree using a *Penicillium expansum* strain (CBS28197, Genbank accession no. AY674401) (Fig. 2C). Successful amplifications were achieved for all the isolates of the collection. The HRM analysis distributed the 90 strains in 6 independent clusters, readily and consistently resolved (Table 2). Six species of *Penicillium s.s.* were confirmed: *P. adametzioides*, *P. brevicompactum*, *P. citrinum*, *P. glabrum*, *P. pagulum*, and *P. johnkrugii*. The *P. glabrum* cluster included 55 isolates, as confirmed by the presence in the cluster of strains G6 and G17 (Table 2). The second most abundant cluster, including 22 isolates, was that of *P. adametzioides,* as confirmed by the presence of strains G9 and G62; the third one was made up of 9 isolates of *P. brevicompactum,* as confirmed by the presence of strains G7 and G14; the fourth one by 3 isolates of *P. jhonkrugiii,* as confirmed by the presence of strains G1 and G31. One isolate of *P. citrinum*

(G73) and one of *P. pagulum* (G47) clustered separately. Comparing the isolate abundance of each cluster, the *P. glabrum* one included 59% of the *Penicillium s.s.* collection, followed by *P. adametzioides*, and *P. brevicompactum*, which included 25 and 18% of tested fungi, respectively. All the other clusters showed incidence ≤10%.

The primer pair design on OTA biosynthetic gene *nps* of *Penicillium s.s.* (Fig. 3A) gave an amplification with a single distinct band of about 250 bp for the *P. nordicum* strain included as positive control (Fig. 3B); instead none of the other tested strains in the pomegranate collection resulted positive (Fig. 3B).

3.2 *Aspergillus* sect. *nigri*

Nine percent of fruit collected in this study showed decay symptoms putatively caused by *Aspergilli* belonging to the section *nigri.* A collection of monoconidial isolates, which were lately grouped according to their micro- and macro-morphological features, was built up. Despite the use of specific media and growth conditions, their characterization at species level needed to be confirmed by sequencing a portion of calmodulin gene for two representative isolates per group (Table 4). Both approaches agreed in identifying the isolates as *A. tubingensis* Mosseray, *A. welwitschiae* (Bres.) Henn., *A. japonicus* Saito, and *A. uvarum* Perrone, Varga & Kozak (Table 4). Thus, a primer pair was designed accordingly to be used in HRM reactions (Fig. 4A). Primer pair specificity was confirmed by derivative melting curve and gel electrophoresis, which showed a definite band of about 205 bp (data not shown). The HRM assay was initially tested on sequenced strains (Fig. 4B) and further strongly validated by a phylogenetic tree using an *Aspergillus niger* strain (CBS 101700, Genbank accession no. GU195633) as outgroup (Fig. 4C). Then it was used to screen all the isolates belonging to this *Aspergillus* section obtained from pomegranate rotted fruit (Table 4). HRM analysis confirmed the presence of four species of *Aspergillus* sect. *nigri*: *A. tubingensis* (56%), *A. welwitschiae* (36%), *A. japonicus,* and *A. uvarum* (4% each). Particularly, *A. tubingensis*

cluster included 14 isolates, whereas *A. welwitschiae* cluster included 9 isolates. The last two clusters were of only one isolate of *A. japonicus* and one of *A. uvarum* (Table 4).

Concerning the evaluation of the presence of genes involved in the biosynthesis of mycotoxins, *ota1* and *ota3* genes were absent in all the examined strains. Instead, *fum8* and *fum15* genes were present in 7 of the 14 tested strains, and they were all *A. welwitschiae* strains. Furthermore, they showed different pattern: three strains (AS16, AS29 and AS30) had only *fum8* and two (AS15 and AS24) *fum15*, whereas two strains (AS6 and AS23) owned both (Table 5).

4. DISCUSSION

Penicillium s.l. and *Aspergillus* genera are widespread etiological agents of postharvest diseases of pomegranate fruit (Palou *et al.*, 2013), whose infectivity is related mainly to the presence of injuries. In the present investigation, *Penicillium s.l.* caused 26% of pomegranate fruit decays in the sampled fruit, whereas, the incidence of rots caused by black *Aspergilli* was lower (15%). Since these fungi are potentiality mycotoxigenic, the rapid identification at the species level is of high relevance. However, the morphological identification alone is not conclusive in the characterization of *Penicillium* and *Aspergillus* genera. As such, molecular tools, as the sequencing of barcoding genes, were used. Concerning *Penicillium s.l.*, the ß-tubulin was confirmed as barcoding gene for the genus; indeed, it allowed not only species discrimination but also to differentiate *Penicillium s.s.* from the sister genus *Talaromyces* to avoid misidentification. In fact, in the present investigation, some of the blue mould symptoms recorded on harvested pomegranate fruit resulted to be caused by *T. albobiverticillius,* whose pathogenicity to pomegranate fruit was previously proved (Mincuzzi *et al.*, 2017). Frisvad and colleagues (2013) reported the main pigments and extrolites produced by *Talaromyces* spp. Particularly, red pigments (azaphilones, monascorubrin, rubropunctatin, derivative of rubropunctatin, monascorubramine, rubropunctamine, N-glutaryl-rubropunctamine, and PP–V) were produced by *T. atroroseus* and *T. albobiverticillius*, so that, these species could be used to produce food dyes (Venkatachalam *et al.*, 2018). The genus-specific primer pair set up in

this study allowed to screen the *Penicillium s.l.* collection, highlighting that 10% of the collection was made up of *Talaromyces* isolates. The occurrence of this genus as pathogen of pomegranate fruit and other crops might have been underestimated in the past. Reasonably, because of the use of improper barcoding gene or lack specialization of operators, since often the morphological features might not be easily interpreted. The recorded *Talaromyces* isolates belonged to the species *T. albobiverticillius*; although several new species of *Talaromyces* were described, such as *T. heiheensis* (Wang *et al.*, 2017) and *T. purgamentorum* (Yilmaz *et al.*, 2016), none of these species produced mycotoxins.

Penicillium s.s. was frequently reported on pomegranate fruit. According to Palou and colleagues (2010), *P. expansum, P. sclerotiorum*, and *P. glabrum* were pathogenic species for this fruit. The latter was reported also in Greece (Bardas *et al.*, 2009) and Italy (Spadaro *et al.*, 2010). Furthermore, *P. implicatum, P. herquei,* and *P. adametzioides* were also described as etiological agents of postharvest pomegranate fruit rots by various researchers (Khokhar *et al.*, 2013; Labuda *et al.*, 2004; Quaglia *et al.*, 2016; Seema and Sharma, 2009). Labuda and colleagues (2004) highlighted the presence of *Penicillium* spp. on decayed stamens; as necrotic masses, they could represent both nutrients for this saprophyte and inoculum for secondary infection. Probably, stamens are infected primarily in the field by conidia carried by the wind, which survive until optimal conditions help them to germinate (temperature between 10 and 30 °C, and high relative humidity). However, *Penicillium* is generally a wound pathogen and cannot infect fruit without injuries (Munhuweyi *et al.*, 2016). In this study, the high variability of the barcoding gene ß-tubulin allowed designing a HRM assay to screen the *Penicillium s.s.* collection from harvested pomegranate fruit to species-level. *P. glabrum* and *P. adametzioides* were identified as the most abundant *Penicillium s.s.* species on pomegranate fruit also in Southern Italy. The frequency of *P. brevicompactum* was also significant; it is reported as causal agent of postharvest rots and producer of mycophenolic acid, a potent immunosuppressant whose presence in commercially marketed produce is a concern (Overy and Frisvad, 2005). Less frequent was *P. citrinum*, whose importance,

however, was considerable, since it produces several compounds such as citrinin, quinolactacins, and citrinadins (Barbosa *et al.*, 2018; Houbraken *et al.*, 2010). Furthermore, it has been recently synonymized with *P. implicatum* (Houbraken *et al.*, 2011), and this ambiguous nomenclature might have caused an underrating incidence of this pathogen. Citrinin is a mycotoxin detected in foods, often joined with OTA, with nephrotoxic properties (Pascual-Ahuir *et al.*, 2014; Pfohl-Leszkowicz and Manderville, 2007), so that the detection of this fungus is important. *P. pagulum* and *P. jhonkrugiii* are rather new species, so there is not much data available on their pathogenicity (Visagie *et al.*, 2013, 2016). None of the tested species seemed able to produce OTA.

Similarly, isolates of *Aspergillus* sect. *nigri* from pomegranate fruit were characterized. The initial identification aimed to separate species according to micro- and macro-morphological characteristics. However, since the species identification within the section is not easy, a rapid, cheap and effective assay based on molecular identification was proposed in the present investigation. Because of its monophyletic nature, species belonging to *Aspergillus* sect. *nigri* are very close, so just a SNP could be enough to separate two species (Samson *et al.*, 2014, 2017). According to this observation and considering that most of the public sequences deposited in GenBank are not certified, the misidentification risk exists (Samson *et al.*, 2014, 2017). For example, *A. awamori* and *A. niger,* in spite of overlapped morphological characteristics and extrolites profile, were recognized as different species (Perrone *et al.*, 2011). Whereas, later on, Hong and colleagues (2013) argued that testing 38 putative strains of *A. awamori,* 24 were reclassified as *A. niger*, 10 as *A. luchuensis*, 2 as *A. tubingensis,* and 2 as *A. welwitschiae* (ex *A. awamori sensu Perrone*); Yamada *et al.* (2011) obtained similar results. Most of *Aspergillus* spp. are producers of secondary metabolites and extrolites, and some others, as *A. welwitschiae,* of mycotoxins (Perrone *et al.*, 2011; Susca *et al.*, 2016). In the present investigation, a HRM assay, based on the high sequence variability in calmodulin gene, permitted to characterize the population of *Aspergillus* sect. *nigri* recorded on harvested pomegranate fruit in Apulia. *A. tubingensis* and *A. welwitschiae* were the most abundant species, followed by *A. japonicus* and *A. uvarum.* These latter

are uniseriate *Aspergillus* spp. morphologically very close to each other, although with a different growth pattern and extrolites profile, thus belonging to the same monophyletic clade (Perrone *et al.*, 2008). *A. japonicus* produces festuclavine and cycloclavin, which are ergot alkaloids of unknown toxicity; instead, *A. uvarum* produces secalonic acids D and F, asterric acid, erdin, geodin, and dehydrogeodin. Secalonic acid D is a mycotoxin (Ehrlich *et al.*, 1982) with cytotoxic and anticancer applications (Zhang *et al.*, 2009); secalonic acid F shows allelochemicals characteristics on higher plants (Zeng *et al.*, 2001). These last two compounds join *A. uvarum* with *A. aculeatus* (Perrone *et al.*, 2008). Asterric acid and geodin are secondary metabolites, typical of *Aspergillus* sect. *terrei*; particularly, the second of these is a well-known mycotoxin (Samson *et al.*, 2011b). The production of asterric acid, erdin, geodin, and dehydrogeodin by *P. glabrum* was noticeable too (Perrone *et al.*, 2008). Confirming the phylogenetic analysis of Perrone and colleagues (2008), *A. uvarum* and *A. japonicus* constituted a well-segregated branch of the phylogenetic tree, although they appeared clearly distinct. Indeed, these species were joined by various deletions, even if the SNPs were different.

Comparing the biseriate branch of *Aspergillus* sect. *nigri*, *A. tubingensis* separated independently from *A. welwitschiae*. None of the tested *A. tubingensis* strains showed the presence of OTA and fumonisin biosynthetic genes, whereas 78% of the tested *A. welwitschiae* strains possessed *fum8* and/or *fum15*. These findings are in agreement with those by Perrone (2005), who indicated that the species closer to *A. niger* had a higher incidence of isolates producing mycotoxins. Similar data were showed by Medina et al. (2005), but because of the molecular characterization based only on ITS region (not barcoding for the genus), the identification could be doubtful. The OTA production in black aspergilli is common, but only two species can produce both ochratoxins and fumonisins, these are *A. niger* and its sister-group *A. welwitschiae* (Susca *et al.*, 2016). In our study, *A. welwitschiae* strains proved to potentially produce fumonisins. For example, *A. welwittschiae* AS6, having both *fum8* and *fum15*, proved to produce 17.73 μg/L and 28,518.75 μg/L of fumonisin B1 (FB1) and B2 (FB2), respectively (T. Gallone, personal communication). Since a regulatory limit

for mycotoxins in pomegranate products and by-products is missing, the threshold for fumonisins in corn, which is 4,000 μg/L (CE 1126/07), could be suggested. Susca and colleagues (2016) supported the hypothesis of host-specific production of mycotoxins. OTA can cause hepato- and nephrotoxicity (Reddy and Bhoola, 2010), and fumonisins are probably teratogenic (Voss and Riley, 2013). OTA and FB1 are grouped in the "possible human cancerogenicity" class (2B) according to IARC classification.

5. CONCLUSIONS

Based on obtained results *T. albobiverticillius* can be considered an important postharvest pathogen of pomegranate fruit, which in the past could have been misidentified with *Penicillium s.s.* genus. *P. glabrum* is the most abundant species within *Penicillium s.s.*, followed by *P. adametzioides* and *P. brevicompactum; P. citrinum* (syn. *P. implicatum*), *P. johnkrugii,* and *P. pagulum* existed occasionally. Specific *Talaromyces* primer pair and *Penicillium s.s.* HRM assay set up herein might allow the rapid, cheap, and efficient identification of *Penicillium s.s.* at the species level, which is particularly useful considering that several of these species produce toxic metabolites and that their identification with traditional approaches is often inconclusive. Similarly, a HRM assay for *Aspergillus* sect. *nigri* species from pomegranate harvested fruit was set up; it stated the absence of *A. niger*, and the abundance of *A. tubingensis* and *A. welwitschiae*. These findings could be ascribed to the recent updates in *Aspergillus* molecular characterization provided by Visagie *et al.* (2014), so that strains of *A. niger* reported in previous studies on pomegranate fruit were reasonably partially misidentified. The analyses of mycotoxin gene clusters showed the presence of fumonisin genes in some *A. welwitschiae* strains, while none of the tested isolates had ochratoxin genes. Finally, two uniseriate black aspergilli were recorded: *A. uvarum* and *A. japonicus*.

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Captions to figures

Fig. 1. Discrimination of *Talaromyces albobiverticillius* within *Penicillium s.l.* collection. A) Alignment of a portion of the β-tubulin sequence of selected *Penicillium s.l.* strains. The specific primers used are highlighted in yellow, while asterisks represent the common positions across the alignment. B) TALF/TALR primer pair applied on *Penicillium s.s.* (-) and *Talaromyces* spp. (+). Ladder: pUC19 (Fermentas).

Fig. 2. Characterization of *Penicillium s.s.* species. A) Alignment of a portion of β-tubulin gene of selected *Penicillium s.s.* strains: HRM primers are highlighted in yellow, while asterisks represent the common positions across the alignment. B) Difference normalized graphs produced by the six *Penicillium s.s.* species: *P. adametzioides* (*P.a.*), *P. brevicompactum* (*P.b.*), *P. citrinum* (*P.c.*), *P. glabrum* (*P.g.*), *P. jhonkrugii* (*P.j.*), and *P. pagulum* (*P.p.*), assigning *P.c.* as reference genotype. C) Phylogenetic tree based on the portion of the β-tubulin amplified in the HRM assay. Numbers on nodes represent the maximum likelihood bootstrap percentages. Branch lengths are proportional to the numbers of nucleotide substitutions and are measured using the bar scale (0.05).

Fig. 3. Evaluation of presence/absence of an OTA biosynthetic gene in *Penicillium s.s.* collection. A) Alignment of a portion of OTA gene of selected *Penicillium s.s.* strains. The primers are highlighted in yellow, while asterisks represent the common positions across the alignment. B) POTAF/POTAR primer pair applied on selected *Penicillium s.s.* strains of the collection (-) and reference *Penicillium nordicum* strain (+). Ladder: 100 bp plus (ThermoFisher).

Fig. 4. Characterization of *Aspergillus* sect. *nigri* species. A) Alignment of a portion of calmodulin gene of selected *Aspergillus* sect. *nigri* strains: HRM primers are highlighted in yellow, while asterisks represent the common positions across the alignment. B) Difference normalized graphs produced by some isolates: *A. japonicus* (*A.j.*), *A. tubingensis* (*A.t.*), *A. uvarum* (*A.u.*), and *A. welwitschiae* (*A.w.*) within *Aspergillus* sect. *nigri* collection, assigning *A.w.* as reference genotype. C) Phylogenetic tree based on the portion of the calmodulin amplified in the HRM assay. Numbers on nodes represent the maximum likelihood bootstrap percentages. Branch lengths are proportional to the numbers of nucleotide substitutions and are measured using the bar scale (0.05).

Table 1. *Penicillium s.l.* primers used in the study: name, sequence (5'-3'), amplicon size (bp), source and targeted gene.

Primer name	Sequence	Amplicon size	Source	Gene
Bt2a	GGTAACCAAATCGGTGCTGCTTTC	330	Glass and Donaldson, 1995	beta-tubulin
Bt2b	ACCCTCAGTGTAGTGACCCTTGGC			
TALF	CGAACAATCGAAAAGCAACC	135	This study	
TALR	TACTTTTTTCATGGTCCTTCG			
PPF1	GAGCGYATGAACGTCTACTT	130	This study	
PPR1	ACVAGGACGGCACGGGGAAC			
P-OTAF	TGTACTCGCCACATCCAACT	257	This study	non-ribosomal peptide synthetase
P-OTAR	CACTTTCGGCCAGACAATGT			

Table 2. *Penicillium s.l.* strains used in the study, accession numbers of their ß-tubulin gene sequences deposited in GenBank, cluster, and number of isolates assigned to each cluster.

Strain	Species	Accession no.	Cluster	Isolate no.
G9	*Penicillium adametzioides*	MK895700	3	22
G62	*Penicillium adametzioides*	MK895701		
G7	*Penicillium brevicompactum*	MK895702	6	9
G14	*Penicillium brevicompactum*	MK895703		
G73	*Penicillium citrinum*	MK895704	5	1
G6	*Penicillium glabrum*	MK895705	1	55
G17	*Penicillium glabrum*	MK895706		
G47	*Penicillium pagulum*	MK895707	2	1
G1	*Penicillium johnkrugii*	MK895708	4	3
G31	*Penicillium johnkrugii*	MK895709		

Table 3. *Aspergillus* sect. *nigri* primers used in the study: name, sequence (5'-3'), amplicon size (bp), source and targeted gene

Primer name	Sequence	Amplicon size	Source	Gene
CMD5	CCGAGTACAAGGARGCCTTC	520	Hong *et al.*, 2005	calmodulin
CMD6	CCGATRGAGGTCATRACGTGG			
HRM-CMDF	ATAGGACAAGGATGGCGATG	205	This study	
HRM-CMDR	AGACTCGGAGGGGTTCTGGC			
FUM8F	TTCGTTTGAGTGGTGGCA	651	Susca *et al.*, 2014	fum8
FUM8R	CAACTCCATASTTCWWGRRAGCCT			
FUM15F	CGATTGGTAGCCCGAGGAA	701		fum15
FUM15R	CTTGATATTGCGGAGTGGTCC			
OTA1F	CAATGCCGTCCAACCGTATG	776	Susca *et al.*, 2016	ota1
OTA1R	CCTTCGCCTCGCCCGTAG			
OTA3F	TTAGACAAACTGCGCGAGGA	613		ota3
OTA3R	GCGTCGCTATGCCCAGATA			

Table 4. *Aspergillus* sect. *nigri* strains used in the study, accession numbers of calmodulin gene sequences deposited in GenBank, cluster, and number of isolates assigned to each cluster.

Strain	Species	Accession no.	Cluster	Isolate no.
AS5	*Aspergillus tubingensis*	MK919489	1	14
AS18	*Aspergillus tubingensis*	MK919490		
AS23	*Aspergillus welwitschiae*	MK919491	3	9
AS28	*Aspergillus welwitschiae*	MK919492		
AS3	*Aspergillus japonicus*	MK919488	2	1
AS13	*Aspergillus uvarum*	MK919493	4	1

Table 5. Presence of fumonisin (*fum8* and *fum15*) and ochratoxin A (*ota1* and *ota3*) biosynthetic genes in selected *Aspergilli* sect. *nigri* strains.

Strain	**Species**	***fum8***	***fum15***	***ota1***	***ota3***
AS17	*A. tubingensis*	-	-	-	-
AS18		-	-	-	-
AS19		-	-	-	-
AS6	*A.welwitschiae*	+	+	-	-
AS15		-	+	-	-
AS16		+	-	-	-
AS20		-	-	-	-
AS23		+	+	-	-
AS24		-	+	-	-
AS28		-	-	-	-
AS29		+	-	-	-
AS30		+	-	-	-
AS3	*A. japonicus*	-	-	-	-
AS13	*A. uvarum*	-	-	-	-

A

```
                          130       140       150       160       170       180
                           |         |         |         |         |         |
G1_johnkrugii             GGGCCAGTAAGTA---TCAATTCGCGTGGAAT--TGGGTGATATGAGAATGGCGGTCTGA
G9_adametzioides          TGGACAGTAAGTTC-TTTGATTCGAGTCGATTGGTATATATGGTGGGAATGGCGGTCTGA
G6_glabrum                TGGACAGTGAGTT--TCCAACATCGATGAGAT----TGCGAGGTGGAAATGGCGGTCTGA
G73_citrinum              TGGACAGTAAGTTCTTTCGACAAAGAAGTGTTTAC-GTTTTTTCGAGAATGGCGGTCTGA
G7_brevicompactum         TGGACAGTAAGTGG--ACGACTGTGTTCGAAT-----T--ACGCGTGGATTG-GGTCTGA
G2_talaromyces            CGGAGTGTGAGTAT-TTCGATACGATTCTCGA-----ACAATCGAAAAGCAACCGTCTGA
                           **   ** ***      *                              ******
Prim.cons.                TGGACAGTAAGTTCTTTCGA22CGG3T2GAAT22T223T2ATGTGAGAATGGCGGTCTGA

                          190       200       210       220       230       240
                           |         |         |         |         |         |
G1_johnkrugii             TATTTTTCTT--AGCTTCACTGGCCAGTCCGACCTCCAGCTCGAGCGCATGAACGTCTAC
G9_adametzioides          TATTTTTTTTCTAGCTTCACCGGCCAGTCCGACCTCCAGCTGGAGCGTATGAACGTCTAC
G6_glabrum                TAAATTT--T--AGCGTCAACGAGGCTTCCGACCTCCAGTTGGAGCGCATGAACGTCTAC
G73_citrinum              TATTTTTGGGC-AGCTACAACGGAACCTCCGATCTCCAGCTGGAGCGCATGAACGTCTAC
G7_brevicompactum         GATCTTG-TT--AGGTACAATGGCACCTCCGACCTCCAGCTCGAGCGTATGAACGTCTAC
G2_talaromyces            CAAAATCC----AGTTACAATGGCTCCTCCGACCTTCAGTTGGAGCGTATGAACGTTTAC
                           *   *      **   **  *     ***** ** *** * ***** ******** ***
Prim.cons.                TATTTTTCTTCTAGCT2CAA2GGC2CCTCCGACCTCCAGCTGGAGCG2ATGAACGTCTAC

                          250       260       270       280       290       300
                           |         |         |         |         |         |
G1_johnkrugii             TTCAACCACGTAAGTG-----TGGAATCAAC--ACTCGATACTCGTCAATC-TCATCTAA
G9_adametzioides          TTCAACCACGTAAGTG-----TGGAATTGAT--CCCTCGAGCCATTCGATA-TTGGCTAA
G6_glabrum                TTCAACGAGGTATGTG-----TGGAATTGA---AGTTAGAAGTATAAAGGC-TTCTCTAA
G73_citrinum              TTCACCCACGTAAGTGATAC-TGACCTCAATGCCATTGGAATCATTATCTAACCAACTAT
G7_brevicompactum         TTCAACCATGTGAGTA-CAA-TGATGTGAAGAACTCTGGTTGTGCATTTTC-TCACCTCA
G2_talaromyces            TTCAACGAGGTGCGTCGAACTTGATACCAAA--ACGAAGGACCATGAAAAAAGTACTCAC
                          **** * * **  **      **      *
Prim.cons.                TTCAACCACGTAAGTG23ACTTG2AATCAAT222CTTGGAAC2ATTAAAT2AT2A2CTAA
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B

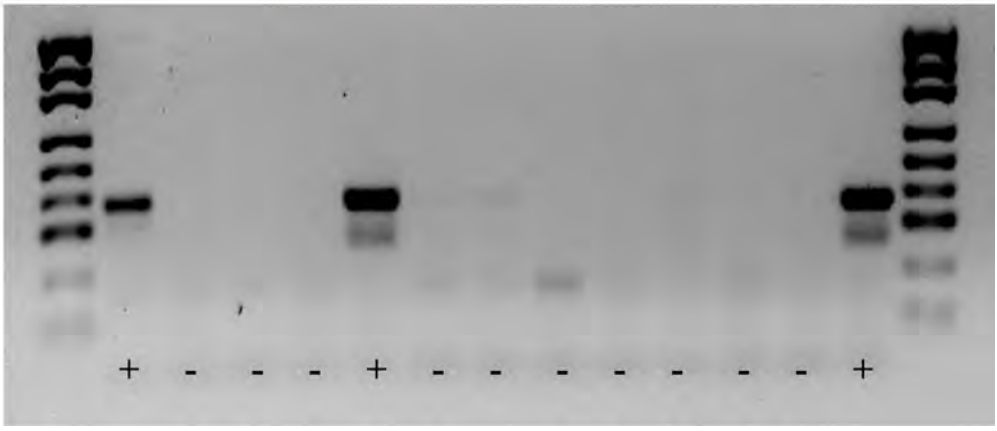
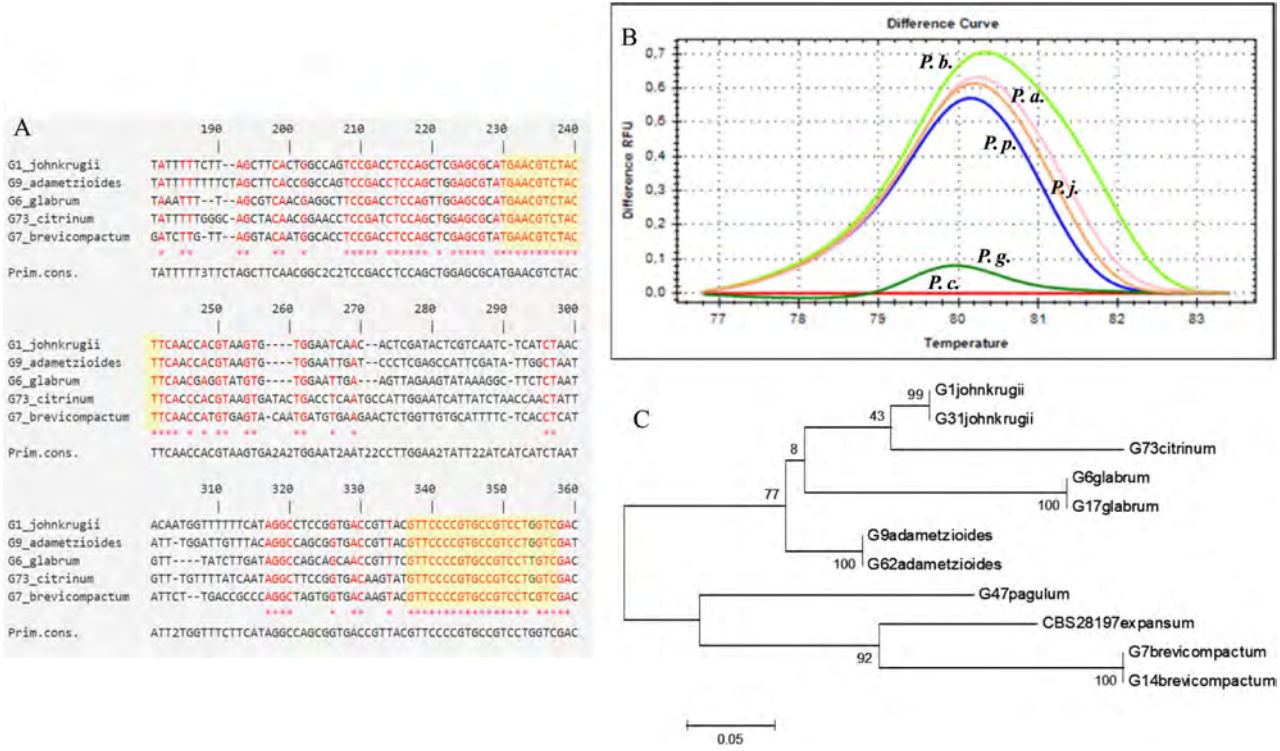


Figure 1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G9_adametzioides TATTTTTTTTCTAGCTTCACCGGCCAGTCCGACCTCCAGCTGGAGCGTATGAACGTCTAC
G6_glabrum TAAATTT--T--AGCGTCAACGAGGCTTCCGACCTCCAGTTGGAGCGCATGAACGTCTAC
G73_citrinum TATTTTTGGGC-AGCTACAACGGAACCTCCGATCTCCAGCTGGAGCGCATGAACGTCTAC
G7_brevicompactum GATCTTG-TT--AGGTACAATGGCACCTCCGACCTCCAGCTCGAGCGTATGAACGTCTAC
Prim.cons. TATTTTT3TTCTAGCTTCAACGGC2C2TCCGACCTCCAGCTGGAGCGCATGAACGTCTAC
250 260 270 280 290 300
G1_johnkrugii TTCAACCACGTAAGTG----TGGAATCAAC--ACTCGATACTCGTCAATC-TCATCTAAC
G9_adametzioides TTCAACCACGTAAGTG----TGGAATTGAT--CCCTCGAGCCATTCGATA-TTGGCTAAT
G6_glabrum TTCAACGAGGTATGTG----TGGAATTGA---AGTTAGAAGTATAAAGGC-TTCTCTAAT
G73_citrinum TTCACCACGTAAGTGATACTGACCTCAATGCCATTGGAATCATTATCTAACCAACTATT
G7_brevicompactum TTCAACCATGTGAGTA-CAATGATGTGAAGAACTCTGGTTGTGCATTTTC-TCACCTCAT
Prim.cons. TTCAACCACGTAAGTGA2A2TGGAAT2AAT22CCTTGGAA2TATT22ATCATCATCTAAT
310 320 330 340 350 360
G1_johnkrugii ACAATGGTTTTTTCATAGGCCTCCGGTGACCGTTACGTTCCCCGTGCCGTCCTGGTCGAC
G9_adametzioides ATT-TGGATTGTTTACAGGCCAGCGGTGACCGTTACGTTCCCCGTGCCGTCCTGGTCGAT
G6_glabrum GTT----TATCTTGATAGGCCAGCAGCAACCGTTTCGTTCCCCGTGCCGTCCTTGTCGAC
G73_citrinum GTT-TGTTTTATCAATAGGCTTCCGGTGACAAGTATGTTCCCCGTGCCGTCCTGGTCGAC
G7_brevicompactum ATTCT--TGACCGCCCAGGCTAGTGGTGACAAGTACGTTCCCCGTGCCGTCCTCGTCGAC
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B
Difference Curve
Difference RFU
Temperature
P. b.
P. a.
P. p.
P. j.
P. g.
P. c.
C
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G73citrinum
G6glabrum
G17glabrum
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G47pagulum
CBS28197expansum
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Figure 2

A

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nordicumCBS110769             TTGTACTCGCCACATCCAACTATCCCCAACCTGTGGAAGTTCCATGGTCGTTTGGACGAT
aurantiogriseum               TTGTACTCGCCACATCCAACTATCCCCAACCTGTGGAAGTTCCATGGTCGTTTGGACGAT
melanoconidiumCBS109605       TTGTACTCGCCACATCCAACTATCCCCAACCTGTGGAAGTTCCATGGTCGTTTGGACGAT
viridicatumCECT2320           TTGTACTCGCCACATCCAACTATCCCCAACCTGTGGAAGTTCCATGGTCGTTTGGACGAT
carneumCBS46895               TTGTACTCGCCACATCCAACTATCCCCAACCTGTGGAAGTTCCATGGTCGTTTGGACGAT
                              ***************************************************************
Prim.cons.                    TTGTACTCGCCACATCCAACTATCCCCAACCTGTGGAAGTTCCATGGTCGTTTGGACGAT

                              430       440       450       460       470       480
                               |         |         |         |         |         |
verrucosumCBS32392            GTGATTGTGCTTAATAATGGCGAGAAGTTCAACCCAGTCACAATGGAGAAGGTTATTGAG
nordicumCBS110769             GTGATTGTGCTTAATAATGGCGAGAAGTTCAACCCAGTCACAATGGAGAAGGTTATTGAG
aurantiogriseum               GTGATTGTGCTTAATAATGGCGAGAAGTTCAACCCAGTCACAATGGAGAAGGTTATTGAG
melanoconidiumCBS109605       GTGATTGTGCTTAATAATGGCGAGAAGTTCAACCCAGTCACAATGGAGAAGGTTATTGAG
viridicatumCECT2320           GTGATTGTGCTTAATAATGGCGAGAAGTTCAACCCAGTCACAATGGAGAAGGTTATTGAG
carneumCBS46895               GTGATTGTGCTCAATAATGGCGAGAAGTTCAACCCAGTCACAATGGAGAAGGTTATTGAG
                              ********** ***************************************************
Prim.cons.                    GTGATTGTGCTTAATAATGGCGAGAAGTTCAACCCAGTCACAATGGAGAAGGTTATTGAG

                              490       500       510       520       530       540
                               |         |         |         |         |         |
verrucosumCBS32392            GGACATCCCTTGGTAGCTCGAGCGGTTGTCGTTGGGCTCGGTAGATTCCAAACAGCCCTG
nordicumCBS110769             GGACATCCCTTGGTAGCTCGAGCGGTTGTCGTTGGGCTCGGTAGATTCCAAACAGCCCTG
aurantiogriseum               GGACATCCCTTGGTAGCTCGAGCGGTTGTCGTTGGGCTCGGTAGATTCCAAACAGCCCTG
melanoconidiumCBS109605       GGACATCCCTTGGTAGCTCGAGCGGTTGTCGTTGGGCTCGGTAGATTCCAAACAGCCCTG
viridicatumCECT2320           GGACATCCCTTGGTAGCTCGAGCGGTTGTCGTTGGGCTCGGTAGATTCCAAACAGCCCTG
carneumCBS46895               GGACATCCCTTGGTAGCTCGAGCGGTTGTCGTTGGGCTCGGTAGATTCCAAACAGCCCTG
                              ************************************************************
Prim.cons.                    GGACATCCCTTGGTAGCTCGAGCGGTTGTCGTTGGGCTCGGTAGATTCCAAACAGCCCTG

                              550       560       570       580       590       600
                               |         |         |         |         |         |
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nordicumCBS110769             TTGATCGAGCCGAGCTGGAATAAATGGGATGCTGTTCCCCCGGGGAATAACTTGATTGAC
aurantiogriseum               TTGATCGAGCCGAGCTGGAATAAATGGGATGCTGTTCCCCCGGGGAATAACTTGATTGAC
melanoconidiumCBS109605       TTGATCGAGCCGAGCTGGAATAAATGGGATGCTGTTCCCCCGGGGAATAACTTGATTGAC
viridicatumCECT2320           TTGATCGAGCCGAGCTGGAATAAATGGGATGCTGTTCCCCCGGGGAATAACTTGATTGAC
carneumCBS46895               TTGATCGAGCCGAGCTGGAATCAATGGGATGCTGTTCCCCCGGGGAATAACTTGATTGAC
                              ********************* ******************************************
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                              610       620       630       640       650       660
                               |         |         |         |         |         |
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nordicumCBS110769             ATTGTCTGGCCGAAAGTGCAGGAGGCTAATCGTATCAGCCCGGCACATGGACATGTCATG
aurantiogriseum               ATTGTCTGGCCGAAAGTGCAGGAGGCTAATCGTATCAGCCCGGCACATGGACATGTCATG
melanoconidiumCBS109605       ATTGTCTGGCCGAAAGTGCAGGAGGCTAATCGTATCAGCCCGGCACATGGACATGTCATG
viridicatumCECT2320           ATTGTCTGGCCGAAAGTGCAGGAGGCTAATGGTATCAGCCCGGCACATGGACATGTCATG
carneumCBS46895               ATTGTCTGGCCGAAAGTGCAGGAGGCTAATCGTATCAGCCCGGCACATGGACATGTCATG
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Prim.cons.                    ATTGTCTGGCCGAAAGTGCAGGAGGCTAATCGTATCAGCCCGGCACATGGACATGTCATG
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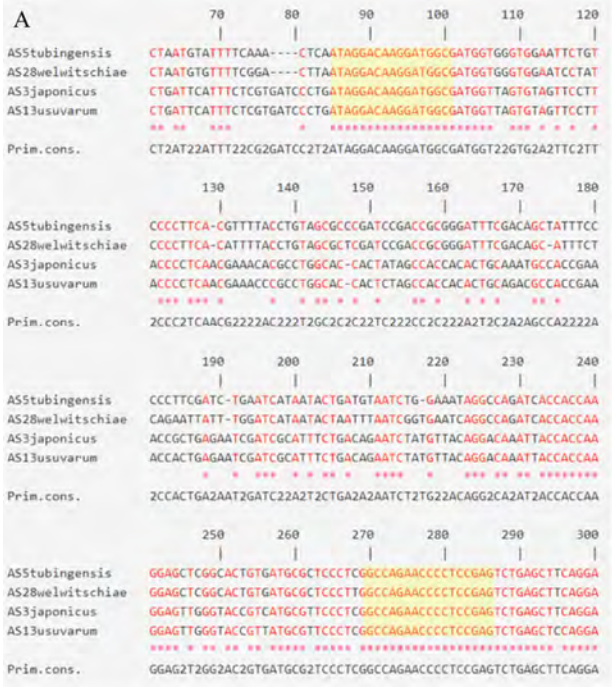
B

Figure 3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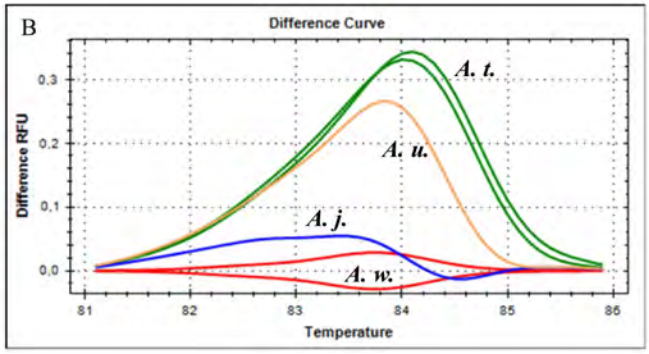
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Difference Curve
Difference RFU
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A. t.
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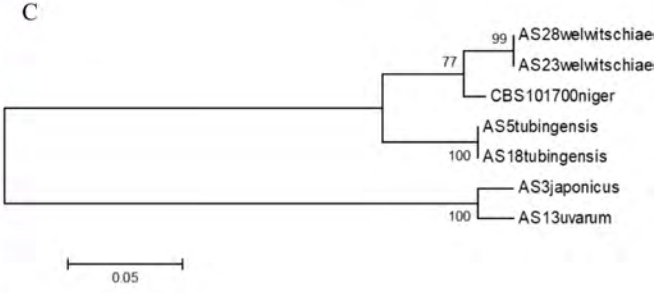
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0.05

Figure 4