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Identification of toxigenic fungal species associated with maize ear rot: Calmodulin as single informative gene

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ABSTRACT

Accurate identification of fungi occurring on agrofood products is the key aspect of any prevention and pest management program, offering valuable information in leading crop health and food safety.

Fungal species misidentification can dramatically impact biodiversity assessment, ecological studies, management decisions, and, concerning toxigenic fungi, health risk assessment, since they can produce a wide range of toxic secondary metabolites, referred to as mycotoxins. Since each toxigenic fungal species can have its own mycotoxin profile, a correct species identification, hereby attempted with universal DNA barcoding approach, could have a key role in mycotoxins prevention strategies. Currently, identification of single marker for species resolution in fungi has not been achieved and the analysis of multiple genes is used, with the advantage of an accurate species identification and disadvantage of difficult setting up of PCR-based diagnostic assays.

In the present paper, we describe our strategy to set up a DNA-based species identification of fungal species associated with maize ear rot, combining DNA barcoding approach and species-specific primers design for PCR based assays. We have (i) investigated the appropriate molecular marker for species identification, limited to mycobiota possibly occurring on maize, identifying calmodulin gene as single taxonomically informative entity; (ii) designed 17 sets of primers for rapid identification of 14 *Fusarium*, 10 *Aspergillus*, 2 *Penicillium*, and 2 *Talaromyces* species or species groups, and finally (iii) tested specificity of the 17 set of primers, in combination with 3 additional sets previously developed.

1. Introduction

Mycotoxins are toxic secondary metabolites, potentially highly harmful for humans, animals and also plants. They are produced by a wide range of toxigenic fungi on several crops of agro-food interest (Bennett and Klich, 2003). Maize (*Zea mays* L.) is one of the most important cereals in the world for animal feed and for human consumption, being the third highest produced grain crop, after wheat and rice. It is estimated that about 65% of the total world maize production is used as livestock feed, 15% for human consumption. The remaining 20% mainly fits industrial purposes (Abassian, 2006). However, maize is frequently contaminated by toxigenic fungi, which represent a danger for the potential accumulation of mycotoxins that they produce in the grains at harvest, and could cause a decrease of the shelf life of the final products. Moreover, during post-harvest, inappropriate storage condition could trigger the development of toxigenic fungi in the grains with consequent production of mycotoxins and further deterioration of maize kernels with low quality and nutritive value (Pitt and Hocking, 1997). On the other hand, also in the field, the ability of toxigenic fungi to colonize ears of maize is strictly related to climatic parameters, which are also key factors for mycotoxin production within the kernels. The mycotoxins that cause major concern on maize for their toxicity and their worldwide distribution are aflatoxins (AFLA), fumonisins (FUM), ochratoxin A (OTA), trichothecenes, and zearalenone (ZEA) (IARC, 2012; Marasas et al., 2008). They can be produced by a wide range of species belonging to three fungal genera: *Aspergillus*, *Fusarium*, and *Penicillium*. A careful and correct identification of any toxigenic fungus is a key issue for a correct risk assessment for consumer's health, because each species usually has its own mycotoxins profile, and each mycotoxins has a specific biological activity and toxicity target. Furthermore, in all above mentioned genera, there are cases of high morphological similarity among species that have a different mycotoxin profile. Many examples of this are available among the species commonly occurring on maize. In the *Aspergillus* genus, the AFLA producing species, *A. flavus* and *A. parasiticus,* can be confused with several other *Aspergillus* species that do not produce AFLA, while in the Black

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Aspergilli group, FUM producing and non-producing species, morphologically often indistinguishable, can co-occur on maize ears at the same time. In *Fusarium*, the FUM producing species, *F. proliferatum* and *F. verticillioides*, can co-exist in the kernels with several other morphologically very similar FUM non-producing species of the same genus. For the trichothecenes mycotoxin family, the species producing and non-producing deoxynivalenol (DON), T-2 toxin, or zearalenone (ZEA) can colonize the same ecological niche in maize. Finally, in *Penicillium*, the OTA producing species *P. verrucosum* can be confused with many other *Penicillium* species that do not produce OTA.

Moreover, early detection of toxigenic fungi, together with a correct species identification, is also an important issue for applying an effective strategy aimed to minimize the further growth of fungi and the production of mycotoxins, thus protecting the maize cultivation and its final product. Therefore, the establishment of an early, accurate, and efficient species-level identification system could significantly enhance our ability to manage fungal diseases caused by toxigenic fungi and to monitor their distributions in terms of spatial and temporal patterns. Indeed, an accurate species identification is the essence of any prevention and pest management program, since errors in identification can impact biodiversity assessment, ecological studies, and management decisions. Species identification based on observation of morphological characters is time consuming and requires expert taxonomists, with high level of expertise in particular species group. Furthermore, it could be unsuccessful in the case of sibling/cryptic species, promoting the use of DNA sequences for species delimitation and identification. Among availableapproaches, the DNA barcoding, which relies on the sequencing of astandardized DNA regions for accurate and rapid species identification, is mostly used to date. The segment of mitochondrial DNA from the cytochrome *c* oxidase gene (COI), proposed as the single standard DNA barcoding region (Hebert et al., 2003), has been demonstrated to be suboptimal for some groups of organisms, including fungi (Seifert et al., 2007; Seifert, 2009). Alternatively, the use of the internal transcribed spacer regions 1 and 2 (ITS) was encouraged (Seifert, 2009) for fungi, even though it is a compromise between universality, resolution, efficiency, and consistency (Vialle et al., 2009). On the other hand, the multilocus fungal identification system provides the most accurate species identification and delimitation (Balajee et al., 2007; Summerbell et al., 2005), but the number of additional loci needed to achieve a high proportion of successful identifications varies between taxonomical groups. Al-Hatmi et al. (2016) analyzed 52 *Fusarium* species by using two recently discovered DNA barcode loci, topoisomerase I (*TOP1*) and phosphoglycerate kinase (*PGK*), in combination with other routinely used markers, such as *TEF*, concluding that all the three above mentioned genes can effectively be used as barcoding markers for precise identification of cultures known to represent *Fusarium* species.

However, the best option of selecting an single marker powerful in discrimination of taxa to species level in fungal genera has not been achieved yet. As a result, multiplegenes are still used for species identification within a single genus (Al-Hatmi et al., 2016; Stielow et al., 2015). Among those, translation elongation factor 1-a (*EF1-α*, or *EF1* or *TEF*), calmodulin (*CaM*) and beta tubulin (*BenA*) provided good resolution at the species level for different fungal genera, such as *Aspergillus*, *Fusarium*, and *Penicillium* (Geiser et al., 2004; Samson et al., 2014; Visagie et al., 2014). Recently, Samson et al. (2011) redefined *Talaromyces* by combining *Penicillium* subgenus *Biverticillium* into *Talaromyces*. Since a recommendation of identification markers has not been suggested yet for *Talaromyces* species, usually the same markers used for identification of *Aspergillus* and *Penicillium* species are applied (Guevara-Suarez et al., 2017; Yilmaz et al., 2014).

The present study has the objective of selecting a single powerful DNA marker for the rapid identification of fungi eventually occurring on a specific crop, namely maize. Several strains representing species belonging to 4 genera, *Aspergillus*, *Fusarium*, *Penicillium*, and *Talaromyces* were analyzed in *BenA, CaM*, and *EF1-α* genes to find discordance in characters that will be useful for accurate species identification. The selected strains belonged to species mainly isolated from maize, and some of them phylogenetically closely related, according to the worldwide literature. The present work has been structured into three consecutive steps: (i) Identification of a single informative gene analyzing sequences of fungal isolates in three loci, (ii) development of primer sets, specific for single species or groups of species, and (iii) optimization of PCR protocols to maximize their specificity and sensitivity.

2. Materials and methods

2.1. Set of fungal strains

The sequences from three loci (*BenA, CaM*, and *EF1-α*) included in this study refer to 28 fungal species, belonging to *Aspergillus* (10 spp.), *Fusarium* (14 spp.), *Penicillium* (2 spp.), and *Talaromyces* (2 spp.). All 28 species were previously reported in literature to occur on maize. Although maize is colonized mainly by some *Fusarium* and *Aspergillus* species, isolates belonging to *Penicillium* and *Talaromyces* (syn. *Penicillium* subgenus *Biverticillium*), detected less frequently, were also included in the current study. The fungal strains were retrieved from the Agri-Food Toxigenic Fungi Culture Collection (ITEM, http://www.ispa.cnr.it/Collection/), where they were deposited after previous isolation from maize kernels collected in the field and identification at a species level by multilocus genotyping (MLGT) approach. Identities of isolates were confirmed by BLAST against the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/). Sequences from strains of species type and reference were downloaded both from internal sequence database held at Istituto di Scienze delle Produzioni Alimentari (ISPA)and from the *Fusarium, Aspergillus,* and *Penicillium* databases hold at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands *(http://www.westerdijkinstitute.nl/aspergillus/)*. A detailed list of species and number of sequences analyzed in this study are shown in Table 1.

2.2. Identification of single gene for species identification of maize pathogens

The assessment of “barcoding” resolution for three loci has been tested on a set of representative strains for each single genus mentioned above, through three analytical methods: pairwise distance method, sequence similarity method, and a tree-based method. Pairwise distances were estimated by TAXONDNA/Species Identifier 1.8 (Meyer and Paulay, 2005), considering multiple representative individuals for each species. The intra- and interspecies variations, as well as the mean of intra- and interspecific distances, the smallest and largest interspecific distances, and the largest intraspecific distances, were calculated with MEGA 7 (Kumar et al., 2016) using the Kimura two-parameters distance model (K2P).

A direct comparison of sequence similarity for each single marker was evaluated using the Best Match (BM) and Best Close Match (BCM) module from the TAXONDNA software package (Meier et al., 2006). Both analyses were carried out with the use of K2P corrected distances and a minimum sequence overlap of 300 bp. The program compared each successive sequence with all the other sequences present in our data set and matched them with the most similar ones. The BM tool assigns a species name to the query sequence, according to the similarity (smallest distance) between the query and the barcode sequences. The BCM tool provided a more robust identification considering the pairwise intraspecific distances and setting the threshold to 95% (Meier et al., 2006). All the results above the threshold were classified as ‘no match’. For the phylogenetic-based method, Maximum Likelihood (ML), Maximum Parsimony (MP), and Neighbor Joining (NJ) trees of all three markers were constructed to assess whether sequences in each genus datasets formed species-specific clusters. Best-fit model of

Table 1
Detailed list of species, number of sequences analyzed in this study, and ITEM strains representative of each species included in the PCR assays.

Species	BenA	CaM	EF1-α	TaxonDNA	n° of different sequences considered for primer design	Representative strains for PCR assay	Possible mycotoxins produced by species
				Aspergillus			
A. flavus	181	181	181	7	6	ITEM 7528	AFLA, CPA, KA, AA
A. niger	97	97	97	13	4	ITEM 15225	OTA,FBs, OXA,
A. ochraceus	5	5	0	2	2	ITEM 10999	OTA, PA
A. oryzae	2	2	2	2	2	ITEM 7529	CPA, BNP
A. parasiticus	7	7	7	2	2	ITEM 7531	AFLA, KA, AA
A. parvisclerotigenus	2	2	1	2	1	ITEM 9594	AFLA, CPA, KA
A. tamarii	22	22	22	3	3	ITEM 7811	AFLA, CPA, KA
A. tubingensis	99	99	99	16	6	ITEM 7040	
A. welwitschiae	44	44	44	14	5	ITEM 15128	OTA,FBs
A. wentii	3	3	1	3	2	ITEM 7899	Emodin, BNP
				Fusarium			
F. avenaceum	6	6	6	4	3	ITEM 2661	ESYN, FusC, MON
F. crookwellense	23	23	23	2	1	ITEM 4332	NIV, ZEA
F. culmorum	6	6	6	2	1	ITEM 13557	DON, NIV, FusC, MON, ZEA
F. equiseti	13	13	13	9	5	ITEM 11363	BEA, DAS, FUSX, NIV, EQUI, ZEA
F. fujikuroi	3	3	3	2	1	ITEM 7584	BEA, FusC, MON, FBs
F. graminearum	27	27	27	7	6	ITEM 12081	DON, NIV, ZEA, FusC
F. langsethiae	1	1	1	2	1	ITEM 11031	T2, HT-2, NEO, FUSX
F. poae	96	96	96	5	2	ITEM 7634	DAS, FUSX, NIV, T-2, BEA,
F. proliferatum	31	31	31	4	1	ITEM 12369	FBs, BEA,FUS,MON
F. sporotrichioides	3	3	3	4	2	ITEM 12168	T-2, HT-2, NEO DAS, FusC, MON, ZEA
F. subglutinans	71	71	71	6	3	ITEM 7582	MON, FA
F. temperatum	97	97	97	7	1	ITEM 10700	MON, BEA, FA
F. venenatum	3	3	3	2	1	ITEM 2495	DAS
F. verticillioides	90	90	90	6	2	ITEM 12050	FBs, FA, FusC
				Penicillium			
P. brevicompactum	7	7	7	3	1	ITEM 15261	OTA
P. oxalicum	9	9	9	3	1	ITEM 7573	OTA
				Talaromyces			
T. amestolkiae	5	5	1	2	2	ITEM 9580	unknown
T. variabilis	3	3	1	2	1	ITEM 9588	Rugulosin

AA (aspergillic acid) AFLA (aflatoxin), BEA (beauvericin), BNP (β-nitropropionic acid),CPA (Cyclopiazonic acid), DAS (4,15-diacetoxyscirpenol),DON (deoxynivalenol), EQUI (equisetin),ESYN (enniatin), FA (fusaric acid), FB (fumonisin), FusC (fusarin C), FUSX (fusarenon X), KA (kojic acid), MON (moniliformin), NEO (neosolaniol), NIV (nivalenol), OTA (ochratoxin),ROC (roquefortine C), ZEA (zearalenone).

molecular evolution for each alignment was selected based on Akaike Information Criterion (AIC) using MEGA 7 software (Kumar et al. 2016). All phylogenies were inferred with the MEGA software and relative support for the branches was assessed via 1000 bootstrap replicates. Species that clustered into a monophyletic group with a bootstrap value above 70% were considered successfully identified.

2.3. DNA extraction

Total genomic DNA of one representative strain for each 28 species belonging to the four genera (Table 1) was extracted using the Wizard Magnetic DNA Purification System for Food kit (Promega, USA), according to the manufacturer's protocol, both from lyophilized and fresh mycelia. Quality of genomic DNA was evaluated by 0,8% agarose gel electrophoresis.

2.4. Design of species-specific primers

Seventeen-specific primers based on calmodulin gene sequences were designed in this study, according to sequence data and their ability to resolve different species. Three other species-specific primers, previously published, were also included in the analyses (Mulè et al., 2004).

Calmodulin gene sequences, previously amplified and sequenced with primer pair CL1/CL2 (O'Donnell et al. 2000) for *Fusarium* and *Aspergillus* and with primer pair CF1/CF4 (Peterson, 2004) for *Penicillium* and *Talaromyces,* were subjected to intraspecific comparison to select representative strains for each species with nucleotide polymorphism. Calmodulin gene sequences of the subset of strains were aligned for each genus separately with Mega 7 using ClustalW, including multiple strains in case of intra-species variability. Based on sequence alignments it was possible to define regions that were highly similar across all species and therefore pinpoint sequence positions that were suitable for the specific primers. Forward and reverse primers were constructed for different species or species group using Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) following the rules for PCR primer design. Candidate primers were checked for their physical features such as melting temperature, self-complementarity, and secondary structures using Primer Express, and cross reactivity with maize genome was also screened by nucleotide BLAST in MaizeGDB (https://www.maizegdb.org) against the sequence database B73 RefGen_v4.

The efficiencies of matching with the target species or species-group, and possible cross reactivity with non-target species were verified empirically by singleplex PCR assays, using pure strains DNAs of species considered in the study (28). In order to check for specificity of PCR amplifications, all primer pairs were tested both by conventional PCR, and real-time PCR. After conventional PCR reactions, the amplicon presence/absence was assessed by electrophoresis in agarose gel and amplification specificity was confirmed when single PCR product of the expected size for the target species was obtained. Using real-time PCR, the specificity of primers has been assessed by melting curve analysis and was subsequently checked electrophoretically for each primer pair. The first derivative of melting curve allows to readily assess the homogeneity of the PCR products, including the presence of primer–dimers, thereby determining the specificity of the PCR reaction.

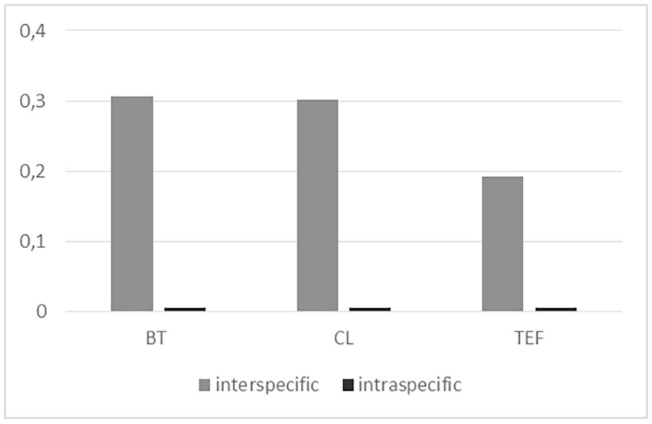

Fig. 1. Mean intra- and inter-specific genetic distances of evaluated DNA regions based on the K2P model of nucleotide substitution in the whole data set. The x-axis reports the different DNA regions used in each genus analyzed and the y-axis refers to mean K2P distance values, reported as the number of base substitutions per site.

2.5. PCR conditions for species specific primers

The primer pairs selected in silico were then tested in PCR assays, by analyzing the isolates listed in Table 1. First, a selectivity test was performed involving: the target strains, few other strains (closely related species), and one non-target strain. Conventional PCRs were performed in an Eppendorf MasterCycler® ep gradient (Hamburg, Germany), involving few target strains for each primer pair (closely related species) and testing different annealing temperatures (59, 60, and 61 °C). All PCR assays were carried out in 15 µL reaction volume containing 20 ng of genomic DNA, 0.2 mM of dNTPs, 1 U of Hot Master Taq DNA Polymerase (5 Prime, Hamburg, Germany), 1× Hot Master Taq DNA Polymerase buffer with 25 mM Mg^{2+}, and 300 nM of each primer. No Template Controls (NTC) using double-distilled sterile water were included in each PCR reaction, as negative control. PCRs were performed under the following conditions: initial denaturation at 95 °C for 2 min; 35 cycles of 95 °C for 50 s, 30 s at the three different Tm selected, 72 °C for 50 s; final extension at 72 °C for 5 min, followed by cooling at 4 °C. The expected amplicons for each primer pairs were confirmed by agarose gel electrophoresis. An annealing temperature of 61 °C was selected for the next qualitative real-time assay. Primer pairs

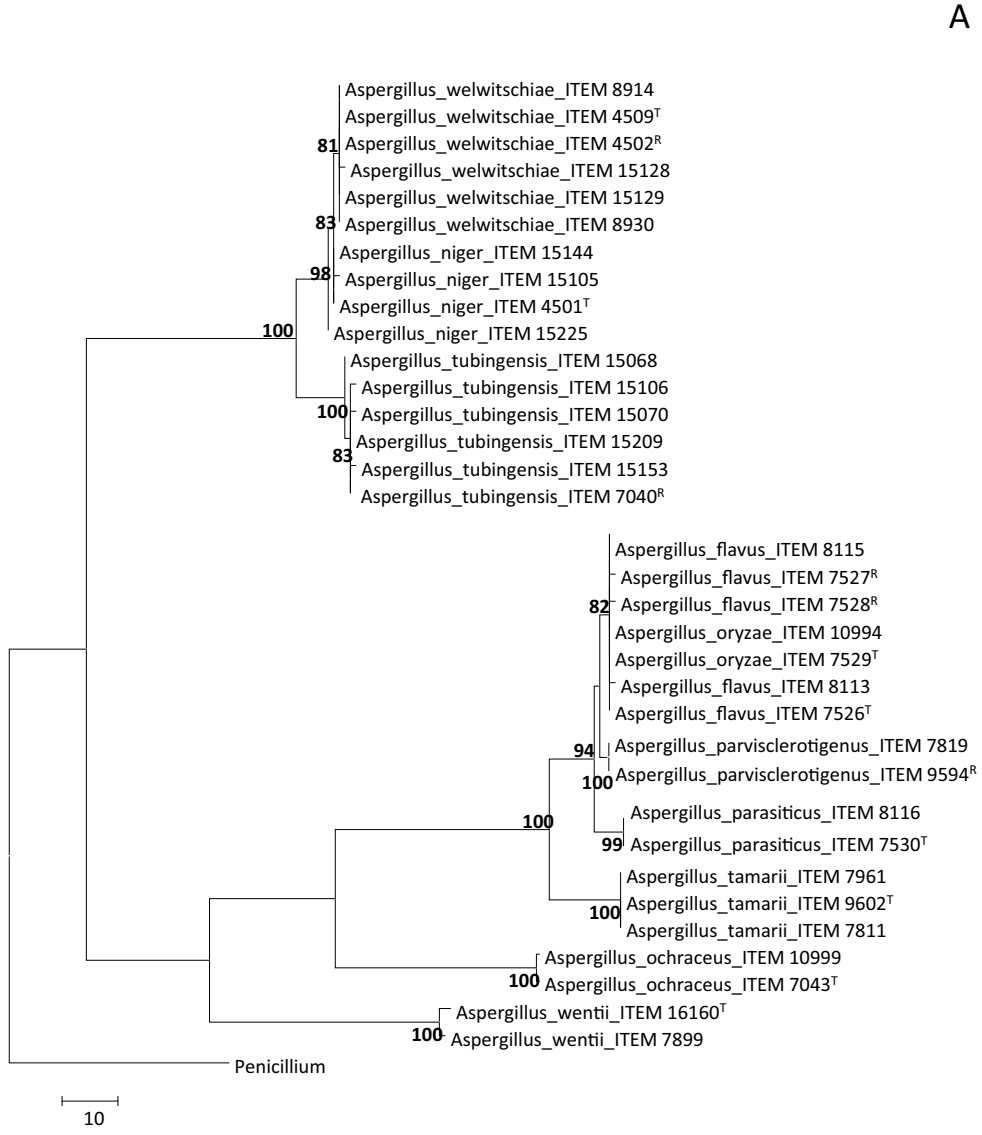

Fig. 2. Maximum Parsimony (MP) trees using *CaM* of *Aspergillus* (A), *Fusarium* (B), and *Penicillium/ Talaromyces* (C). Numbers on branches are bootstrap values based on 1000 pseudoreplicates. Bootstrap values ≥70% were considered significant.

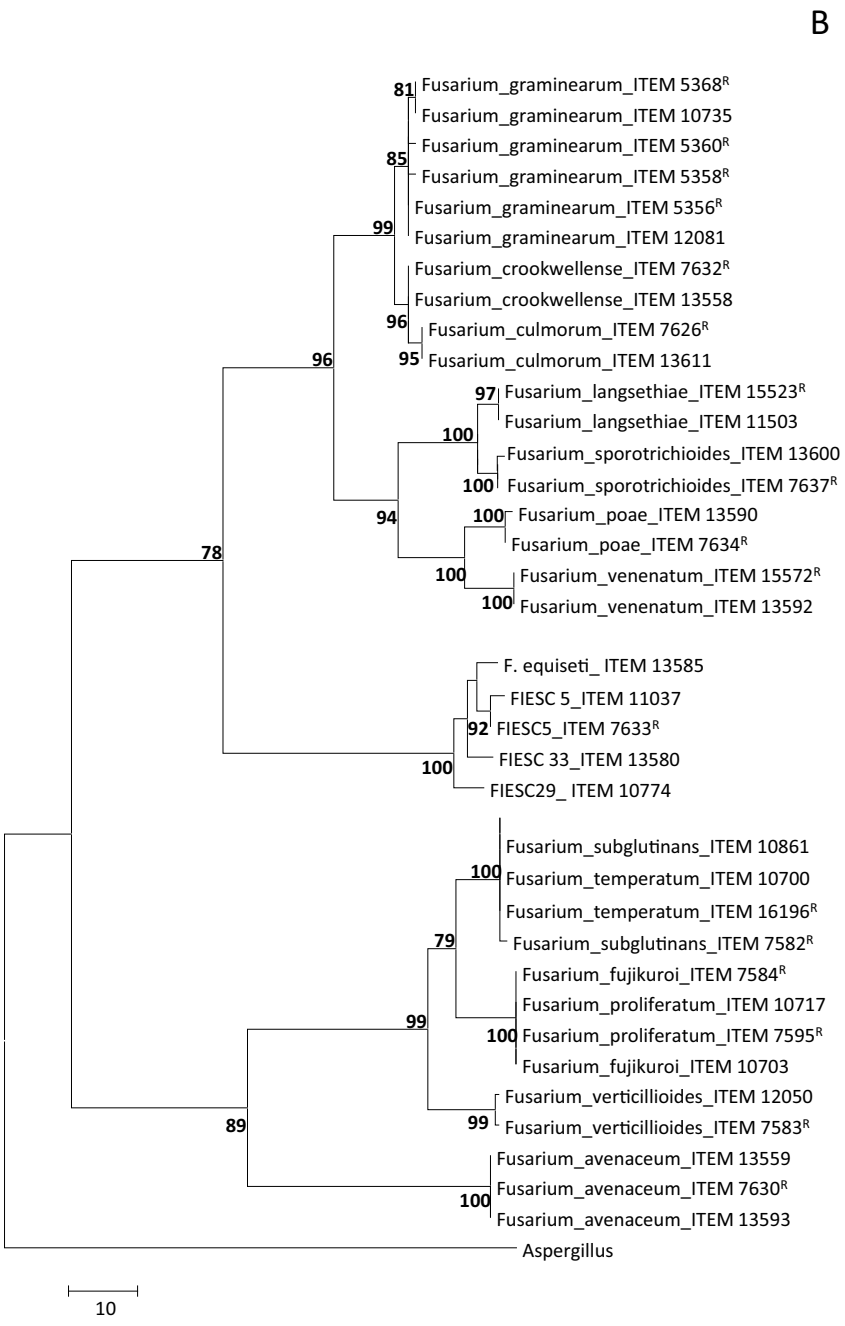

Fig. 2. (*continued*)

amplifying only the target strains were tested for the following analysis. A deeper selectivity test, including all 28 strains belonging to different species and genera, was performed through a qualitative SYBR® Green real-time PCR analysis. Reactions were carried out in the 7500 Fast Real-Time PCR System (Applied Biosystems) with MicroAmp® Fast Optical 96-Well Reaction Plate with barcode (Applied Biosystems). Assays were set up using the Real Master SYBR ROX mix (5 Prime) and dissociation curves were analyzed to avoid non-specific amplification. Each PCR reaction (20 µL) consisted of 9 µL of 2.5× Real Master Mix SYBR ROX, 0.6 µL of each primer (10 µM), and 2 µL of DNA (20 ng).

2.6. Primer specificity

The melting protocol included 95 °C for 1 min, followed by 35 cycles of 95 °C for 10 s, 61 °C for 30 s and 68 °C for 50 s, building a dissociation curve at the end of each PCR experiment, as follows: 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s and 60 °C for 15 s. Fluorescence detection was performed at the end of each extension step. In addition, the same PCR products were also analyzed by electrophoresis in 2% agarose gels. The fluorescent reporter signal was normalized against the internal reference dye (ROX) signal and No Template Controls (NTC), set up with sterilized water, were included in each PCR reaction to assess primer dimer formation or non-specific amplification.

3. Results

A total of 2572 sequences from the partial region of *BenA* (862), *CaM* (862), and *EF1-α* (848) genes obtained from different species of *Aspergillus*, *Fusarium*, *Penicillium*, and *Talaromyces* were analyzed (Table 1). According to previous species identification by using the MLGT approach, a set of 68 sequences (Table 2), representative for intra-species variability observed in all the 3 loci, was selected to

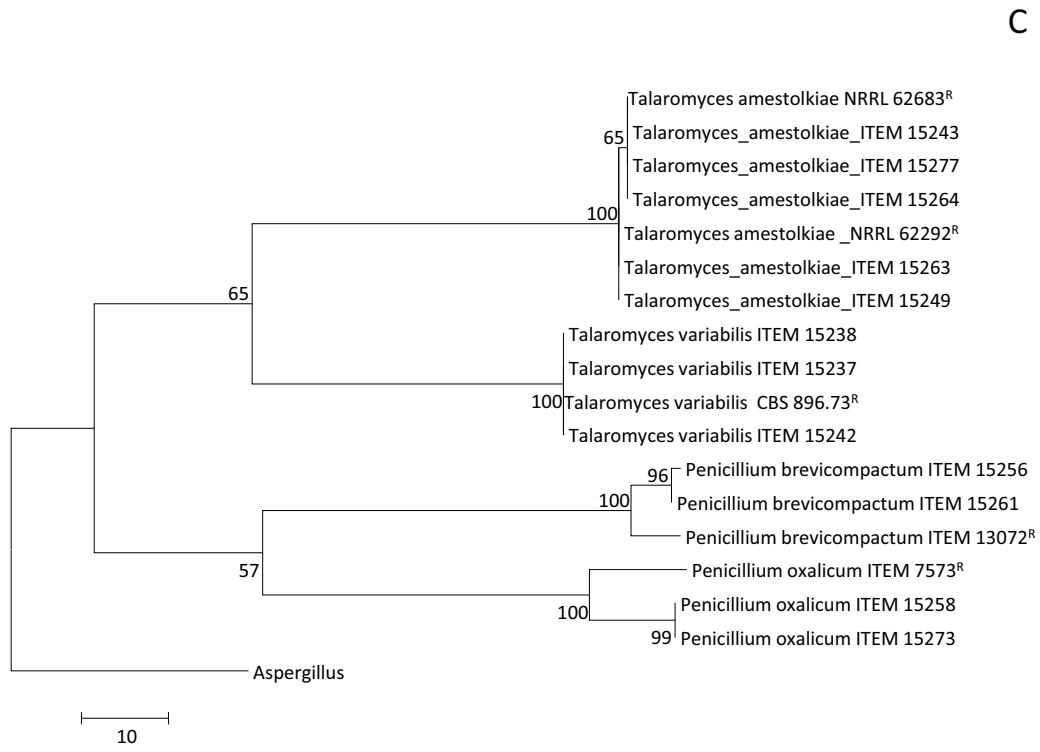

Fig. 2. (*continued*)

identify the suitable gene for species-specific primer development. The length of aligned DNA regions ranged from 280 bp to 799 bp, for *BenA* and *EF1-α* in *Talaromyces,* respectively (Table 3). Overall, the *CaM* coding gene was found to be generally the most variable among all the four genera, while *EF1-α* was found to be the most variable only in the genus *Fusarium*, but the most conserved among the other genera. In the genus *Aspergillus*, the sequenced *CaM* gene fragment had the highest number of parsimony informative sites (42%), followed by *BenA* (32%) and *EF1-α* (16%). In the genus *Fusarium*, *EF1-α* had the highest number of variable sites (42%), followed by *CaM* (35%) and *BenA* (25%). In the genus *Penicillium, BenA* had the highest number of parsimony informative sites (17%), followed by *CaM* (16%) and *EF1-α* (4,26%). In the genus *Talaromyces, CaM* had the highest number of parsimony informative sites (20%), followed by *BenA* (16%) (Table 3). Mean intra- and inter-specific genetic distances of all the tested regions using Kimura's model are shown in Fig. 1 and Supplementary Fig. S1. Since no EF1-α sequence is available to date for *A. ochraceus*, and only one sequence of *EF1-α* was available for some *Aspergillus* species (*A. parvisclerotigenus* and *A. wentii*) and all *Talaromyces* species (*T. amestolkiae* and *T. variabilis*), it was not possible to define the intra-specific distance for those species. Our analysis showed overlap between intra- and interspecific distances in all three loci investigated among *Fusarium* and *Aspergillus* species, while *BenA* and *CaM* showed a significant barcode gap among *Penicillium* and *Talaromyces* species (Supplementary Fig. S1). At the interspecific level, the highest divergence was provided both by *BenA* and *CaM* within all the genera (0,307 and 0,302, respectively), while the lowest was found in *EF1-α* (0,192) (Fig. 1).Among *Aspergillus*, *Fusarium*, and *Talaromyces* species, *CaM* showed the highest interspecific genetic distance (0,728, 0,439, and 0,364, respectively), while among *Penicillium*, *BenA* showed the highest interspecific genetic distance (0,430), and *EF1-α* the lowest (0,064). In most cases, intraspecific distance was zero, and generally, minimum interspecific distances were larger than corresponding intraspecific distances (Table 4). The pairwise distance analysis showed *CaM* as the most divergent gene at interspecific level among *Aspergillus*, *Fusarium*, and *Talaromyces* species, while in *Penicillium* the highest interspecific divergence was obtained in *BenA*. The lowest divergent gene at interspecific level was *EF1-α* (Table 4).

3.1. Sequence similarity

The discriminatory power of the three genes as barcode evaluated by tree-based method and TAXONDNA is reported in Table 5. In the *EF1-α* region, six species were removed from the data set prior to determining the match, because not available (*A. ochraceus*) or represented by a single individual (*A. parvisclerotigenus*, *A. wentii, T. amestolkiae*, and *T. variabilis*). The BM and BCM analysis showed similar identification percentages for all the tested loci (Table 5). Using *BenA*, the BCM criterion yielded an identification success ranging from 71,42% (*Talaromyces*) to 84,37% (*Fusarium*). Using *CaM*, the BCM criterion yielded an identification success ranging from 71.87% (*Fusarium*) to 100% (*Talaromyces*). Using *EF1-α*, the BCM criterion yielded an identification success ranging from 83.05% (*Aspergillus*) to 100% (*Penicillium*). The sequences analysis generated with three phylogenetic methods showed similar tree topologies, in agreement with results based on pairwise distances and sequence similarity (Table 5, Fig. 2, Supplementary Fig. S2). For ML, the Kimura 2 parameter with Gamma distribution (K2 + G) was the best fit model for *BenA* and *EF1- α* in *Aspergillus* and *Penicillium/Talaromyces* phylogenies, the Kimura 2 parameter with Invariant sites (K2 + I) was the best fit model for *CaM,* whereas the Hasegawa–Kishino–Yano parameter with Gamma distribution (HKY + G) was the best fit model for *EF1-α* in *Fusarium*. *BenA* and *CaM* had good levels of species discrimination, ranging from 43% (*BenA*) to 100% (*BenA* and *CaM*) (Table 5). The highest species resolution was obtained by *BenA* and *CaM*, as 20 out of 28 species were successfully identified (71%), while *EF1-α* had the highest species resolution only within the *Fusarium* genus, where 13 out of 14 species were successfully identified (Fig. 2). Within *Aspergillus* genus, *CaM* gene was the unique gene able to discriminate between *A. niger* and *A. welwitschiae*, while within *Penicillium* and *Talaromyces* genera, both *BenA* and *CaM* regions were able to discriminate all species analyzed (Fig. 2, Supplementary Fig. S2).

Table 2
Fungal strains used for phylogenetic analyses of *Fusarium*, *Aspergillus*, *Penicillium,* and *Talaromyces* species and for primer design.

Organism	Strain	Country	Host	GenBank Acc. N°
Fusarium avenaceum^{R}	ITEM7630			LR215911
Fusarium avenaceum	ITEM13559	The Netherlands	maize	LR215888
Fusarium avenaceum	ITEM13593	The Netherlands	maize	LR215892
Fusarium crookwellense^{R}	ITEM7632			LR215912
Fusarium culmorum^{R}	ITEM7626			LR215910
Fusarium equiseti	ITEM10774	Turkey	maize	LR215898
Fusarium equiseti	ITEM11037	Italy	maize	LR215868
Fusarium equiseti	ITEM13580	The Netherlands	maize	LR215889
Fusarium equiseti	ITEM13585	The Netherlands	maize	LR215890
Fusarium fujikuroi^{R}	ITEM7584			LS423026
Fusarium graminearum^{r}	ITEM5356			LR215902
Fusarium graminearum^{R}	ITEM5358			LR215903
Fusarium graminearum^{R}	ITEM5360			LR215904
Fusarium graminearum^{R}	ITEM5368			LR215905
Fusarium graminearum	ITEM10735	Turkey	maize	LR215897
Fusarium graminearum	ITEM12081	Italy	maize	LR215870
Fusarium langsethiae^{R}	ITEM15523			LR215915
Fusarium poae^{r}	ITEM7634			LR215913
Fusarium poae	ITEM15390	Iowa	maize	LR215862
Fusarium proliferatum^{R}	ITEM7595			LS423028
Fusarium sporotrichioides^{r}	ITEM7637			LR215914
Fusarium sporotrichioides	ITEM13600	The Netherlands	maize	LR215893
Fusarium subglutinans^{r}	ITEM7582			LR215906
Fusarium subglutinans	ITEM10861	South Africa	maize	LR215894
Fusarium subglutinans	ITEM16125	Italy	maize	LR215884
Fusarium temperatum	ITEM10700	Turkey	maize	LR215896
Fusarium venenatum	ITEM13592	Olanda	maize	LR215891
Fusarium verticillioides ^{R}	ITEM7583			LS423025
Fusarium verticillioides	ITEM12050	Italy	maize	LR215869
Aspergillus flavus ^{T}	ITEM7526		Cellophane	EF202063
Aspergillus flavus	ITEM7527	Uk	brazil groundnuts	LR215899
Aspergillus flavus	ITEM7528	Turkey	pistachio nuts	LR215895
Aspergillus flavus	ITEM10992	Egypt	maize	LR215859
Aspergillus flavus	ITEM8113	Italy	maize	LR215863
Aspergillus flavus	ITEM8115	Italy	maize	LR215864
Aspergillus niger^{T}	ITEM4501			AJ964872
Aspergillus niger	ITEM15105	Italy	maize	LR215873
Aspergillus niger	ITEM15144	Italy	maize	LR215877
Aspergillus niger	ITEM15225	Italy	maize	LR215880
Aspergillus ochraceus^{T}	ITEM7043			FJ491537
Aspergillus ochraceus	ITEM10999	Egypt	maize	LR215861
Aspergillus oryzae^{T}	ITEM7529			EF202055
Aspergillus oryzae	ITEM10994	Egypt	maize	LR215860
Aspergillus parasiticus^{T}	ITEM7530			EF202043
Aspergillus parasiticus	ITEM8116	Italy	maize	LR215865
Aspergillus parvisclerotigenus^{R}	ITEM9594			EF202077
Aspergillus tamari^{T}	ITEM9602			EF202034
Aspergillus tamari	ITEM7811	Nigeria	maize	LR215885
Aspergillus tamari	ITEM7961	Nigeria	maize	LR215887
Aspergillus tubingensis^{R}	ITEM7040			AJ964876
Aspergillus tubingensis	ITEM15068	Italy	maize	LR215871
Aspergillus tubingensis	ITEM15070	Italy	maize	LR215872
Aspergillus tubingensis	ITEM15106	Italy	maize	LR215874
Aspergillus tubingensis	ITEM15153	Italy	maize	LR215878
Aspergillus tubingensis	ITEM15209	Italy	maize	LR215879
Aspergillus welwitschiae^{T}	ITEM4509			AJ964874
Aspergillus welwitschiae	ITEM8914	Italy	maize	LR215866
Aspergillus welwitschiae	ITEM8930	Italy	maize	LR215867
Aspergillus welwitschiae	ITEM15128	Italy	maize	LR215875
Aspergillus welwitschiae	ITEM15129	Italy	maize	LR215876
Aspergillus wentii^{T}	ITEM16160			FJ531116
Aspergillus wentii	ITEM7899	Nigeria	maize	LR215886
Penicillium brevicompactum^{R}	ITEM13072			LR215901
Penicillium oxalicum^{R}	ITEM7573			LR215900
Talaromyces amestolkiae	ITEM15249	Italy	maize	LR215882
Talaromyces amestolkiae	ITEM15264	Italy	maize	LR215883
Talaromyces variabilis	ITEM15238	Italy	maize	LR215881

^{R}: reference species strain ^{T}: type species strain.

Table 3
Summary of sequence characteristics of three loci analyzed in this study in the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Talaromyces*. n.a. not applicable.

Gene	Sequences alignment lenght (bp)	No. of conserved sites (%)	No. of Parsimony-informative sites (%)	No. of Singleton sites (%)
Aspergillus				
BenA	468	316 (67)	150 (32)	2 (0,4)
CaM	648	369 (57)	273 (42)	6 (0,9)
EF1-α	729	579 (79)	116 (16)	33 (4,5)
Fusarium				
BenA	302	223 (74)	75 (25)	4 (1,3)
CaM	585	372 (63)	207 (35)	4 (0,7)
EF1-α	663	362 (55)	278 (42)	22 (3,3)
Penicillium				
BenA	338	265 (78)	57 (17)	12 (3,5)
CaM	568	460 (81)	94 (16)	14 (2,5)
EF1-α	633	593 (94)	27 (4,26)	12 (1,89)
Talaromyces				
BenA	280	230 (82)	45 (16)	5 (1,78)
CaM	482	386 (80)	96 (20)	0
EF1-α	799	490 (61)	n.a	n.a

3.2. Species-specific primers design

The analysis of the barcoding resolution, conducted on private and public DNA sequences (3 loci), suggested the calmodulin gene as DNA locus for discrimination purposes among *Aspergillus*, *Fusarium*, *Penicillium*, and *Talaromyces* species isolated from maize, and therefore for designing species-specific PCR primers. A set of 68 strains has been considered in this work, including 33 strains belonging to 10 *Aspergillus* species (*A. flavus*, *A. niger*, *A. ochraceus*, *A. oryzae*, *A. parasiticus*, *A. parvisclerotigenus*, *A. tamari*, *A. tubingensis*, *A. welwitschiae*, *A. wentii*), 30 strains belonging to 14 *Fusarium* species (*F. avenaceum*, *F. crookwellense*, *F. culmorum*, *F. equiseti*, *F. fujikuroi*, *F. graminearum*, *F. langsethiae*, *F. poae*, *F. proliferatum*, *F. sporotrichioides*, *F. subglutinans*, *F. temperatum*, *F. venenatum*, *F. verticillioides*), 2 strains belonging to 2 *Penicillium* species (*P. brevicompactum*, *P. oxalicum*), and 2 strains belonging to 2 *Talaromyces* species (*T. amestolkiae*, *T. variabilis*). The nucleotide sequences of the calmodulin gene were aligned for each genus, respectively, allowing to design species-specific primers from regions that were conserved between strains but differed intraspecifically. In primer designing, mismatches were located preferentially near the 3′ end of the primer, to hinder extension 10 of non-target DNA templates thereby increasing specificity of the primers to their respective target DNA. For the detection of the selected fungal species potentially occurring on maize, seven primer pairs were designed to selectively identify 10 *Aspergillus* species, 9 primer pairs, among which 3 retrieved from literature, Mulè et al. (2004), to specifically amplify 14 species within *Fusarium* genus, and 4 primer pairs to selectively identify 4 *Penicillium*/*Talaromyces* species (Table 6). The newly designed primers are characterized by melting temperatures in the range of 55–60 °C, GC content in the range of 40–60%, and the generation of amplicons ranging from 99 bp to 631 bp (Table 6).

3.3. Specificity of primers

Analysis of PCR reactions through agarose gel electrophoresis showed that all the tested primer pairs amplified a single PCR product of the expected size, respectively. However, some of them were able to amplify closely related non-target species. Among the three primer pairs retrieved from literature (Mulè et al., 2004), we showed that FSUB1/2 and FPRO1/2 are able to amplify, as expected on the basis of sequences analysis, also *F. temperatum* and *F. fujikuroi*, respectively. The primer pair FGRAM1/2, designed to detect *F. graminearum*, showed the production of an amplicon also for *F.crookewellense* and *F. culmorum*. Similarly, the primer pair FCUCR1/2, that was designed to detect *F.crookewellense* and *F. culmorum* showed the production of an amplicon

Table 4
Results of pairwise distance analysis based on MEGA 7.0-Compute Pairwise Distance.

	Minimum interspecific distance			Maximum interspecific distance			Maximum intraspecific distance		
	BenA	CaM	EF1- α	BenA	CaM	EF1- α	BenA	CaM	EF1- α
A. flavus	0	0	0	0,493	0,661	0,135	0,003	0,013	0,004
A. niger	0,003	0,004	0,001	0,593	0,625	0,136	0,006	0,013	0,009
A. ochraceus	0,412	0,332	n.a.	0,49	0,504	n.a	0	0,015	n.a.
A. oryzae	0	0	0	0,48	0,666	0,135	0	0,009	0
A. parasiticus	0,009	0,024	0,026	0,49	0,641	0,142	0,003	0	0
A. parvisclerotigenus	0,009	0,01	0,006	0,522	0,653	0,135	0,009	0	n.a.
A. tamarii	0,073	0,072	0,072	0,563	0,728	0,149	0,003	0,002	0,006
A. tubingensis	0,309	0,055	0,013	0,577	0,634	0,149	0,006	0,024	0,01
A. welwitschiae	0,003	0,004	0,001	0,593	0,641	0,141	0,006	0,004	0,012
A. wentii	0,412	0,418	0,125	0,593	0,728	0,138	0,012	0,004	n.a.
F. avenaceum	0,186	0,243	0,002	0,313	0,385	0,361	0,003	0	0,02
F. crookwellense	0,007	0,004	0,004	0,233	0,345	0,322	0	0	0
F. culmorum	0,025	0,004	0,004	0,252	0,359	0,314	0	0	0,002
F. equiseti	0,007	0,009	0,05	0,217	0,411	0,346	0,034	0,021	0,071
F. fujikuroi	0,003	0	0,018	0,233	0,423	0,26	0	0	0,002
F. graminearum	0,007	0,007	0,012	0,233	0,352	0,32	0,014	0,004	0,014
F. langsethiae	0,003	0,007	0,034	0,238	0,431	0,318	0	0	0,002
F. poae	0,055	0,038	0,095	0,313	0,371	0,335	0	0	0,004
F. proliferatum	0,003	0	0,018	0,235	0,431	0,267	0	0,004	0,008
F. sporotrichioides	0,003	0,007	0,034	0,24	0,439	0,316	0,003	0,006	0,004
F. subglutinans	0,003	0	0	0,224	0,439	0,316	0	0,002	0,043
F. temperatum	0,003	0	0	0,233	0,431	0,316	0	0	0,008
F. venenatum	0,046	0,038	0,095	0,268	0,383	0,361	0	0	0
F. verticillioides	0,054	0,043	0,079	0,255	0,42	0,28	0	0	0,01
P. brevicompactum	0,399	0,341	0,059	0,430	0,370	0,064	0,028	0,008	0,002
P. oxalicum	0,399	0,341	0,059	0,430	0,370	0,064	0,012	0,022	0,002
T. amestolkiae	0,274	0,358	0,063	0,317	0,364	0,063	0,005	0,008	n.a
T. variabilis	0,274	0,358	0,063	0,317	0,364	0,063	0,020	0,006	n.a

Table 5
Identification success rate using TAXONDNA tool under Best match and Best close match methods, and NJ, MP, and ML method. n.a not applicable. * Percentage of taxa monophyly with 70% bootstrap.

Gene	Best match			Best close match				Threshold (%)	NJ/MP/MLtrees (%)*
	Correct (%)	Ambiguous (%)	Incorrect (%)	Correct (%)	Ambiguous (%)	Incorrect (%)	No match (%)		
Aspergillus									
BenA	84.84	15.15	0	81.81	15.15	0	3.03	0.5	43/60/70
CaM	86.36	6.06	7.57	86.36	6.06	7.57	0	2.25	60/70/70
EF1-α	83.05	10.16	6.77	83.05	10.16	5.08	1.69	1.26	14/28/28
Fusarium									
BenA	84.37	15.62	0	84.37	15.62	0	0	1.69	57/71/64
CaM	71.87	26.56	1.56	71.87	26.56	1.56	0	2.67	50/64/57
EF1-α	89.06	4.68	6.25	89.06	4.68	6.25	0	7.19	57/93/86
Penicillium									
BenA	100	0	0	83,33	0	0	16,66	3,24	100/100/100
CaM	100	0	0	83,33	0	0	16,66	3,63	100/100/100
EF1-α	100	0	0	100	0	0	0	1,05	100/100/100
Talaromyces									
BenA	100	0	0	71,42	0	0	28,57	1,90	100/100/100
CaM	100	0	0	100	0	0	0	1,16	100/100/100
EF1-α	n.a	n.a	n.a	n.a	n.a	n.a	n.a		n.a

Table 6
Sequence of oligonucleotide primers used in this study.

Genus	Primers	5′ > 3′ nucleotide sequence	Exp. size	Tm(°C)[b]	Species-specificity
Fusarium	FSUB1[a]	CTGTCGCTAACCTCTTTATCCA	631	86	*F. subglutinans/F. temperatum*
	FSUB2[a]	CAGTATGGACGTTGGTATTATATCTAA			
	FPRO1[a]	CTTTCCGCCAAGTTTCTTC	585	85.5	*F. proliferatum/F. fujikuroi*
	FPRO2[a]	TGTCAGTAACTCGACGTTGTTG			
	FVER1[a]	CTTCCTGCGATGTTTCTCC	578	86.5	*F. verticillioides*
	FVER2[a]	AATTGGCCATTGGTATTATATATCTA			
	FGRAM1	GTTTGTAAGCCAACCTTCCTCG	169	84.5–85	*F.crookwellense/F. culmorum/*
	FGRAM2	GCGATTGCGTGTAGTCATTCA			*F. graminearum*
	FCUCR1	CCTGTTTGTAAGTCAACCTTCCTTG	397	85–86	*F.crookwellense/F. culmorum/*
	FCUCR2	GTTAGCGTTGCCACTCTACGG			*F. graminearum*
	FEQUI1	TGGTGAGTTATGCTCCCCTTTTC	581	86.5	*F. equiseti*
	FEQUI2	ATCTTGATTGTCCAACGCAGAA			
	FPOAE1	TCGCATCCGCATTCTTTTG	482	86	*F. poae*
	FPOAE2	TCAGCAACCGTGCACATTG			
	FVEN1	ACTCCCTTCCTTGTGCCTTTC	202	85	*F. venenatum*
	FVEN2	TTATCGGCTACGTGTGCCC			
	FSPLAN1	GATCGTYAAGTTCATCTTAAAAAAGTCCC	289	85	*F. sporotrichioides/*
	FSPLAN2	TCTTTTCTCCAATGTCAGTGTCGT			*F. langsethiae*
	FAVEN1	GTAATTCTCCAAGACGACAGGGTAC	295	84.5	*F. avenaceum*
	FAVEN2	GTGCTTTATCGCCCAATCTGA			
Aspergillus	ANIG1	GTGGAATTCTATCCCCTTCACATTAT	99	80	*A. niger*
	ANIG2	AAATTAGTATTATGATCCAAATCGTTCTGGA			
	AWEL1	GGGATTTCGACAGCATTTCTC	351	87	*A. welwitschiae*
	AWEL2	ACCATTGTTGTCGCGGTCA			
	ATUB1	GGCTCATAATGCTAATGTATTTTCAAAC	600	88	*A. tubingensis*
	ATUB2	GGGCATATCAGGAATGGGAA			
	ATAM1	GGACGGTGATGGTTAGTACAGTCTC	101	80	*A. tamari*
	ATAM2	GCCGATCGAACAGAAAATGG			
	AFLA1	CTGAAGTCTTGGCATTGATGAATT	401	85–86	*A. flavus/A. oryzae/*
	AFLA2	TCGCGGATCATCTCATCAACT			*A.parvisclerotigenus/A.parasiticus*
	AOCH1	ATTCCCTTCCAGTTCGTCGA	311	85.5	*A. ochraceus*
	AOCH2	CAGCTGGGTTTCAGGATCGT			
	AWEN1	AGTGCCACTTGCTTCCAACG	271	86.5	*A. wentii*
	AWEN2	TTCGCATCGACGCAAACTT			
Penicillium	PBR1	AATGAAATCCTAATGCACCTCCC	220	86.5	*P. brevicompactum*
	PBR2	ATGGTCAGGAATTCTGATCATTTG			
	POX1	GGCGATGGTGAGATTCTTCCT	189	84	*P. oxalicum*
	POX2	GCGAGCGCATGACAGTACC			
Talaromyces	TAM1	CAATGATGGCCCGCAAAA	217	83	*T. amestolkiae*
	TAM2	GGAGGAAACTCACAGTCAATCCTT			
	TVAR1	TGGGCTATGGCTGATATTTTATTTTT	391	86.5	*T. variabilis*
	TVAR2	GCGGATCATCTCGTCGACTT			

[a] Mulè et al., 2004.
[b] Amplicon melting temperature.

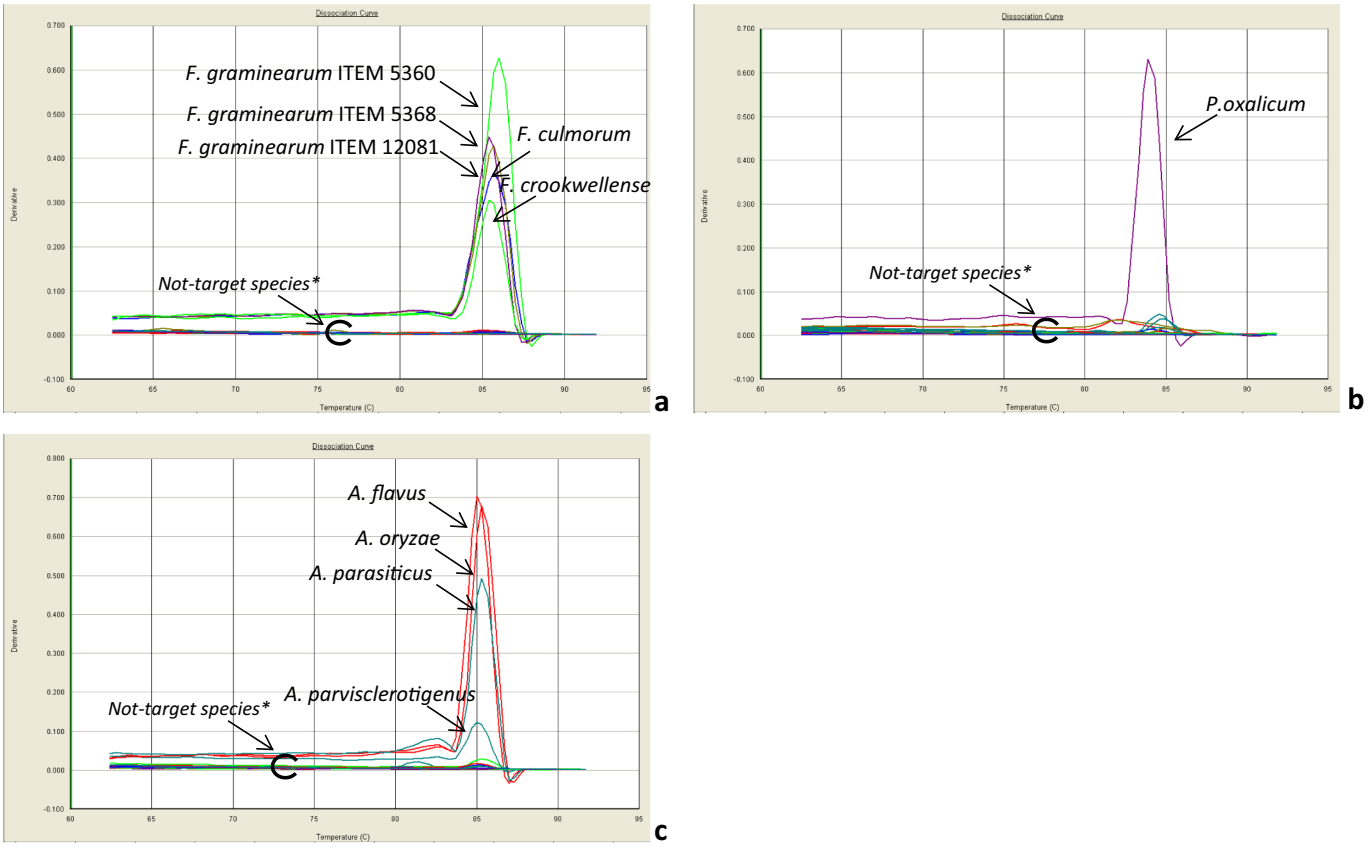

Fig. 3. Specificity of real-time PCR amplification analyzing CaM Dissociation curves of representative primers: a) Fcucr, b) Pox, c) Afla. : Single peaks are shown for representative strains (*) Table 1. Y-axis: derivative of normalized fluorescence signal to ROX™; x-axis: temperature (60° ÷ 95 °C).

also for *F. graminearum*.

The melting-curve analysis (Fig. 3) showed the presence of single peaks for 11 out of 20 primer pairs (*F. avenaceum*, *F. graminearum/ crookwellense/culmorum*, *F. sporotrichioides/langsethiae*, *A. flavus/oryzae/parasiticus/ parvisclerotigenus*, *A. ochraceus*, *A. wentii*, *A. tubingensis*, *P. oxalicum*, *P. brevicompactum*, *T. amestolkiae*, and *T. variabilis*,) and the presence of additional primer-dimers for 8 out of 20 primer pairs (*F. equiseti*, *F. poae*, *F. proliferatum/fujikuroi*, *F. venenatum*, *F. verticillioides*, *A. niger*, *A. tamari*, *A. welwitschiae*). However, the presence of primer-dimers does not affect the assessment of presence/absence of and calculation of Tm for species-specific amplicon (see Table 6).

Finally, possible matches with the maize genome have also been investigated using web-based Nucleotide Basic Local Alignment Search Tool (BLASTn) at Maize GDB (https://www.maizegdb.org) against the sequence database B73 RefGen_v4. Blast search revealed no evidence of cross-reactivity with maize genomic DNA because no good hits were found: in some cases both forward and reverse primers align on the same strand or matched on different chromosomes or genomic context (Supplementary Table S1).

4. Discussion

The early detection of fungal plant pathogens in the field, eventually before symptoms became visible, is a crucial step for disease management and mycotoxin risk prevention. The use of molecular markers in the last two decades has greatly enhanced the sensitivity and specificity of detection, compared to morphology-based techniques (Geiser et al., 2004; Taylor et al., 2000; Tsang et al., 2018). Moreover, technological advances in PCR-based methods, such as real- time PCR, allow fast and accurate detection of fungal pathogens and can be applied to practical management of the diseases in the field (Brandfass and Karlovsky, 2006; Johnson et al., 2012; Suanthie et al., 2009). DNA-based taxonomic studies have benefits; i. they resolve evolutionary relationships in fungi by a phylogenetic approach that is based on the concordance of multiple gene genealogies (Taylor et al., 2000); ii. by generating sequences for identification of single DNA markers that are useful as tools for universal identification of fungal species, based on the DNA barcode approach. In 2012, the International Fungal Barcoding Consortium formally recommended the ITS regions of the nuclear ribosomal RNA gene cluster as the primary fungal barcode (Schoch et al., 2012). Although routinely used in diagnostic laboratories, the ITS regions do not always provide resolution at species level in some genera, such as *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Trichoderma*, which lead to consideration of other genes for the purpose. To date, some protein-coding genes have been suggested as secondary fungal DNA barcodes, i.e. *EF1-α*, selected as the best candidate gene in *Fusarium* genus (Al-Hatmi et al., 2016; Geiser et al., 2004), or *BenA* and *CaM* genes, suggested for *Aspergillus* and *Penicillium* genera (Samson et al., 2014). Those 3 genes were considered in the current study, with the aim of identifying the best candidate gene with a barcode power for the rapid identification of fungi potentially occurring on maize. We first analyzed sequences of several representatives strains belonging to 28 species in four genera, some of which phylogenetically closely related, and that are frequently isolated from maize, at worldwide level. Calmodulin and *EF1-α* genes showed a similar inter-specific divergence variation in *Fusarium*, which was higher than the one shown by the *BenA* gene. On the other hand, in *Aspergillus*, *Penicillium*, and *Talaromyces*, *EF1-α* showed the lowest range of inter-specific distances compared with the range of the other two loci. Also, most of the *Penicillium* and *Talaromyces* have not been sequenced for the *EF1-α* region and annotations in public DNA repositories are insufficient, forming a tangible obstacle to sequence-based species identification. Although

BenA was proved to be the best gene in distinguishing species in *Aspergillus*, *Penicillium* and *Talaromyces* genera, the presence of divergent paralogs in some species of *Aspergillus* and *Fusarium* (Hubka and Kolarik, 2012; O'Donnell et al., 2015; Peterson, 2008) may render this gene ineffective as barcode. Therefore, we have selected the *CaM* gene as best barcode candidate for the differentiation of the *Aspergillus*, *Fusarium*, *Penicillium*, and *Talaromyces* species included in this study.

Additionally, species-specific primers, for rapid sensitive simultaneous and PCR-based identification of fungal species, were designed and tested for their specificity. The alignment of the *CaM* sequences for the species included in the present study allowed the selection of species-specific pairs of primers for PCR-based molecular diagnostic assays. The effort in primer design consisted in determining a set of forward and reverse primers that will amplify one group of sequences (the target species) and excluding others (the non-target species), through integration of terminal mismatches. The chosen primers have been tested both by using conventional and real-time PCR, which is more sensitive than electrophoresis. Moreover, the method allows testing of amplicon specificity by curve analysis. The sets of primers were designed and optimized for a unique annealing temperature of 61 °C, allowing simultaneous test for multiple species, possibly with further optimizations also in multiplexed reactions. The here proposed assays consist of 20 singleplex PCR reactions, using a unique DNA template, the total DNA isolated from a maize, and targeting *CaM* as single species-specific polymorphic gene. The PCR test is the opportunity to set a standardized species recognition, suitable for a high-throughput DNA processing workflow that results in early diagnosis of toxigenic and phytopathogenic fungi on maize. Results of the analysis offer valuable information for the selection of appropriate management strategies to protect crop health and food safety. Direct amplification of maize sample DNA, with 20 different primer pairs, may offers information about the possible presence of 28 different fungal species (or species-groups) belonging to *Fusarium* (14), *Aspergillus* (10), *Penicillium* (2), and *Talaromyces* (2). Most of the tested primers are able to specifically identify one species. In some cases *CaM* was not able to distinguish closely related species (Fig. 3 and Table 6). In particular, in *Fusarium*, the following species are identifiable in combinations: *F. proliferatum/fujikuroi*, *F. graminearum/crookwellense/culmorum*, *F. langsethiae/sporotrichioides*, and *F. subglutinans/temperatum*, and single species could not be distinguished. However, the developed primers represent a valid and useful tool for risk assessment. *Fusarium proliferatum* and *F. fujikuroi* are considered sibling species (Leslie and Summerell, 2006; O'Donnell et al., 1998), being not only phylogenetically closely related, but also having natural intercross fertility, nevertheless producing dramatically different levels of FB. On the other hand, the occurrence of *F. fujikuroi* on maize is extremely rare if ever recorded, since this fungus is a strict pathogen of rice (Leslie and Summerell, 2006.). On the contrary, *F. proliferatum*, together with *F. verticillioides*, is the main FB producing species occurring on maize, from which source it is usually isolated. Therefore we assume that the lack of distinction of *F. fujikuroi* and *F. proliferatum* by using *CaM* marker would not affect the efficacy of FB risk assessment on the maize crop. Similarly, for the combination *F. subglutinans/temperatum*, the two species represent sibling species phylogenetically strictly related and having intercross fertility in the lab experiments (Scauflaire et al., 2011; Steenkamp et al., 2002). Both species can colonize maize, but usually have a different pattern of environmental conditions. Both species produce a range of mycotoxins of minor concern, which are not regulated by any Institution at worldwide level. Therefore, although they are indistinguishable by using *CaM* marker, the risk management related to the occurrence of these two species on maize would not be affected. In the unsolved combinations *F. graminearum/crookwellense/culmorum*, and *F. langsethiae/sporotrichioides*, within each of them, the mycotoxin profile is similar for the respective combinations: DON can be produced by *F. graminearum*, *F. crookwellense*, and *F. culmorum*, while T-2 toxin and HT-2 are produced by both *F. langsethiae* and *F. sporotrichioides*. In addition, in both combinations, the species can be easily identified morphologically in a further step. In the genus *Aspergillus*, the unique combination where species could not be distinguished, by the *CaM* marker, was *A. flavus/oryzae/parvislclerotigenus/parasiticus*. All those species, with the exception of *A. oryzae*, can produce carcinogenic aflatoxins. On the other hand, while *A. flavus*, *A. parvislclerotigenus*, and *A. parasiticus* are morphologically identical, *A. oryzae* is very easy to be distinguished from the other three aflatoxigenic species by a simple analysis under microscope. Therefore, also in this case, after a first and rapid alert by using *CaM* marker, the possibility of having a correct risk management approach would be of easy approach, in a second step if required. In *Penicillium* and *Talaromyces* genera, the main concern related to their occurrence on maize is related to the kernel contamination by OTA producing species.

The added value of the proposed method of this work relies on the possibility for non-experts of this field to perform easy and identification of toxigenic and phytopathogenic fungi at species level, obtaining quickly an alert for fungi and related mycotoxin risk, driving samples towards further analyses (further specific PCR assays, observation of morphological characters of cultures, etc), with the advantage of reducing the number of further expensive investigations (e.g. multitoxins determination in maize matrix). Indeed, these primers has the potential to be incorporated in rapid and highthroughtput screening systems, i.e. array cards, for the detection of multiple fungi by simultaneous PCR reactions for each maize sample, generating in the time of a single reaction the information related to multiple species potential presence.

In conclusion, in this work we have (i) investigated firstly the appropriate molecular marker for fungal species identification, limiting focus to fungi potentially occurring on maize, (ii) designed 17 sets of primers for rapid, sensitive and simultaneous identification of fungal species from pure culture, and finally (iii) tested species specificity of newly designed primers and 3 additional species-specific primer pairs, designed in a previous work (Mulè et al., 2004), against a set of 28 fungal species. Most of the primer pairs are able to specifically identify a single species and therefore provide a useful tool for risk assessment. However, the results obtained as a whole allow us to give a barcode ability for detection of a wide range of toxigenic and phytopathogenic fungi as starting tool for further and more accurate development.

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