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Detection of *Aspergillus carbonarius* and other black aspergilli from grapes by DNA OLISA™ microarray

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5 1 **Detection of *Aspergillus carbonarius* and other black aspergilli**
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8 2 **from grapes by DNA OLISA™ microarray**
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4 **Abstract**
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9 Black aspergilli, and particularly *Aspergillus carbonarius*, are responsible for ochratoxin A
10 production in grapes. Correct identification of these species is essential for toxicological risk
11 assessment in grape and wine. Low-complexity oligonucleotide microarray (OLISA™,
12 Apibio, F) based on DNA oligonucleotides probes, obtained from sequences of the
13 calmodulin gene, was set up in order to detect *A. carbonarius*, *A. japonicus/A. aculeatus* and
14 *A. ibericus* isolated from grape. The designed microarray distinguished all *Aspergillus* species
15 and the detection limit for *A. carbonarius* was 3.2 pg of DNA, as template for the PCR
16 reaction. This microarray offers a quick and parallel analysis to detect individual *Aspergillus*
17 species in pure cultures and in naturally contaminated grapes samples.
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35 **Keywords:** microarray, *Aspergillus carbonarius*, grape, oligonucleotides, ochratoxin A,
36 DNA microarray
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1 Introduction

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9 The presence of black aspergilli in grape is usually linked to the occurrence of ochratoxin A
10 (OTA) in grapes and wine. *Aspergillus carbonarius* is highlighted as the main species
11 responsible for OTA production in grapes with a high percentage of OTA-producing strains
12 (Bau et al. 2005; Leong et al. 2004; Serra et al. 2003; Sage et al. 2002, 2004). Ochratoxin A
13 production was also found in *A. niger* aggregate, but at a much lower rate, whereas *A.*
14 *japonicus* and *A. aculeatus* do not produce OTA (Abarca et al. 2004; Perrone et al. 2006a).
15 Recently *A. ibericus* also belonging to the *Nigri* section, not producing OTA and
16 morphologically similar to *A. carbonarius*, has been characterized and isolated from wine
17 grapes and/or dried vine fruit in Portugal and Spain (Serra et al. 2006). Due to the different
18 ability of the *Aspergillus* species to produce OTA, an accurate identification is very important
19 particularly for epidemiological studies and for toxicological risk assessment in grapes and
20 wine.

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40 In recent years, numerous DNA-based methods have been developed to improve the
41 identification of ochratoxigenic fungi occurring in foodstuffs (Abarca et al. 2004; Niessen et
42 al. 2005; Castella et al. 2002): in particular techniques based on polymerase chain reaction
43 (PCR) mostly investigating on multiple gene sequences or anonymous fragments (Perrone et
44 al. 2006 a,b), on rDNA, including internal transcribed spacers (ITS) (Accensi et al. 1999;
45 Martinez-Culebras et al. 2007), or on structural genes (Perrone et al. 2004, Susca et al. 2007
46 a,b). Real-time PCR systems for quantitative analysis of ochratoxigenic *A. carbonarius* have
47 also been developed (Mulè et al. 2006; Atoui et al. 2007). These PCR detection methods were
48 designed to detect single or very few species.

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7 2 Alternatively, microarray technology may be a useful technique for identification and
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9 3 differentiation of high numbers of microorganisms in parallel (Call et al. 2003). In arrays of
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11 4 genus-, species- or subspecies specific oligonucleotides are immobilised on a solid support in
12
13 5 order to bind its complementary sequence, included in a DNA fragment such as a PCR
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15 6 product, in a highly specific and reversible manner.
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21 8 This technology was originally developed as a technique to screen for human genetic
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23 9 disorders (Saiki et al. 1989), but has also been successfully applied to detect and identify
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25 10 human and animal pathogens (Fiss et al. 1992; Anthony et al. 2000; González et al. 2004). In
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27 11 plant pathology, DNA microarray technology has been successfully applied to identify
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29 12 oomycete, nematode and bacterial pathogen DNA from pure cultures (Lévesque et al. 1998;
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31 13 Uehara et al, 1999).
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37 15 Recently Nicolaisen et al. (2005) and Kristensen et al. (2007) designed and tested an
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39 16 oligonucleotide microarray, based upon *Fusarium* ITS2 rDNA sequences, for identification
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41 17 and differentiation of trichothecene producing and non-producing *Fusarium* species occurring
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43 18 on cereal grain and for detection of toxigenic *Fusarium* species, respectively. The aim of this
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45 19 study was to set up a new microarray format to identify and detect most black aspergilli
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47 20 occurring on grapes. To this end the Apibio biochip OLISA™ (OLigo Sorbent Arrays),
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49 21 usually applicated for medical diagnosis, in monitoring and testing gene expression,
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51 22 genotyping studies (Bufflier et al. 2003; Guerniou et al. 2004) and protein identification
52
53 23 (Perrin et al. 2003), was tested for diagnosis of *Aspergillus* spp. mainly occurring in the
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55 24 grapes: *Aspergillus carbonarius*, *A. japonicus/A. aculeatus* and *A. ibericus*.
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7 2 In particular OLISA™ are microarrays including of up to 17 probes arrayed on the well
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9 3 bottom of a 96-well microtiter plate, using a proprietary surface chemistry. DNA could be
10
11 4 simplex or multiplex PCR amplified to generate biotin-labelled fragments and revealed by
12
13 5 colorimetric detection, analyzed by the automated Apimager™ system (Apibio, Grenoble,
14
15 6 France), giving an easy-to-use final report of results.
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21 8 **Material and methods**

23 9 *Fungal strains and sequence analyses*

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26 10 All strains were obtained from the culture collection (ITEM) of the National Council of
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28 11 Research - Institute of Sciences of Food Production. Further information about the strains
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30 12 (year of isolation, depositor, toxin production, etc.) are available at the ITEM web site:
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32 13 <http://www.ispa.cnr.it/Collection>.
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37 15 Calmodulin nucleotide sequences of the thirty strains, belonging to four *Aspergillus* species
38
39 16 mainly occurring on grapes (ten of *A. niger* group, ten of *A. carbonarius*, seven of *A.*
40
41 17 *japonicus* and three of *A. aculeatus*) previously sequenced by Perrone et al. (2004), were
42
43 18 analyzed. Moreover three strains of *A. ibericus* (ITEM 4776, ITEM 6601, ITEM 6602) were
44
45 19 sequenced and included in this study. Total DNA extracted from pure fungal culture: ITEM
46
47 20 4500 (*A. tubingensis*, belonging to Niger group), ITEM 4502 (*A. niger*), ITEM 4503 (*A.*
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49 21 *carbonarius*), ITEM 5019 (*A. japonicus*) and total DNA extracted from two natural samples
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51 22 (red grapes) were tested for specificity and the sensitivity of the selected probes.
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59 24 *DNA isolation*

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4 1 Protocols used for DNA extraction from pure fungal culture, the calmodulin gene
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6 2 amplifications and sequencing are the same as previously described by Perrone et al. (2004).
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9 3 Purification of DNA from natural samples (grapes berries) was set up combining the
10
11 4 extraction methods of Steenkamp et al. (1994), Kim et al. (1997) and Loureiro (1998). In
12
13 5 detail: a portion of fresh and frozen berries (300 mg) was weighed and incubated with 1.5 ml
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15 6 extraction buffer (1M Tris-HCl Ph 8, 1.4 M NaCl, 20 mM EDTA, 3% CTAB) and 15 µl β-
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17 7 mercaptoethanol for 90 min. at 65°C under constant agitation. After incubation, samples were
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19 8 centrifuged at 6500 rpm in a Biofuge Fresco centrifuge (Heraeus) for 5 min. at 4°C and the
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21 9 supernatant was collected in a fresh tube. One volume of chloroform/isoamyl alcohol (24:1)
22
23 10 was added and samples were mixed and centrifuged at 6500 rpm for 20 min. at 4°C. The
24
25 11 upper aqueous phase was transferred into another tube, adding 0.1 volume of 10% CTAB.
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27 12 Again, 1 volume of chloroform/isoamyl alcohol (24:1) was added and samples were mixed
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29 13 and centrifuged at 6500 rpm for 20 min. at 4°C. The upper aqueous phase was transferred into
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31 14 another tube, adding 0.1 volume of cold 2-propanol. Samples were then incubated for 60 min
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33 15 at -80°C and centrifuged for 20 min at 13000 rpm. The pellet was dissolved in 300 µl of
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35 16 sterile H₂O and processed according to the EZNA Fungal DNA Miniprep Kit protocol,
36
37 17 starting from step 8 of protocol B. The nucleic acid purification kit is based on a Hi-bond[®]
38
39 18 spin column technology to eliminate polysaccharides, phenolic compounds and enzyme
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41 19 inhibitors from lysed tissues. In the final steps, DNA was eluted in 100 µl of deionised H₂O.
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51 *Polymerase Chain Reaction amplifications*

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53 22 Two amplification strategies were compared to produce DNA target, useful for discrimination
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55 23 of different *Aspergillus* species simultaneously:
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5 1 - The generic PCR approach: amplification of all *Aspergillus* spp. using consensus
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7 2 conserved PCR primers (CL-1/CL-2A, O'Donnell et al. 2000). This approach allows the
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9 3 setting up of a PCR reaction with a single primer pair. An incorrect evaluation can occurs
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11 4 with this method when target is present at lower level than others, because it could suffers
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13 5 for primers competition and be down-amplified compared to predominant targets.
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16 6 - The specific PCR approach: amplification of single target species, using species-specific
17
18 7 PCR primers (CARBO-1/2 and JAPO1/2, Perrone et al. 2004). This approach necessitates
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20 8 availability of as many primer pairs as target species to be investigated.
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23
24 9 The primers sequences used are summarized in Table 1. PCR amplification conditions
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26 10 were as follows: up to 10 ng DNA was amplified in 100µL final volume using 5'biotine-
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28 11 labelled oligonucleotides primers. The reaction mix contained 30 pmol of each primer,
29
30 12 200µM dNTPs and 2.5 U Taq polymerase (Takara Taq) from Cambrex, with the associated
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32 13 buffer. The program was set up on the Applied Biosystem 2700 Thermocycler as follows: 10
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34 14 min. at 94°C, followed by 35 cycles of [50 sec. at 94°C, 50 sec. at 55°C (generic
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36 15 primers)/58°C (specific primers), 1 min. 72°C] and a final elongation of 7 min. at 72°C.
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42 *DNA hybridization of PCR amplicons*

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44 18 The OLISA™ biochip consists of a series of 17 oligonucleotide probes deposited as spots on
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46 19 the bottom of each of 96-wells in microtiterplate (Figure 1), spotted in duplicate
47
48 20 symmetrically on the right and left hand side. The specimen is the amplicon amplified with
49
50 21 biotin labelled primers, denaturated and hybridised on the chip. Short oligonucleotide probes
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52 22 are designed and optimized to hybridize specifically to any single nucleotide polymorphism
53
54 23 of an amplified DNA target. The pattern of spots allows automated identification of the
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56 24 species present in the specimen. Detection is based on hybridization between probe and
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1 biotin-labelled target (amplicon). Hybridization is detected through streptavidin-horse radish
2 peroxidase conjugate that binds to the hybridised target, and subsequently create a coloured
3 precipitate at the position of the probe (Apibio SAS, Grenoble, F).

4 The PCR products (5 μ L) were denaturated for 5 min. with 0.2 M NaOH (OLISA™
5 denaturation solution), and hybridised 1 hour at 37°C on the chip together with OLISA™
6 hybridisation buffer. OLISA™ biochips were then washed 4 times with OLISA™ washing
7 buffer using a microtiterplate washer (Colombus, Tecan, CH). After washing, the hybridised
8 probes were detected by mean of a streptavidin-HRP conjugate, combined with a colorimetric
9 substrate (TMB) which generate a blue spot where hybridisation occurred. A streptavidin-
10 HRP conjugate (OLISA™ detection solution) was incubated on the chip at room temp for 10
11 min., and then washed. Finally, 50 μ L of TMB substrate (OLISA™ staining solution) was
12 added on the chip, and incubated for 10 min. to at 25°C. Finally images were taken using the
13 Apimager System (Apibio, Grenoble, F). Spot density is analysed by calculation of the
14 average grey level of each spot minus that of the average well-bottom background, and the
15 result is expressed in Arbitrary Unit (A.U.).

17 *Design of specific probes*

18 Probes (Table 2) were designed based on phylogenetic analyses of the calmodulin gene in 33
19 strains belonging to different *Aspergillus* spp., sequenced in the present study and previously
20 by Perrone et al. (2004). As the species-specificity in the calmodulin gene of *Aspergillus* spp.
21 was already established in Perrone et al. (2004 and 2006 a,b), nucleotide sequences were
22 compared using the Clustal W method, in order to select appropriate species-specific
23 oligonucleotides. The visual inspection of the aligned sequences allowed identification of
24 unique regions, useful to design oligonucleotides specific at the species level. Eight probes

1 were designed based on the alignment, two for each *Aspergillus* species mostly occurring in
2 grapes (Table 2), and adapted in OLISA™ microarray: JA1 and JA2 (positions 2 and 16,
3 figure 1) specific for the group *A. japonicus/A. aculeatus*, C1 and C2 (positions 6 and 7)
4 specific for *A. carbonarius*, aC1 and aC2 (positions 8 and 9) specific for *A. ibericus*, CaC1
5 and CaC2 (positions 4 and 5) specific for the group *A. carbonarius/A. ibericus* (Table 2).
6 Oligonucleotides for *A. niger* was not designed because sequences of calmodulin gene
7 showed an high grade of variability, reflecting its complex taxonomy.

8
9 Probes range from 16 to 27 nucleotides in length and present a species specific nucleotide
10 variability at different levels: unique polymorphisms (at least one mismatch) were localized
11 manually at 5'-end, 3'-end, centred, and distributed (data not shown). The length of
12 oligonucleotides was adjusted to give a Tm in the wide range 44°C-72°C (Table 2), calculated
13 using the Primer Express software (Applied Biosystems, Foster City, CA, USA). The ability
14 to form dimers and hairpin structures was checked using the same program, and
15 oligonucleotides with the lowest tendency to form such structures were chosen. Blast
16 similarity searches were conducted with Genbank program BLAST (Altschul et al. 1990) by
17 using all of the probes from the array as queries against the the National Center for
18 Biotechnology Information (NCBI) database, to further ensure that the proposed probes were
19 complementary with the target species.

20 21 *Specificity of DNA hybridization assay*

22 Hybridizations between probes and amplicons or primers were done as previously described.
23 Hybridization were done at least three-time to confirm the result and specificity of the probes.

24

1 To evaluate the variability in hybridization patterns against other species, PCR amplicon of *A*
2 *japonicus* (ITEM 5019), *A. carbonarius* (ITEM 4503), *A. niger* (ITEM 4502) and *A.*
3 *tubingiensis* (ITEM 4500) were hybridized to the DNA array. Amplifications were performed
4 according to the specific and generic approach.

6 *Environmental sample testing*

7 For a preliminary test of the ability of the microarray to detect individual *A. carbonarius*, *A.*
8 *japonicus/A. aculeatus* and *A. ibericus* species in environmental samples, two natural grapes
9 samples were investigated according to the generic and specific PCR approach. Two different
10 amounts, 1/20 and 1/10 of total nucleic acids isolated from 300 mg of grapes, were evaluated
11 as template in the PCR step, and used for detection on the OLISA™ array.

12
13 *Aspergillus* spp. qualitative contamination was also evaluated by microbiological analysis,
14 observing fungal growth in 3 berries half-cut and placed on Dichloran-rose Bengal (King et
15 al. 1979), for 5 days in the dark.

17 **Results**

19 *Specificity of DNA hybridization assay*

20 Sequence variability analysis of the calmodulin gene in *Aspergillus* spp. (sect. *Nigri*) mostly
21 occurring in grapes allowed identification of unique regions specific at the species level. Two
22 probes for each species were designed and adapted in the OLISA™ microarray (Table 2). To
23 identify sources of potentially false positive signals the BLAST search was done for all
24 proposed probes. The results showed highest hits with target species.

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7 2 Figure 2 shows specificity test results obtained from amplifications of *A. carbonarius* and *A.*
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9 3 *japonicus* DNA separately, using the two different approaches. Using *A. carbonarius* specific
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11 4 primers (A column), typing was correct, except for probe CaC1, specific for *A. carbonarius*
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13 5 and *A.ibericus*, which cross reacted with *A. japonicus* PCR product. Specifically, PCR
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15 6 reaction in that case was set up with *A. japonicus* DNA as template, but no PCR product was
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17 7 expected for it. Therefore a cross-hybridization occurred between probe CaC1 and primers
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19 8 (Carbo-1 or Carbo-2). This is confirmed by the same result in the no template PCR control.
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21 9 Moreover a weak signal was also observed between primers (Carbo-1 or Carbo-2) and probe
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23 10 C2. In that case the result was not obtained in the control, giving evidence of the non
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25 11 specificity and irreproducibility of that binding. Using *A. japonicus* specific primers (B
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27 12 column), typing was correct for both probes (JA1 and JA2), but no specificity appeared for
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29 13 probe JA2, which cross reacted with primers (Japo-1 or Japo-2), as demonstrated by the
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31 14 signals that appeared in the hybridization with no product of PCR set up without template or
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33 15 *A. carbonarius* DNA. Using generic primers (C column), DNA can be correctly typed, except
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35 16 for the unspecific typing due to hybridization between *A. japonicus* specific PCR product and
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37 17 probe aC1, specific for *A. ibericus*. The same signal was also observed when testing the no
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39 18 template control, proving that the cross-hybridization was due to interaction with primers
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41 19 (CL-1 or CL-2A).
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52 21 These results are summarized in Table 3 and confirmed by the cross hybridization test
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54 22 between each primer and probes. Each primer was tested as a single target (in an identical
55
56 23 amount to the PCR products) on the OLISA™ biochip. Primer CL-1 (generic forward primer)
57
58 24 cross hybridized with probe aC1. Primer Japo-2 (*A. japonicus* specific primer) cross
59
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1 hybridized with probe JA2. Primer Carbo-2 (*A. carbonarius* specific primer) cross hybridized
2 with probe CaC1, which was expected due to sequence complementarity. Therefore it was
3 found that probe aC1 would not be suitable with generic primers, and probes JA2 and CaC1
4 would not be suitable with specific primers (Figure 3).

5
6 In conclusion, using the generic PCR approach the microarray was set up with JA2, CaC1,
7 CaC2, C1, C2 and aC2 probes; using specific PCR approach with Carbo-1/2 and Japo-1/2
8 primers, the microarray was set up with JA1, CaC2, C1, aC1 and aC2.

9 10 *Interspecificity evaluation*

11 Using the specific PCR approach 10 ng DNA of each species were PCR with *A. carbonarius*
12 specific primers and PCR product detected on the biochip. The results showed that the two
13 probes CaC2 and C1 specifically hybridized with *A. carbonarius* (respectively 9553 A.U. \pm
14 1476, 23565 A.U. \pm 3412). No hybridization signals were observed between them and *A.*
15 *japonicus*, *A. niger* or *A. tubingensis*, confirming specificity of primers Carbo-1 and Carbo-2,
16 able to amplify only *A. carbonarius* DNA. These two probes were thus good candidates for
17 the *A. carbonarius* specific amplification approach.

18
19 Using the generic PCR approach 10 ng DNA of each species was PCR amplified separately
20 using generic primers and detected on the biochip. Probes CaC1, CaC2, C1 and C2 hybridized
21 with PCR amplicon obtained by amplifying *A. carbonarius* DNA, while JA1 and JA2
22 hybridized with PCR amplicon obtained by amplifying *A. japonicus* DNA. Probes CaC2, C2
23 and JA1 weakly hybridized with the PCR amplicon obtained by amplifying *A. niger* DNA.
24 Probe JA1 hybridized with PCR product obtained by amplifying *A. tubingensis*. These results

1 suggest that JA1 should be excluded for the generic PCR approach because it hybridized with
2 *A. japonicus* (15182 A.U. \pm 1613), but also with *A. niger* and *A. tubingensis* (respectively 918
3 A.U. \pm 378 and 2986 A.U. \pm 253). Probes C2 and CaC2 non specifically hybridized with *A.*
4 *niger* (respectively 1227 A.U. \pm 384 and 448 A.U. \pm 237), therefore it could be considered if a
5 threshold value is defined at about at 2000 A.U. In practice, it could be valid for pure culture
6 identifications, because in natural samples DNA levels of fungal contaminations are
7 unpredictable, thus threshold cannot be established and CaC2, C2 and JA1 should be
8 excluded.

10 *Sensitivity of A. carbonarius detection*

11 Using the generic primers approach the detection limit (DL) of the method was determined by
12 testing a series of 5-fold dilutions of *A. carbonarius* DNA in PCR reactions: 2 ng, 0.4 ng, 0.08
13 ng, 16 pg, 3.2 pg, 0.64 pg, 128 fg, 25.6 fg, 5.12 fg and 0 mixed with 2 ng of total DNA of
14 each of *A. japonicus*, *A. niger* and *A. tubingensis*. A PCR negative control, without DNA
15 templates, was included. All probes tested gave a signal decreasing with the dilution factor
16 (Figure 4). In particular CaC1 and C1 probes showed hybridization signals up to 3.2 pg,
17 establishing their sensitivity. Probes CaC2 and C2 gave a signal for all concentrations tested
18 and also for the negative DNA control of *A. carbonarius* DNA, due to the previously proved
19 cross hybridizations with *A. japonicus*, *A. niger* and *A. tubingensis* DNA.

21 *Detection of A. carbonarius in environmental samples*

22 Qualitative microbiological analysis on the two environmental samples showed sample 1
23 uncontaminated by *Aspergillus* spp. and sample 2 contaminated by *A. carbonarius*. The two
24 samples were also tested using the generic and the specific approaches for a preliminary

1 evaluation of *Aspergillus* spp. OLISA™ microarray application on food samples. The DNA
2 inputs for the PCR were two different amounts: 1/20 and 1/10 of total nucleic acids isolated
3 from 300 mg of grapes. A volume 5µL of PCR product was then hybridized for both on the
4 OLISA™ microarray, as described previously. The results are shown in Figure 5. No signal
5 was detected for sample 1 (with either amount of DNA template in PCR), as expected for an
6 *A. carbonarius* uncontaminated sample. No *A. japonicus* DNA was detected in either the
7 samples. While in the sample 2 only *A. carbonarius* was detected , with both the generic and
8 specific PCR approaches.

9
10 In conclusion probes CaC2, C1, aC1, aC2, JA1 and JA2 are suitable for use in the *A.*
11 *carbonarius* specific PCR approach for identification of fungal DNA in pure cultures and
12 natural samples; probes JA1, CaC1, CaC2, C1, C2, aC1 and aC2,are suitable for use in the *A.*
13 *japonicus* specific PCR approach for identification of fungal DNA in pure cultures and
14 natural samples; probes JA2, CaC1, CaC2, C1, C2 and aC2 are suitable only for use in the
15 generic PCR approach for identification of fungal DNA in pure cultures, while JA2, CaC1,
16 C1 and aC2 are suitable in natural samples.

17 18 **Discussion**

19 The increasing use of molecular methods in fungal diagnosis has provided tools for answering
20 various taxonomic questions, that morphological procedures have left unsolved. In particular,
21 a number of PCR based methods have emerged which are sensitive, accurate and rapid for the
22 identification of toxigenic fungi. PCR identification of consensus sequences obtained from
23 specific phylogenetic information can be inferred for all taxonomic groups of mycotoxigenic
24 fungi. The most common is based on exploiting the interspecies differences between

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5 1 sequences of nuclear ribosomal intergenic spacer DNA (IGS rDNA), beta-tubulin,
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7 2 calmodulin, elongation factor and AFLP analysis (Schmidt et al. 2003; Mule` et al. 2004a, b).
8
9 3 Other researchers have designed PCR oligonucleotide primers for the amplification of genes
10
11 4 involved in the biosynthesis of some toxins (Seo et al. 2001; Sidhu 2002; Chandler et al.
12
13 5 2003). These molecular markers can be used for the detection of toxigenic fungal species by
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15 6 implementing them in DNA biochips. Microarray technology can be used for detecting
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17 7 toxigenic species belonging to the same or a different genus. This is particularly important
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19 8 since generally it is not a single species but a complex of species, often phylogenetically
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21 9 closely related but toxicologically different, that causes plant disease (e.g. *Fusarium* head
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23 10 blight on wheat; *Fusarium* ear rot on maize, *Aspergillus* black rot on grapes) (Nicolaisen et al.
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25 11 2005; Schmidt-Heydt, Geisen 2007).
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33 13 Oligonucleotide microarray technology is a rapid and cost-effective method that has potential
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35 14 for parallel analysis of a large number of target organisms such as *Aspergillus* spp. species
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37 15 from grape. This is a significant improvement to other methods such as morphological,
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39 16 ELISA and PCR based methods that are either time consuming or directed against a single or
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41 17 few target(s). Because it is based on a preliminary PCR amplification, oligonucleotide
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43 18 microarray technology is rapid, highly specific and can be used to detect minute quantities of
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45 19 fungal DNA from environmental samples, allowing implementation of early control methods
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47 20 before symptoms occur, and direct testing of environmental samples, without pure fungal pure
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49 21 culture preparation.
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56 23 OLISA™ is an example of an oligonucleotide DNA microarray which will avoid the
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58 24 development of as many single tests as there are species to detect and identify, allowing
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1 simultaneous detection and identification of all targeted species of *Aspergillus* by a molecular
2 method combining multiple amplification and multiple detection. In the generic and specific
3 PCR approaches the OLISA™ biochip allowed the subsequent detection of the PCR products
4 and the simultaneous identification of the *Aspergillus. carbonarius*, *A. japonicus/A. aculeatus*
5 and *A. ibericus* DNA.

6
7 Both generic and specific *A. carbonarius* amplifications signals can be distinguished from the
8 background, although the specific amplification gives a much higher signal than the generic
9 amplification, with no significant difference related to the PCR input amount. On the
10 corresponding agarose gel for PCR product analysis, the generic product could not be seen,
11 while the specific product could be seen for both amount tested.

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13 The results of this study proved that in the specific amplification approach the sensitivity and
14 specificity are due to primers and PCR itself, while in the generic approach, the specificity
15 depends on probe sequences, and sensitivity is subjected to the prevalence of target species. In
16 conclusion, both approaches could be used, with different probes, allowing monitoring of *A.*
17 *carbonarius* (mainly ochratoxigenic species), *A. japonicus/A. aculeatus* and *A. ibericus* ((not
18 ochratoxigenic species) DNA occurring. The assay developed in this study provides a further
19 valuable tool in multiple identification of the main *Aspergillus* species occurring on grapes.
20 This study allowed to chose specific probes among well defined *Aspergillus* species occurring
21 in grapes. Since the array format allows to immobilize up to 16 oligonucleotides, further
22 probes, representing other toxigenic fungi, occurring on grapes, could be included, in order to
23 have a wide view of potential toxigenic risk.

24

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Table 1 : Primers used for specific and generic PCR approaches.

Name	Sequence (5' - 3')	Amplicon length	PCR approach	References
Carbo-1	AAGCGATCGATAGTCCACAAGAATA	322 bp	Specific for <i>A. carbonarius</i>	Perrone <i>et al.</i> , 2004.
Carbo-2	TCTGGCAGAAGTTAATATCCGGTT			
Japo-1	TTACTGATTCATTTCTCGTGATTCCT	563 bp	Specific for <i>A. japonicus</i>	
Japo-2	TCAAGCAGGGAATGTGTGGA			
CL-1	GARTWCAAGGAGGCCTTCTC	700 bp	Generic	O'Donnell <i>et al.</i> , 2000.
CL-2A	TTTTGCATCATGAGTTGGAC			

W corresponds to A or T; R corresponds to A or G (IUPAC code)

Table 2. Species-specific probes designed in calmodulin gene.

Spot # on OLISA™	Probe name	Sequence (5'-3')	Length	Tm (°C)	Species-specificity
1/17	Control	(GACT) ₅ -biotine	20	45	none
2/16	JA1	TAGTGTAGTTCCTTACCCCTCAACGAA	27	61	<i>A. japonicus</i> and <i>A. aculeatus</i>
3/15	JA2	TAGCCACCACACTGCA	16	48	
4/14	CaC1	GATATTA ACTTCTGCCAGA	19	44	<i>A. carbonarius</i> and <i>A. ibericus</i>
5/13	CaC2	CTGGGCACTGTCATGCGCTCGCTC	24	72	
6/12	C1	GGGCTTGTGTTACACGAGGCAAG	24	68	<i>A. carbonarius</i>
7/11	C2	AGCCGTGATACTGATGTGACA	21	55	
8/10	aC1	TCTCCGACCGACTTGA ACTTAAT	24	59	<i>A. ibericus</i>
9	aC2	GTGTTGATATTTATAGGAAGAGGG	24	52	

Probes were spotted in duplicate symmetrically on right and left part of the OLISA™ array.

Table 3 : Hybridization results between probes and amplicons obtained with specific (for *A. carbonarius* and *A japonicus*) and generic PCR approach.

Probe	<i>A. carbonarius</i> specific primers	<i>A. japonicus</i> specific primers	Generic primers
JA1	–	+	X (with <i>A. niger</i> and <i>A. tubingensis</i>)
JA2	–	X (with Japo-2)	+
CaC1	X (with Carbo-2)	–	+
CaC2	+	–	+ ^Y
C1	+	–	+
C2	X	–	+ ^Y
aC1	–	–	X (with CL-1)
aC2	–	–	–

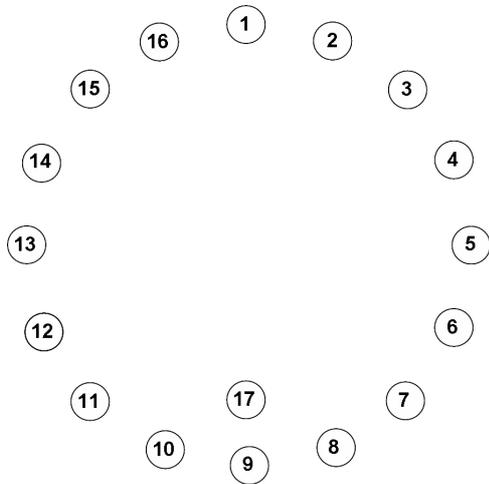
+ corresponds to specific hybridization;

– corresponds to none hybridization;

X corresponds to unspecific hybridization.

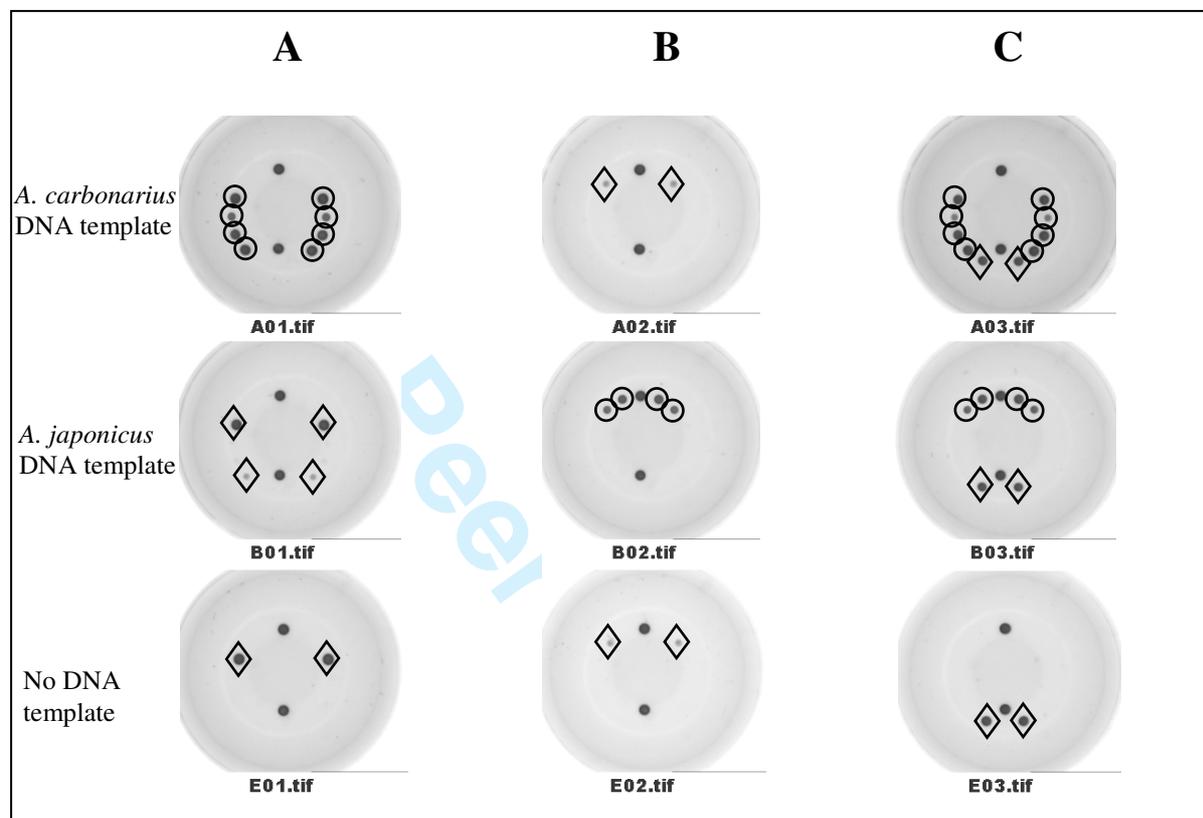
^Y corresponds to unspecific hybridization with *A. niger*, in pure culture (below the threshold value 2000A.U.) and in environmental samples identification assays.

Figure 1. DNA spots positions in each of 96 wells of microtiter plate in OLISA™ array.



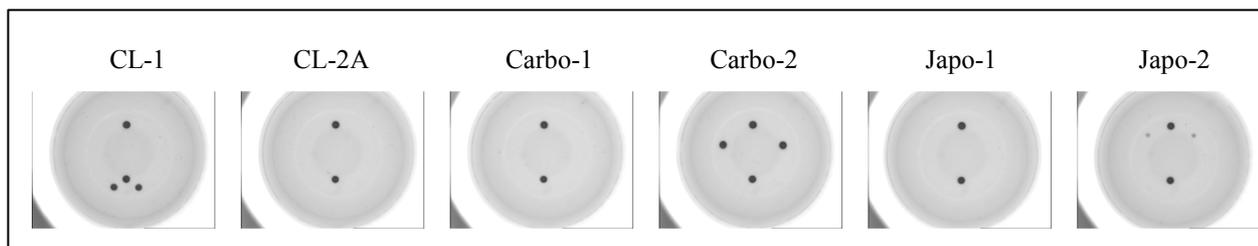
Circles represent probes spot positions in the bottom of each well. Reciprocal relations between numbers and spotted probes are listed in table 2.

Figure 2. Specificity hybridization test between probes and amplicons obtained with specific (A, B) and generic (C) PCR approach .



Dark spots indicate hybridization signals. A: *A. carbonarius* specific amplification. B: *A. japonicus* specific amplification. C: Generic amplification. Amplifications were set up using as target: *A. carbonarius* DNA (top), *A. japonicus* DNA (middle), and no DNA (bottom). Expected specific signals are marked with a circle, non specific signals are marked with a rhombus.

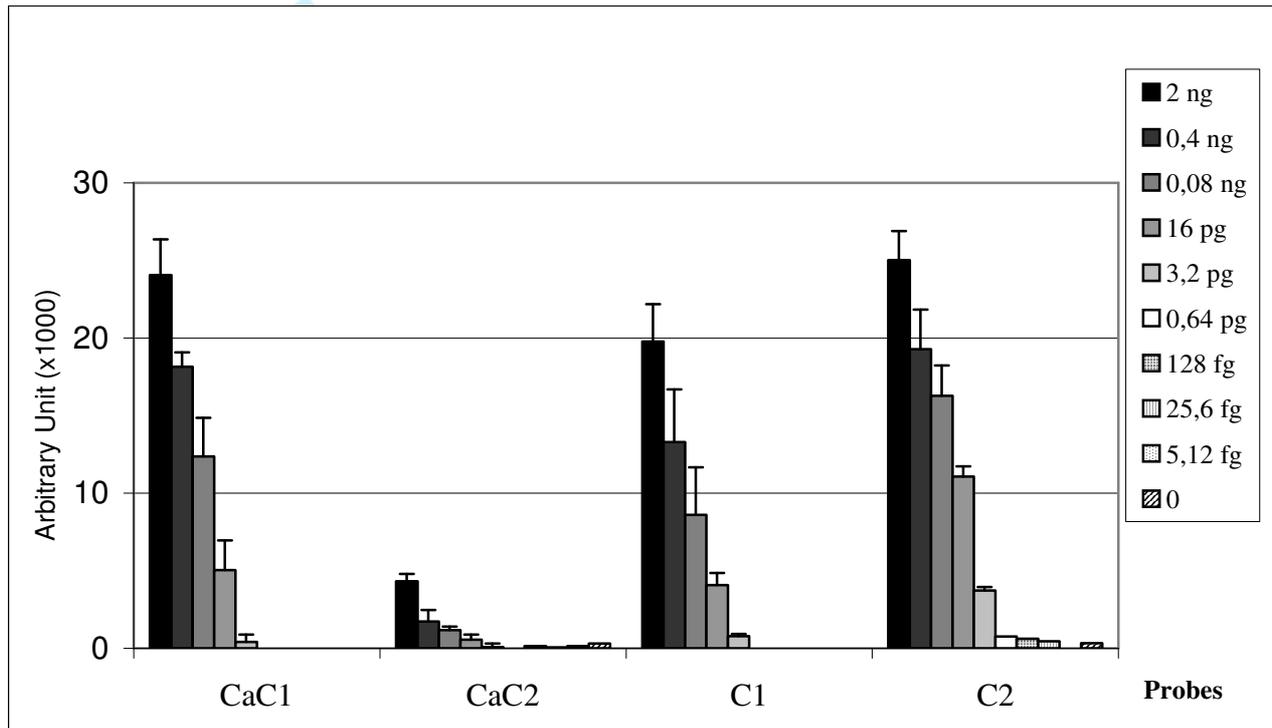
Figure 3. Cross-hybridisation test between probes and PCR primers (generic and specific).



Dark spots indicate hybridization signals. CL1, Carbo-2, and Japo-2 (Table1) cross-hybridized respectively with aC1 (positions 8/10 on array, Figure 1), CaC1 (positions 4/14) and JA2 (positions 3/15).

Figure 4. Sensitivity of *A. carbonarius* detection using generic PCR approach by OLISA™ array.*

A 5-fold dilution series of genomic DNA from *A. carbonarius* was amplified mixed to *A. niger*, *A. tubingensis* and *A. japonicus* DNA (2ng each). Hybridization signals intensity (A.U.) between probes and amplicons are showed. PCR negative control showed no signal for all probes.



*: standard deviations refer to 3 replicates.

1 Figure 5. Detection of *A. carbonarius* DNA in natural grapes samples by OLISA™
 2 microarray. The histogram shows hybridization signals intensity (A.U.) between probes and
 3 amplicons obtained with generic and specific PCR approach. Sample 1: uncontaminated;
 4 sample 2: contaminated by *A. carbonarius* (according to microbiological analyses). Two
 5 different amounts, 1/20 and 1/10 of total nucleic acids isoalted from 300 mg of grapes, were
 6 evaluated.

