European Journal of Plant Pathology **110:** 641–649, 2004.

PCR assay for identification of *Aspergillus carbonarius* and *Aspergillus japonicus*

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Key words: Aspergillus aculeatus, calmodulin gene, grapes, PCR-assay, specific primer

Abstract

Black Aspergilli, and in particular *Aspergillus carbonarius*, are the main causes of contamination of grapes and their by-products by ochratoxin A. A PCR-based method was developed to detect DNA of *A. carbonarius* and *A. japonicus*. Two pairs of primers (CARBO1/2 and JAPO1/2) designed from the calmodulin gene, produced PCR products of 371 and 583 bp for *A. carbonarius* and *A. japonicus*, respectively. Primer specificity was tested with DNA of 107 strains belonging to *Aspergillus* section *Nigri* isolated mostly from grapes in Europe. The sensitivity of primers CARBO1/2 and JAPO1/2 was 12.5 pg when using pure total genomic DNA of the two species. The developed primers provide a powerful tool for detection of the main ochratoxigenic producing *Aspergillus* species in grapes.

Introduction

Species of *Aspergillus* belonging to section *Nigri* (Gams et al., 1985) are commonly known as black Aspergilli and have a significant impact on modern society through their ability to produce different hydrolytic enzymes (amylases, lipases) and organic acids (citric acid, gluconic acid) which are useful in the fermentation industry. These species also cause food spoilage and produce mycotoxins (Kozakiewicz, 1989; Bennet and Klich, 1992). The taxonomy of *Aspergillus* section *Nigri* has been studied by means of morphological and cultural criteria (Raper and Fennel, 1965; Al Musallam, 1980; Klich and Pitt, 1988; Kozakiewicz, 1989; Samson, 1992), but it is still controversial and problematic. For example, *A. aculeatus* and *A. japonicus* are considered by some authorities to be the same species. In this respect, according to studies reported in literature (Parenicova et al., 2000; Varga et al. 2001), *A. japonicus* and *A. aculeatus* are not distinguishable by their sequence data. Identification of the *A. niger* group still has unsolved problems of classification. *A. carbonarius* and *A. niger* aggregate were reported as ochratoxin A (OTA) producers (Abarca et al., 1994; Téren et al., 1996; Varga et al., 1996), whereas *A. japonicus* and *A. aculeatus* do not produce these OTA. Ochratoxin A is a highly harmful metabolite classified in 1993 by the International Agency for Research on Cancer as a possible human carcinogenic toxin (group 2B) (IARC, 1993).

Recently, OTA has emerged as a serious problem because of the contamination of grapes and their by-products (Zimmerli and Dick, 1996; Otteneder and Majerus, 2000). A recent review on the presence of ochratoxin A in grapes and wine underlined the importance of *A. carbonarius* as the main cause of OTA accumulation in wine (Battilani and Pietri, 2002). The accurate identification of *Aspergillus* species in the section *Nigri* is of great importance because the toxic profile of the single species could be different and could expose the contaminated food commodities to different toxicological risks.

The increasing use of molecular methods in fungal diagnosis has provided tools for answering taxonomic questions that morphological procedures have left unsolved (Taylor et al., 1999; Parenicova et al., 2000). A promising and informative technique for characterizing *Aspergillus* and *Penicillium* species at the intra- and interspecific level is related to the sequence analysis of variable conserved DNA regions (28S, ITS-1/ITS2, β-tubulin,

calmodulin and elongation factor) (Geiser et al., 2000), since from such sequences it is possible to design primers and generate molecular markers.

The aim of this study was to design species specific primers for *A. carbonarius* and *A. japonicus* which could be used to identify OTA-producing species on grapes.

Materials and methods

Fungal strains

One hundred and seven strains belonging to *Aspergillus* section *Nigri* and six strains belonging to other fungal genera were studied to verify the specificity of the designed primers. All strains were obtained from the culture collection of the Institute of Sciences of Food Production, with accession number ITEM. Further information about the strains (year of isolation, depositor, toxin production, etc.) are available in the ITEM web site: http://www.ispa.cnr.it/Collection. Eighty-nine strains belong to the three main species occurring on grapes in Europe were used as listed in Table 1.

Furthermore 14 referenced strains from CAB International Mycological Institute, Kew, Surrey, UK belonging to the *Aspergillus* section *Nigri* are used as listed in Table 2. Finally six out-group fungal strains were also tested in the PCR assay: *A. ochraceus* (ITEM 4537, 4549); *Botrytis cinerea* ITEM 5154; *Saccharomyces cerevisiae* DSM 70451 (Deutsche Sammlung von Mikro-organismen, Göttingen, Federal Republic of Germany); *Fusarium culmorum* ITEM 627 and *Fusarium proliferatum* ITEM 1475.

DNA extraction

Fungal strains were grown in shaken cultures (150 rpm) in Wikerman medium (40 g of glucose, 5 g of peptone, 3 g of yeast extract, 3 g of malt extract and water up to 1 l). About 40 mg of filtered, frozen and lyophilized mycelium from each strain were used for total genomic DNA extraction using the EZNA. Fungal DNA Miniprep Kit (Omega Bio-tek, Doraville, USA). DNA was recovered and dissolved in sterile water. Concentrations of DNA were determined by gel electrophoresis, and by measuring the ultraviolet-induced

Table 1. Aspergillus section *Nigri* strains isolated from grapes in Europe and examined by PCR reaction with the species-specific primers for *A. carbonarius* (CARBO) and *A. japonicus* (JAPO)

Species	Strains Tested	Origin	Primer CARBO	Primer JAPO	ITEM[1] Accession number
Aspergillus carbonarius	4	Portugal	+	−	4555, 4556, 4770, 4776
	6	France	+	−	4784, 4786, 4792, 4807, 4808, 4810
	8	Italy	+	−	4722, 4724, 4729, 4838, 4849, 4854, 5002, 5005
	4	Spain	+	−	4934, 4935, 4936, 4937
	4	Israel	+	−	4976, 4977, 4978, 4979
	4	Greece	+	−	5223, 5297, 5298, 5301
Aspergillus japonicus	1	Portugal	−	+	4865
	6	France	−	+	4814, 5322, 5328, 5334, 5336, 5340
	8	Italy	−	+	4690, 4691, 4716, 4733, 4848, 4857, 5013, 5019
	6	Spain	−	+	4957, 4958, 4959, 4960, 4961, 4962
	7	Israel	−	+	4995, 4996, 4997, 4998, 5348, 5349, 5350
	2	Greece	−	+	5246, 5287
Aspergillus aculeatus	3	Italy	−	+	4727, 4833, 4844
	1	Israel	−	+	4999
Aspergillus niger	2	Portugal	−	−	4550, 4541
	5	France	−	−	4815, 4816, 4820, 4821, 4822
	7	Italy	−	−	4720, 4725, 4836, 4837, 4840, 4846, 4859
	4	Spain	−	−	4950, 4951, 4952, 4954
	5	Israel	−	−	4982, 4983, 4984, 4985, 4986
	6	Greece	−	−	5253, 5254, 5257, 5265, 5272, 5277

[1] ITEM indicates culture collection of agri-food important toxigenic fungi, ISPA-CNR, Bari, Italy.

Table 2. List of CABI referenced strains examined by PCR reaction with the species-specific primers for *A. carbonarius* (CARBO) and *A. japonicus* (JAPO)

Species	Origin	Host	Primer CARBO	Primer JAPO	Accession number[1]
Aspergillus japonicus	Panama	Soil	–	+	IMI 211387, ITEM 4497
Aspergillus phoenicis	Japan	Kuro-koji	–	–	IMI 211395, ITEM 4498
Aspergillus helicothrix	Costa Rica	Contaminant of *A. ellipticus*	–	–	IMI 278383, ITEM 4499
Aspergillus tubingensis	–	–	–	–	IMI 172296, ITEM 4500
Aspergillus niger	Turkey	*Ficus carica*	–	–	IMI 388198, ITEM 3856
Aspergillus niger	–	Tannin gallic acid fermentation	–	–	IMI 50566, ITEM 4501
Aspergillus niger	–	–	–	–	IMI 091881, ITEM 4502
Aspergillus carbonarius	–	Paper	+	–	IMI 16136, ITEM 4503
Aspergillus carbonarius	Brazil	–	+	–	IMI 41875, ITEM 4504
Aspergillus ellipticus	Costa Rica	Soil	–	–	IMI 172283, ITEM4505
Aspergillus foetidus	–	–	–	–	IMI 15954, ITEM 4506
Aspergillus foetidus var. *acidus*	Japan	–	–	–	IMI 104688, ITEM 4507
Aspergillus foetidus var. *pallidus*	–	–	–	–	IMI 175963, ITEM 4508
Aspergillus awamori	Japan	–	–	–	IMI 211394, ITEM 4509

[1] IMI: CAB International Mycological Institute, Kew, Surrey, UK; ITEM: culture collection of agri-food important toxigenic fungi, ISPA-CNR, Bari, Italy.

fluorescence emitted by ethidium bromide molecules intercalated into DNA, and comparing the fluorescent yield of the samples with a standard.

Fungal DNA amplification and sequencing

Amplifications of the partial calmodulin gene were set up with 2.5 U of *Taq Gold* DNA polymerase (Applied Biosystems) in 100 µl reaction mixtures, containing 30 pmol of each outside primer, 12.5 µm of each deoxynucleoside triphosphate (Applied Biosystems), and 1 µl (approximately 10 ng) of fungal template DNA. All isolates analyzed were amplified using primers CL1 and CL2A (O'Donnell et al., 2000). The reactions were performed using the following PCR conditions: denaturation at 94 °C for 10 min; 35 cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 50 s, extension at 72 °C for 1 min; final extension at 72 °C for 7 min, followed by cooling at 4 °C until recovery of the samples. After amplification, PCR products were purified by agarose gel-electrophoresis and excised from agarose gel using spin columns (Gene Elute Agarose spin columns-SIGMA). Sequence analysis was performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit for both strands, and the sequences were aligned with the MT Navigator software (Applied Biosystems). All the sequencing reactions were purified by gel filtration through columns containing Sephadex G-50 (Pharmacia) equilibrated in double-distilled water and were analyzed on the 310 Genetic Analyzer (Applied Biosystems) on ABI 310 automatic sequencer (Applied Biosystems). The resulting calmodulin region sequences of all the isolates were aligned by the clustal method with the DNAMAN programme (Lynnon BioSoft).

Primer design and testing

The species-specific primers were designed using Primer Express software (Applied Biosystems). PCR products were resolved in 2% Tris–acetate–EDTA–agarose gel and were visualized with ethidium bromide and ultraviolet illumination. Images were captured and stored using a Kodak EasyShare DX3215 Zoom Digital Camera. The PCR containing species-specific primers were set up with 1.25 U of *Taq Gold* DNA polymerase (Applied Biosystems) in 50 µl reaction mixtures, containing 15 pmol of each outside primer, 12.5 µm of each deoxynucleoside triphosphate (Applied Biosystems), and 1 µl (approximately 10 ng) of fungal template DNA. Reactions were performed using the following PCR conditions: denaturation at 95 °C for 5 min; 35 cycles of denaturation at 94 °C for 50 s; annealing at 58 °C for 50 s; extension at 72 °C for 1 min; final extension at 72 °C for 7 min, followed by cooling at 4 °C until recovery of the

samples. Amplification products were checked on 2% agarose gel stained with ethidium bromide.

Results

Sequence alignment

In order to select appropriate variability in nucleotide sequences among the main *Aspergillus* species (*Nigri* section) occurring on grapes, 30 strains were sequenced: 10 of *A. niger* group, 10 of *A. carbonarius* and seven of *A. japonicus* and three of *A. aculeatus* from different geographical areas.

DNA extracted from the 30 strains was amplified by PCR with calmodulin CL1 and CL2A primers (O'Donnell et al., 2000) resulting in approximately 700 bp fragments (data not shown). The amplification products of all isolates were sequenced in both directions. The visual inspection of the aligned calmodulin partial gene sequences readily identified unique regions within the amplified fragment. In the calmodulin sequences, we observed 99.98% identity for strains of *A. carbonarius*, and 99.40% identity for strains of *A. japonicus* and *A. aculeatus*, although only one strain of *A. japonicus* (ITEM 4497) differed from the others with a homology of 96% (Figure 1). Moreover, the results of sequencing showed regions useful for designing specific primers for *A. carbonarius* and *A. japonicus*/*A. aculeatus* group (Figure 2). Among the *A. niger* aggregate, sequence alignment of the calmodulin partial gene did not reveal good regions for designing a specific primer due to the high sequence variability found for this species/group (89% of identity among the *A. niger* strains analyzed).

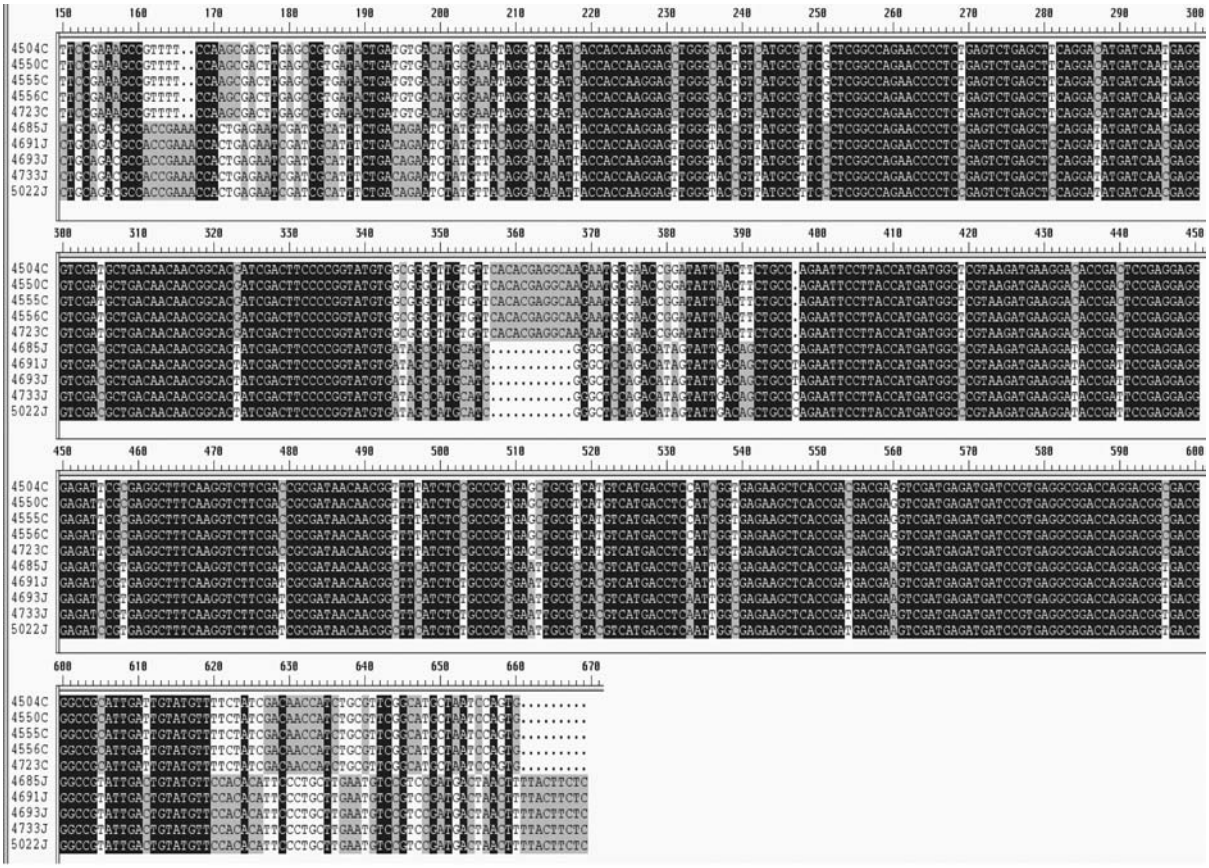

Figure 1. Alignment of partial calmoduline gene sequences of 10 representative strains of *Aspergillus carbonarius* and *Aspergillus japonicus*: black background represents region showing 100% of homology.

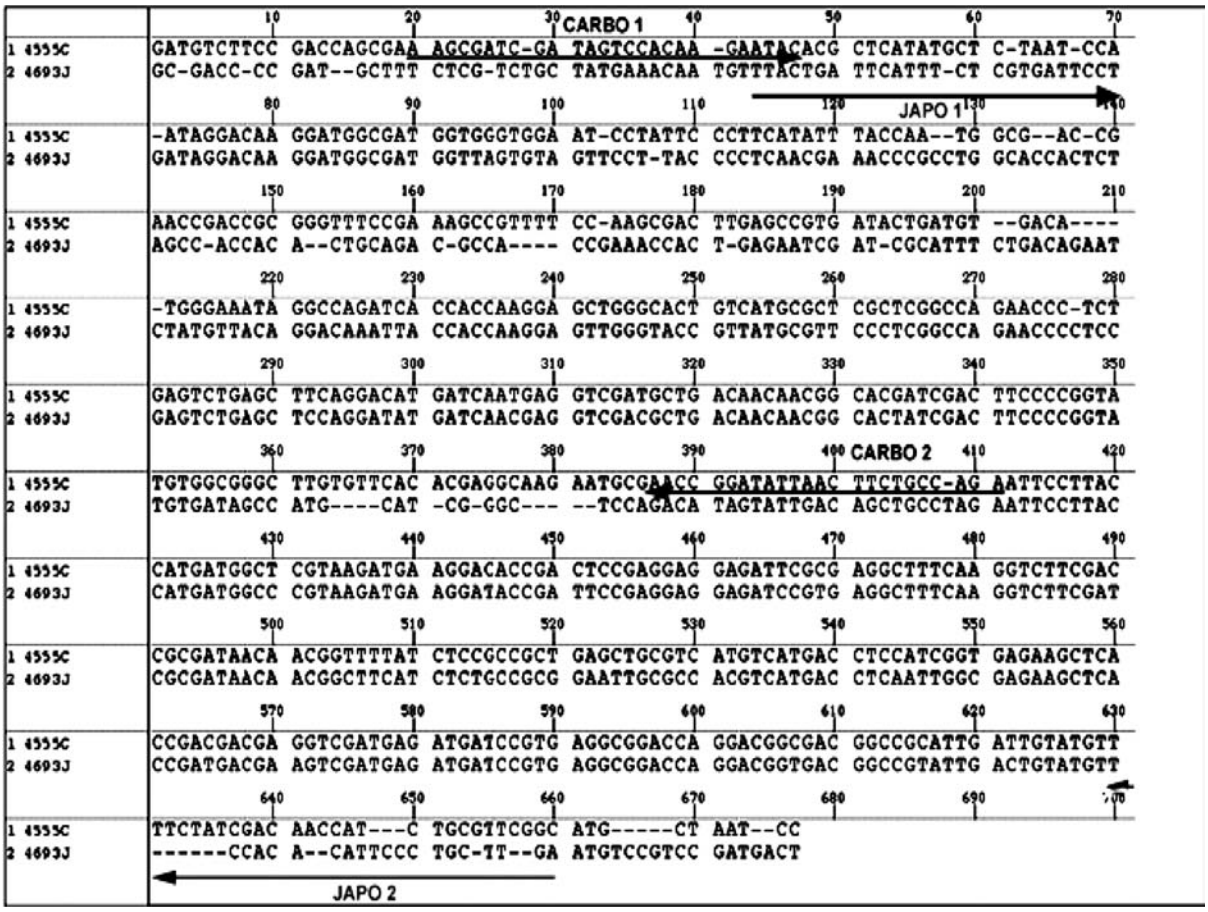

Figure 2. Alignment of partial calmoduline gene sequences from *Aspergillus carbonarius* (4555C) and *Aspergillus japonicus* (4693J), with locations of primer sites specific for the two species: arrows represent the site of primer sequences and are named respectively CARBO1/2 and JAPO1/2.

Primer design and testing

Using the sequence variation founded in the calmoduline region (Figure 1), a specific set of primers for *A. carbonarius* and *A. japonicus/aculeatus* was derived. All primers were designed to operate at high annealing temperatures (58 °C), thereby preventing the co-amplification of non-specific target of DNA. Species-specific primers were chosen with divergency located in 3′ among species considered in this study. In particular for primer JAPO1 a specific primer was chosen with a mismatch located in 3′.

The species-specific primers designed for *A. carbonarius* and *A. japonicus/aculeatus* are listed in Table 3. Specific primer pairs CARBO1/2 amplified a fragment of 371 bp in *A. carbonarius* and JAPO1/2 a fragment of 583 bp in *A. japonicus* (Figure 3a and b). The specificity of the primer pairs was tested against isolates of the target species (30 strains each of the two species studied) and a range (42 strains) of other closely related species within the black Aspergilli. Strains of *Aspergillus japonicus* were not amplified by the *A. carbonarius*-specific PCR primers (CARBO1/2) (Figure 3a), and strains of *A. carbonarius* were not amplified by *A. japonicus*-specific PCR primers (JAPO1/2) (Figure 3b). None of the *Aspergillus* section *Nigri* were amplified by the two sets of primers, with the exception of the four strains of *A. aculeatus* which were amplified by the specific primers JAPO1/2 (Figure 3b). No amplifications were obtained with the other main fungal/yeast species occurring on grapes, including *Botrytis cinerea*, *Saccharomyces*

Table 3. Sequences of the two designed primer sets specific for *A. carbonarius* and *A. japonicus* respectively

Primer name	Primer sequences	Species-specificity
CARBO1	5′-AAGCGAATCGATAGTCCACAAGAATAC	*A. carbonarius*
CARBO2	5′-TCTGGCAGAAGTTAATATCCGGTT -3′	
JAPO1	5′-TTACTGATTCATTTCTCGTGATYCCT -3′	*A. japonicus*
JAPO2	5′-TCAAGCAGGGAATGTGTGGA-3′	

cerevisiae and several toxigenic fungi such as some Fusarium species (Figure 3).

In order to confirm primer specificity, fragments derived from the species studied were sequenced. Comparison of the sequences obtained with the previous calmodulin sequences that had been used for primer design were 100% homologous, thus confirming the species-specificity of the primers.

Finally, the calmodulin sequences were deposited at the EMBL nucleotide sequence database and the relative accession numbers were obtained for the following strains: *A. carbonarius* ITEM 4556 (AJ582714) as a representative strain of ITEM 4166, 4200, 4209, 4504 (IMI 4185), 4550 and 5723, because they share 100% identity; *A. carbonarius* ITEM 4555 (AJ582715) that had unique sequences; *A. japonicus* ITEM 4693 (AJ582716) as a representative strain of ITEM 4691, 4833, 4685, 4733, 4998 and 4844 that share 100% identity; *A. japonicus* ITEM 4497 (AJ582717) that had unique sequences.

Sensitivity of detection of A. carbonarius *and* A. japonicus/A. aculeatus

The sensitivity of the PCR using the CARBO1/2 and JAPO1/2 primer pairs was examined using DNA from a pure fungal culture of *A. carbonarius* (ITEM 4722) and *A. japonicus* (ITEM 4690). In order to determine the minimum amount of fungal DNA needed to produce a visible band after PCR and gel electrophoresis a two-fold dilution series ranging from 20 to 1.25 ng and a ten-fold dilution series ranging from 1.25 to 12.5 fg per 25 µl PCR were performed. The smallest amount of fungal genomic DNA which led to a detectable product was 12.5 pg (Figure 4).

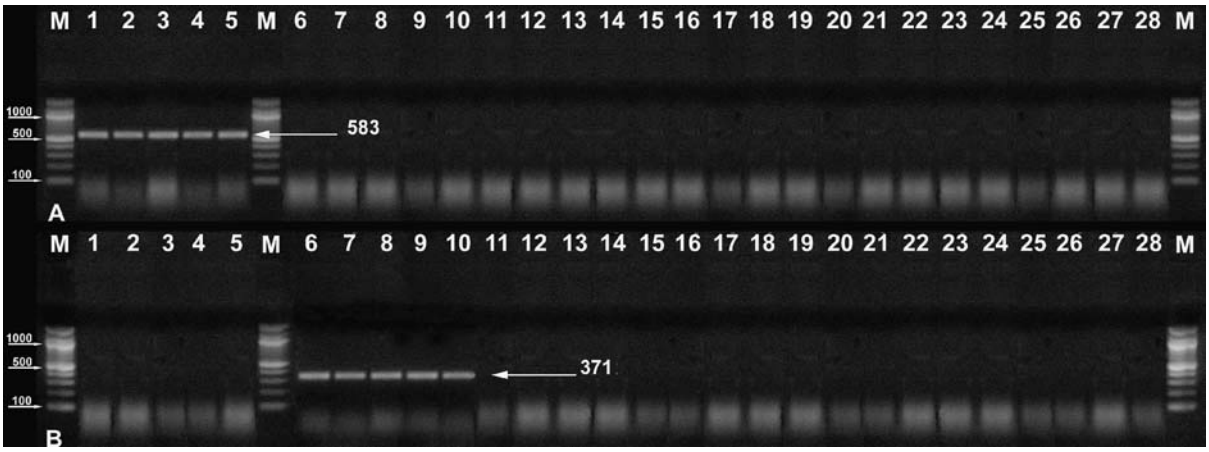

Figure 3. Rapid identification of *Aspergillus japonicus* (A) and *A. carbonarius* (B) using PCR primers (CARBO1/2 and JAPO1/2) designed from calmoduline gene sequences. Lane 1–3: *Aspergillus japonicus*; 4–5: *Aspergillus aculeatus*; 6–10: *Aspergillus carbonarius*; 11–14: *Aspergillus niger*; 15: *Aspergillus phoenicis*; 16: *Aspergillus helicothrix*; 17: *Aspergillus tubingenesis*; 18: *Aspergillus ellipticus*; 19: *Aspergillus foetidus*; 20: *Aspergillus foetidus* var. *acidus*; 21: *Aspergillus foetidus* var. *pallidus*; 22: *Aspergillus awamori*; 23: *Aspergillus ochraceus*; 24: *Botrytis cinerea*; 25: *Saccharomyces cerevisiae*; 26: *Fusarium culmorum*; 27: *Fusarium proliferatum*; 28: negative control without DNA; M: Marker DNA Ladder Bio Labs New England Inc.

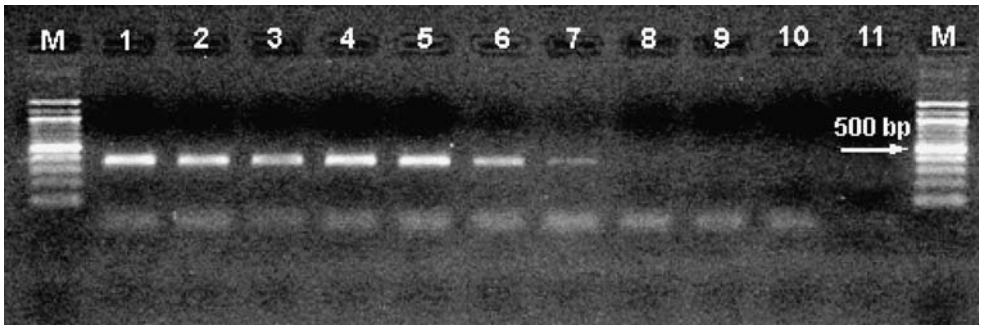

Figure 4. PCR with primers CARBO1/2 on a dilution series of *A. carbonarius* genomic DNA; lane 1: 20 ng, lane 2: 10 ng, lane 3: 5 ng, lane 4: 2.5 ng, lane 5: 1.25 ng, lane 6: 125 pg, lane 7: 12.5 pg, lane 8: 1.25 pg, lane 9: 125 ft, lane 10: 12.5 ft, lane 11: negative control (no DNA). Lane M: molecular mass marker (100-bp DNA Ladder Bio Labs New England Inc.).

Discussion

This study was focused on *A. carbonarius*, *A. aculeatus* and *A. japonicus* in order to develop molecular markers useful to discriminate these species. Our primers proved to be a useful tool for rapid and correct identification within this complex *Aspergillus* taxonomic section. The results obtained showed a high specificity of the CARBO primers in identifying *A. carbonarius* strains, since none of the other closely related species was amplified. These findings could be very important because *A. carbonarius* is considered the main cause of OTA in grapes and wine (Battilani and Pietri, 2002). The PCR assay can be used to identify these species without the need for morphological analysis and consequent risks of misidentification. In addition, this method is suitable for investigations that involve a large number of isolates because the DNA extraction is rapid and PCR amplification is simple. Serial dilutions of fungal genomic DNA revealed that at least 12.5 pg of fungal DNA is necessary to produce a visible product after PCR and electrophoresis. This amount corresponded to about 260 haploid fungal genomes. This calculation was based on the estimate that the size of a haploid genome of the *A. niger* is 37.5 Mbp, corresponding to a theoretical genomic weight of 4.5×10^{-8} μg (van Peij et al., 2002).

The JAPO primer set was shown to be highly specific since it did not amplify closely related *Aspergillus* section *Nigri* species, while such primers amplified the *A. aculeatus*. *Aspergillus aculeatus* was previously described as a variety of *A. japonicus* (Al Musallam, 1980); however, the further division of these two taxa into separate species has been debated (Kozakiewicz, 1989; Samson, 1992). Molecular analysis such as RFLP on rDNA, sequences of internal transcribed spacers (ITS1, ITS2) and 5.8 S rRNA gene sequences were not able to distinguish strains of these two species (Parenicova et al., 2001). In contrast, mtDNA RFLP patterns of *A. japonicus* and *A. aculeatus*, and their secondary-metabolites profiles, could differentiate the two taxa (Parenicova et al., 2001; Hamari et al., 1997). Our results support the view that these two taxa could be the same species since we obtained a high sequence similarity of the partial calmodulin gene (99.4% homology in sequence alignment) among strains of *A. japonicus* and *A. aculeatus*. It is unambiguous that the JAPO primers discriminate these two taxa from other members of the *Aspergillus* section *Nigri* species, and together with the CARBO primers could be a useful tool for investigations of a high number of unidentified strains isolated from grapes. As far as we are aware, this is the first report on species-specific primers for these two *Aspergillus* species obtained from DNA sequences of a calmodulin gene. The availability of reliable molecular markers for *Aspergillus* section *Nigri* occurring on grapes is of great interest since an early diagnosis of *A. carbonarius* occurrence in the field would provide important information on possible OTA contamination in grapes. In fact, all the *A. carbonarius* strains tested were OTA producers (data unpublished). Moreover, in this respect, such primers could be of a great use for further development of PCR assays from vegetative material. Finally, the two sets of primers that we developed, CARBO and JAPO, providing a rapid

and robust tool for a correct evaluation of the black Aspergilli occurrence on grapes and the prediction of the related risks of OTA contamination on grapes and by-products.

In addition, the PCR products amplified by these two sets of primers have a different molecular weight (372 and 583 bp). This is an indispensable characteristic for possible development of a multiplex PCR assay.

Acknowledgements

This work was supported by the European Union through project QLK1-CT-2001-01761. We thank Filomena Epifani, for her valuable technical assistance, Zofia Kozakiewicz (CAB International Mycological Institute, Kew, Surrey, UK) for morphological identification of some strains; Paola Battilani (Catholic University Piacenza, Faculty of Agriculture, Italy), Javier Cabañes (Facultat de Veterinaria, Universitat Autonoma de Barcelona, Spain), Armando Venancio (Departamento Engenharia Biologica, Universidade do Minho Portugal), Eleftherios Tjamos (Agricultural University of Athens, Faculty of Crop Sciences and Production, Greece), Amnon Lichter (Department of Postharvest Science, ARO The Volcani Center, Israel), Vicente Sanchis (Food Technology Department, Universitat de Lleida, Spain) and Ahmed Lebrihi (Ecole Nationale Supérieure Agronomique de Toulouse, France) for providing the strains used.

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