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**A rapid PCR-SSCP screening method for identification of *Aspergillus Sect. Nigri* species by the detection of calmodulin nucleotide variations**

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Additives/Contaminants:	Mycotoxins - fungi, Mycotoxins – ochratoxin A
Food Types:	Grapes, Coffee, Dried fruit, Cocoa

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1 **A rapid PCR-SSCP screening method for identification of *Aspergillus***

2 ***Sect. Nigri* species by the detection of calmodulin nucleotide variations**

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4 **ABSTRACT**  
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9 Single-stranded conformational polymorphism (SSCP) analysis for genetic diversity  
10 studies has been widely applied to indirectly detect sequence differences up to a single  
11 base in amplified DNA fragments of the same length, representing an alternative to  
12 gene-sequencing. In this study SSCP analysis was used to detect sequence variations  
13 contained in an about 180 bp-region of the calmodulin gene in order to identify  
14 *Aspergillus* Sect. *Nigri* species. The method described shows that fluorescence-based  
15 SSCP analysis by capillary electrophoresis (SSCP-CE) is cheaper and faster than direct  
16 sequencing, and suitable for computer-assisted analyses allowing discrimination  
17 between the *Aspergillus* species belonging to the *Nigri* Section: *A. aculeatus*,  
18 *Aspergillus* “atypic uniseriate”, *A. brasiliensis*, *A. carbonarius*, *A. ellipticus*, *A.*  
19 *foetidus*, *A. heteromorphus*, *A. ibericus*, *A. japonicus*, *A. niger*, and *A. tubingensis*.  
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40 **KEYWORDS:** *Aspergillus* Section *Nigri*, SSCP, species detection, calmodulin gene,  
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## 1 Introduction

2 *Aspergillus* species belonging to the *Nigri* section, commonly known as “black  
3 aspergilli” are distributed worldwide, many species cause food spoilage, and several are  
4 used in the fermentation industry (Bennett and Klich, 1992). Species within this Section  
5 have been widely studied in recent years since they were identified as the main species  
6 responsible for ochratoxin A (OTA) accumulation in grapes and wine (Cabanés et al.  
7 2002; Da Rocha Rosa et al., 2002; Battilani and Pietri, 2002). However, the taxonomy  
8 of this Section is still not completely resolved and the species distribution in food is  
9 often not clearly reported, as it is mainly based on morphological traits. This is more  
10 evident within the *A. niger* “aggregate” in which molecular studies support the division  
11 of this “aggregate” into two to four morphologically indistinguishable species: *A. niger*,  
12 *A. tubingensis*, *A. foetidus* and *A. brasiliensis* (Kuster van Someren et al., 1991; Accensi  
13 et al., 1999; Parenicova et al., 2000; Abarca et al., 2004). As *A. tubingensis* and *A.*  
14 *niger*, together with *A. carbonarius*, are the three species within the “black aspergilli”  
15 known to produce OTA, their exact identification is very important to avoid  
16 overestimation of specific contamination and toxicological risk problems (Perrone *et*  
17 *al.*, 2006). Molecular analysis on fungal DNA is indispensable to identify species  
18 within this Section clearly. In this respect, Single-Stranded Conformational  
19 Polymorphism (SSCP) analysis has been widely applied to detect Single-Nucleotide  
20 Polymorphisms (SNPs), including point variability, and consider useful polymorphic  
21 markers for genetic studies. First announced in 1989 as a new means of detecting DNA  
22 polymorphisms, or sequence variations, SSCP analysis offers an inexpensive,  
23 convenient, and sensitive method for determining genetic variation (Orita et al., 1989;  
24 Rath and Ansorg, 2000; Sunnucks et al., 2000).

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5 1 Although the exact identity of the polymorphism cannot be determined, SSCP can be  
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7 2 employed to detect and map unknown polymorphisms as they are co-dominant and can  
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9 3 be as robust as other sequence specific markers. SSCP indirectly detects sequence  
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11 4 differences to a single base in amplified DNA fragments of the same length. The SSCP  
12  
13 5 method requires no *a priori* knowledge of SNPs and can be applied to identify  
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15 6 polymorphisms in unknown strains. Polymorphisms are detected as alterations of  
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17 7 mobility induced by nucleotide differences that cause stable changes in the  
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19 8 conformation of the ssDNA. Therefore a single nucleotide change could dramatically  
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21 9 affect the strand's mobility through a gel by altering the intra-strand base pairing and its  
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23 10 resulting 3D conformation (Melcher, 2000).  
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30 12 The use of a fluorescence-based capillary electrophoresis system for SSCP analysis has  
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32 13 also contributed to the efficiency of the methodology and reliability of analysis.  
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35 14 The usefulness of the SSCP analysis for the identification of *Aspergillus* sect. *Nigri*  
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37 15 occurring on food has not been investigated until now, even if it was set up and  
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39 16 described by Rath and Ansorg (2000) for other medically important *Aspergillus*  
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41 17 species, analyzing the PCR-amplified intergenic spacer region.  
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47 19 The aim of this study is to provide a rapid, computer assisted, and relatively low cost  
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49 20 tool for the identification of *Aspergillus* species belonging to the *Nigri* Section by  
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51 21 amplifying, with unique pairs of fluorescent primers and PCR conditions, a species-  
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53 22 specific variable region of the calmodulin gene.  
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## 57 23 **Materials and methods**

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1 **Sequencing analysis.** The Calmodulin gene was previously sequenced in 107 (Perrone  
2 et al., 2004) and 16 (the standard strains in Perrone et al., 2006) isolates of *Aspergillus*  
3 spp., obtained from the ITEM culture collection from different geographical areas and  
4 hosts, belonging to *A. aculeatus*, *Aspergillus* “atypic uniseriate”, *A. brasiliensis*, *A.*  
5 *carbonarius*, *A. ellipticus*, *A. foetidus*, *A. heteromorphus*, *A. ibericus*, *A. japonicus*, *A.*  
6 *niger*, and *A. tubingensis*. Further information about the strains (year of isolation,  
7 depositor, toxin production, etc.) are available at the ITEM web site:  
8 <http://www.ispa.cnr.it/Collection>.

9  
10 Sequences were aligned using the Clustal-W method. A generic primer pair, able to  
11 amplify about 180 bp fragments in all *Aspergillus* spp. belonging to the *Nigri* section,  
12 was designed with Primer Express software version 2.0 (Applied Biosystems, Foster  
13 City, CA, USA): SSCPCL1 (5'-FAM-AGTCTGAGCTKCAGGACATGATCAA-3')  
14 and SSCPCL2 (5'-JOE-CGGAGTCGGTYTCCTTCATCTT-3'). The primer pair  
15 amplifies a species-specific variable region (figure 1).

16  
17 **SSCP conditions.** In this study 50 isolates were analysed: 11 type-strains (tab.1) as  
18 reference and 39 *Aspergillus* spp. strains to test the method (3 *A. aculeatus*, 6  
19 *Aspergillus* “atypic uniseriate”, 2 *A. brasiliensis*, 8 *A. carbonarius*, 2 *A. foetidus*, 2 *A.*  
20 *ibericus*, 2 *A. japonicus*, 7 *A. niger*, and 7 *A. tubingensis*).

21  
22 A single PCR reaction was performed for each strain using DNA purified from a pure  
23 fungal culture as template, with the EZNA Fungal DNA Miniprep Kit. PCR reactions  
24 were carried out on a 9700 thermalcycler (Applied Biosystems, Foster City, CA, USA)

1 in 20  $\mu$ l volume. Each mixture contained 5 pmol of each labelled primer, 0.5 U of DNA  
2 Taq-Gold polymerase (Applied Biosystems, Foster City, CA, USA), 1X PCR buffer,  
3 0.1 mM for each dNTP, approximately 3 ng of fungal template DNA. Amplification by  
4 PCR was optimized under standard reaction conditions: denaturation at 95 °C for 5 min;  
5 35 cycles of denaturation at 94 °C for 50 s; annealing at 60 °C for 50 s; extension at 72  
6 °C for 1 min; final extension at 72 °C for 7 min.

7  
8 Samples were analyzed on an ABI 310 Genetic Analyzer (Applied Biosystems, Foster  
9 City, CA, USA). All runs were conducted on a 36 cm capillary, containing 5%  
10 GeneScan polymer, with 10% glycerol in Tris-borate-EDTA (TBE), and TBE as  
11 running buffer. Injection was for 22 s at 1 kV and electrophoresis was at a constant  
12 voltage of 15 kV. The electrophoretic runs were conducted at 35°C. Each amplification  
13 product was diluted 1:20 with distilled water, denatured at 95°C for 5 min and snap-  
14 cooled on ice.

#### 16 **Data analysis.**

17 GeneScan Analysis software version 2.5 (Applied Biosystems, Foster City, CA, USA)  
18 was used to align peaks to the internal standard, ROX 500 (Applied Biosystems, Foster  
19 City, CA, USA), included in each run. Mobility values were assigned to internal  
20 standard fragments, which encompassed the peak region of interest. Analysis  
21 parameters were set to 4400–5500 scan numbers. The software then analyzed the  
22 unknown sample peaks and assigned them values based on the internal standard  
23 fragment migration rates. Eleven species type-strains were run 3 times each, to ensure  
24 run-to-run reproducibility. SSCP analysis patterns were exported to Genotyper software



1 (Applied Biosystems, Foster City, CA, USA), used to automate the data analysis of  
2 SSCP. The SSCP patterns for each species, which consisted of at least one retention  
3 time representative of each primer-labelled PCR product, were entered into a database.  
4 Type-strains (table 1) belonging to 11 species within the *Aspergillus* sect. *Nigri* were  
5 used as reference samples in identification. The type-strain SSCP patterns were defined  
6 as the average retention times relative to the 3 runs. Peaks with similar retention times  
7 were placed in the same category on the basis of a 0.1% position tolerance, as standard  
8 deviation within a species ranged from 0.3 to 2. This allows the retention times for an  
9 unknown organism to be classified into similar categories. Because of overlapping  
10 confidence intervals, some organisms could be classified together in the same  
11 categories, but this problem was resolved by considering matches of both strands which  
12 consist of unique combinations: e.g. The *A. carbonarius* and *A. ellipticus* reverse  
13 strands have very close retention times, and the *A. carbonarius* forward strand is more  
14 than 0.1% position tolerance from *A. ellipticus* (figure 2).

## 16 Results

17 In this study a generic primer pair was designed in the calmodulin gene for SSCP  
18 application considering that: (i) primers should bind in highly conserved regions of the  
19 gene and (ii) the sequence that lies within the forward and reverse primers should  
20 contain mismatches between the different targets to be differentiated. Using SSCPCL1  
21 and SSCPCL2 primers, 50 pure cultures of *Aspergillus* spp. sect. *Nigri* species were  
22 analyzed with fluorescence-based SSCP by capillary electrophoresis (CE). Because of  
23 their different sequences each strand is expected to yield different peak upon SSCP  
24 analysis.

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4 Figure 2 shows the electropherograms for 11 *Aspergillus* species belonging to sect.  
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1 Figure 2 shows the electropherograms for 11 *Aspergillus* species belonging to sect.  
2 *Nigri* (Table 1) amplified with SSCPCL1 and SSCPCL2. The *x*-axis shows peak  
3 mobility and the *y*-axis relative fluorescent intensity. A single peak for 6 of 11 species,  
4 2 peaks for 4 species and 3 peaks for *Aspergillus* “atypic uniseriate” were observed in  
5 the forward strand; a single peak for 10 of 11 species and 3 peaks for *Aspergillus*  
6 “atypic uniseriate” were observed in the reverse strand. Results were similar for other  
7 strains analyzed, therefore electropherograms of the remaining 39 strains are not shown.  
8 Identifications were made by visually and computer assisted comparing peak mobility.  
9 Data analysis was performed with Genotyper software which allowed fully automated  
10 comparison of blind sample mobility to reference samples (type-strains). To this  
11 purpose a macro in Genotyper software was set up on analysis of the mobility of both  
12 strands.

13  
14 Using this protocol all 39 genotypes belonging to 11 of the 16 *Aspergillus* Sect *Nigri*  
15 were correctly identified, referring to ITEM collection related species information and  
16 to their previous molecular identification (Perrone et al., 2006).

## 17 18 **Discussion**

19 We report the application of a single-stranded conformation polymorphism/capillary  
20 electrophoresis (SSCP/CE) assay for the rapid identification of *Aspergillus* species of  
21 the *Nigri* Section.

22  
23 Until now the species identification in the *Nigri* Section has been still essentially based  
24 on observation of macroscopic and microscopic features, which is time-consuming and

1 requires expertise. Some molecular tools for PCR-based diagnosis have been developed  
2 to detect and identify isolates of *A. ochraceus* (Schmidt et al., 2003), *A. carbonarius*  
3 (Schmidt et al., 2004, Pellegrinelli-Fungaro et al., 2004; Mulè et al., 2006), *A.*  
4 *carbonarius*, *A. japonicus/aculeatus* (Perrone et al., 2004), *A. carbonarius/A. ochraceus*  
5 (Patino et al., 2005; Gonzales-Salgado et al., 2005). Since they consist of species-  
6 specific assays, which should be performed individually for each species under different  
7 PCR conditions, they are not always suitable for investigation of multiple species  
8 contamination. Species-specific assays have the advantage that they can be utilized to  
9 directly analyze environmental samples, but they are not yet available for all *Aspergillus*  
10 sect. *Nigri* species. In contrast, the SSCP protocol set up in this study can only be  
11 utilised to analyze pure cultures, but is rapid tool to identify closely related species that  
12 are morphologically indistinguishable. The results of SSCP analysis can be obtained  
13 within 30 min of PCR, and with the new capillary electrophoresis systems on the  
14 market, 8 or 16 samples can be run at one time, further increasing the turnaround time.  
15 Moreover some preliminary tests, made starting from environmental samples, were  
16 successful (data not shown), but PCR condition optimization is needed.

17

18 Finally, the automated sequencer enables quick and easy computer analysis, allowing  
19 the generation of a database for comparison. SSCP analysis, excluding common initial  
20 purchase of the equipment, is a cheaper and faster alternative to sequencing in order to  
21 estimate sequence variation useful for genotype identification. It consists of a simple  
22 PCR reaction with labelled generic primers amplifying DNA from all target species  
23 under unique conditions.

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1 SSCP analysis takes information from the mobility of single stranded DNA in gel  
2 electrophoresis which is dependent on strand size and length and is noticeably affected  
3 by very small changes in nucleotide sequences, possibly with a resolution of one  
4 changed nucleotide out of several hundred.

5  
6 The success of this method was demonstrated by its ability to identify organisms  
7 comparing SSCP patterns of blind samples to others previously entered in the reference  
8 database and its ability to recognize new patterns, further investigable by sequencing  
9 only few representative samples. Therefore most DNA sequence variations can be  
10 detected with relatively little sequencing.

11  
12 The CE-SSCP protocol set up in this study allows the rapid and reliable identification of  
13 the different *Aspergillus* spp., based on analysis of the calmodulin gene sequence.

14 The SSCP assay set up in this study offers a potential diagnostic test able to identify  
15 11/16 of the *Aspergillus* Sect *Nigri* species by a single reaction with a single primer pair  
16 under the same PCR conditions. The 5 species (*A. costaricaensis*, *A. homomorphus*, *A.*  
17 *lacticoffeatus*, *A. sclerotioniger*, *A. vadensis*) recently identified by Samson et al. (2004)  
18 inside the Section *Nigri* rarely occur in food and therefore have not been included in this  
19 study. However, a species-specific variability for them has also been found in the  
20 calmodulin nucleotide sequence (Varga et al., 2007).

21  
22 In conclusion, the SSCP protocol offers a sensitive, inexpensive, rapid, and convenient  
23 method to determine differences in sequences in a high number of samples, so that only  
24 a few informative samples need be sequenced. Moreover it should be a useful tool for

1 the rapid screening of a large number of *Aspergillus* spp. strains and for the assessment  
2 of their potential ochratoxigenic risk in food.

3

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9

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**Table 1** Type-strains of *Aspergillus* Sect. *Nigri* used.

ITEM**	Species	Calmodulin gene (Acc. Number)
4497	<i>A. japonicus</i>	AJ582717
7040	<i>A. tubingensis</i>	AJ964876
4502	<i>A. niger</i>	AJ964872
4503	<i>A. carbonarius</i>	AJ964873
4505	<i>A. ellipticus</i>	AM117809
4507	<i>A. foetidus</i>	AM419749
4693	<i>A. atypic</i> "uniseriate" *	AJ582716
6600	<i>A. ibericus</i>	AJ971806
7046	<i>A. aculeatus</i>	AJ964877
7045	<i>A. heteromorphus</i>	AM421461
7048	<i>A. brasiliensis</i>	AM295175

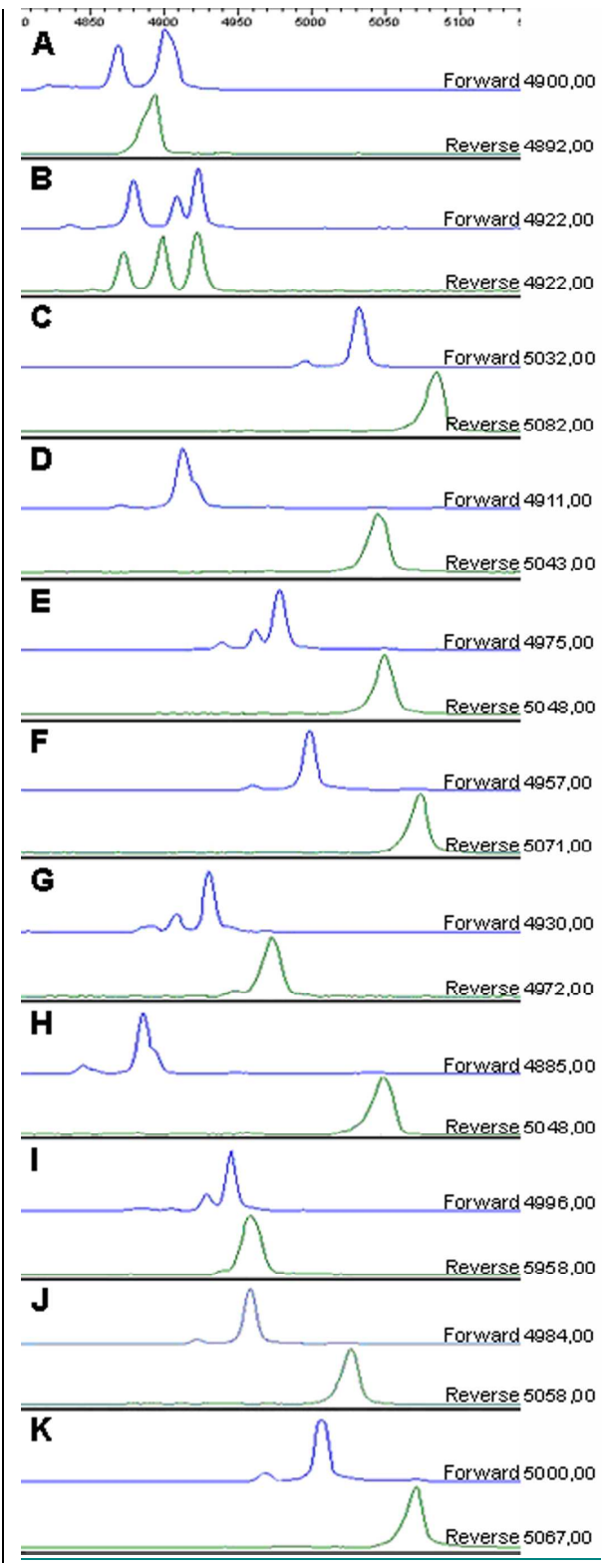
\*(Perrone et al., 2006)

\*\*ITEM: Culture Collection of Agri-Food Important Toxigenic Fungi, ISPA-CNR.



38 **Fig. 1** Multiple alignment of PCR product amplified with primer SSCPCL1 and SSCPCL2, including species –specific variable region in the calmodulin gene.  
39 Only nucleotide differences are shown. Arrows correspond to the annealing-regions of primers.  
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4 **Fig. 2** Panels show SSCP-profiles of the 11 *Aspergillus* species identifiable by the SSCP approach.  
5 Strains evidencing clearly different migrations due to variations in sequence. The profiles were  
6 respectively of *A. aculeatus* (A), *A. atypic* “uniseriate” (B), *A. brasiliensis* (C), *A. carbonarius* (D),  
7 *A. ellipticus* (E), *A. foetidus* (G), *A. heteromorphus* (G), *A. ibericus* (H), *A. japonicus* (I), *A. niger*  
8 (J), *A. tubingensis* (K). Values on the right indicate retention time of discriminant peaks.

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