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Simultaneous detection of the main black aspergilli responsible for OTA contamination in grapes by multiplex real-time PCR

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3 **1 Simultaneous detection of the main black aspergilli responsible for OTA**
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6 **2 contamination in grapes by multiplex real-time PCR**
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56 23 **Abstract**

7
8 24 In this paper we report a duplex real-time PCR assay for the simultaneous detection of
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10 25 members of the *A. niger* aggregate and *A. carbonarius*, which are the main responsible
11
12 26 species for ochratoxin A (OTA) contamination in grapes. This single tube reaction targets
13
14 27 the β -ketosynthase and the acyl transferase domains of the poliketide synthase of *A.*
15
16 28 *carbonarius* and the *A. niger* aggregate, respectively. Melting curve analysis using a SYBR
17
18 29 Green I real-time PCR approach showed characteristic T_m values demonstrating the
19
20 30 specific, efficient and balanced amplification of the two PCR fragments. Subsequently, a
21
22 31 TaqMan real-time PCR approach was settled, using FAM and VIC labeled specific probes
23
24 32 for an automated detection. Results indicated no differences in sensitivity when using either
25
26 33 the two sets of primers and probes in separate or in the same reaction. However, when both
27
28 34 targets are in very different amounts, there is a preferential amplification of the target which
29
30 35 is in more concentration. C_T values obtained in the presence of grape DNA were very
31
32 36 similar to those observed when only fungal purified DNA was present, indicating that the
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34 37 grape DNA does not interfere in the real-time PCR reaction. This procedure provides a fast
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36 38 and accurate tool to monitor, in a single reaction, the presence of OTA-producing species in
37
38 39 grapes which, to some extent, will facilitate OTA contamination surveys to guarantee food
39
40 40 safety in the wine industry.

41

42 **Keywords:** *Aspergillus carbonarius*; *Aspergillus niger* aggregate; *Aspergillus tubingensis*;43 *grapes*; *Mycotoxin*; *Ochratoxin A*; *Polyketide synthase*; *Real-time PCR*; *Wine*

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47 **Introduction**

48 Ochratoxin A (OTA) is a mycotoxin with nephrotoxic, carcinogenic, immunotoxic,
49 genotoxic and teratogenic effects, which has been associated with Balkan Endemic
50 Nephropathy (Krogh 1978; Kuiper-Goodman & Scott 1989; Abouzieed et al. 2002). In the
51 European diet, wine and especially red wine, has been identified as the second major source
52 of human exposure to OTA, following cereals (Anonymous 1998). As a consequence, the
53 European Commission has considered the need of imposing regulatory limits and has
54 established 2 ng g⁻¹ as the maximum level of OTA in wine and grape products
55 (Commission regulation No 123/2005 amending Regulation No 67 446/2001 as regards
56 ochratoxin A) (Varga & Kozakiewicz 2006).

57 *Aspergillus* species belonging to section *Nigri*, commonly known as black aspergilli in
58 particular, *Aspergillus carbonarius* and species belonging to the *Aspergillus niger*
59 aggregate, have been identified as the main fungi responsible for OTA contamination in
60 grapes and wine (for an overview see Abarca et al. 2003; Samson et al. 2004; Battilani et al.
61 2006). *A. carbonarius* is considered the mayor responsible for OTA contamination in
62 grapes because the reported percentages of OTA-producing strains in this species are higher
63 than those reported for members of the *A. niger* aggregate. Nevertheless, the black
64 aspergilli most frequently isolated from grapes are species belonging to the *A. niger*
65 aggregate, mainly *A. niger* and *Aspergillus tubingensis* (Cabañes et al. 2002; Abarca et al.
66 2003; Battilani et al. 2003; Bellí et al. 2004; 2006; Martínez-Culebras & Ramón 2007).
67 Recently, Martínez-Culebras & Ramón (2007) reported the existence of a new OTA
68 producing group of strains, frequently found in grapes, which is related to *A. tubingensis*
69 and might be considered within the *A. niger* aggregate. These data provide strong evidence

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2
3 70 of an important contribution to OTA contamination in grapes by species belonging to the *A.*
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5 71 *niger* aggregate. Therefore, a quick assay capable of quantifying the presence of both *A.*
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7 72 *carbonarius* and the *A. niger* aggregate in grapes would improve OTA risk assessment.
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10 73 Recent advances in DNA-based techniques such as real-time PCR are providing new
11
12 74 tools for fungal detection and quantification by detecting and quantifying their DNA.
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14 75 Multiplex real-time PCR allows the simultaneous amplification of more than one target
15
16 76 sequence in a single reaction. This saves considerable time and effort, and decreases the
17
18 77 number of reactions that need to be performed to detect fungal pathogens in vegetable
19
20 78 samples (Brandfass & Karlovsky 2006). Several real-time PCR assays have proven useful
21
22 79 in monitoring and quantification of OTA-producing fungi such as *Penicillium nordicum*
23
24 80 (Geisen et al. 2004) and *Aspergillus ochraceus* (Schmidt et al. 2004). Regarding black
25
26 81 aspergilli, single real-time PCR assays for the detection and quantification of *A.*
27
28 82 *carbonarius* have been described (Mulè et al. 2006; Atoui et al. 2007; Selma et al. 2008).
29
30 83 However, no real-time PCR assay is currently available for the simultaneous quantitative
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32 84 detection of *A. carbonarius* and species belonging to the *A. niger* aggregate.
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39 85 A specific real-time PCR assay for the quantitative detection of *A. carbonarius* in
40
41 86 combination with a rapid and efficient fungi DNA extraction procedure has already been
42
43 87 developed (Selma et al. 2008). This assay targets a 161 bp fragment in the β -ketosynthase
44
45 88 domain of a polyketide synthase (PKS) from *A. carbonarius*. DNA extraction procedure
46
47 89 includes a pulsifier equipment to remove conidia from grapes which prevents releasing of
48
49 90 PCR inhibitors. It is highly selective, sensitive and suitable for the accurate quantification
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51 91 of all strains of *A. carbonarius*. In the present study, we describe a duplex real-time PCR
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53 92 assay that combines the *A. carbonarius*-specific β -ketosynthase domain assay and the
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3 93 amplification of the acyl transferase (AT) domain of the species belonging to the *A. niger*
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5 94 aggregate.

7
8 95 **Materials and Methods**

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10 96 *Fungal strains and growth conditions*

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12 97 Black *Aspergillus* strains were isolated from Spanish vineyards and are held in the Institute
13
14 of Agrochemistry and Food Technology (IATA-CSIC). They were previously identified by
15
16 98 ITS-RFLP profiles and tested for OTA-production (Martínez-Culebras & Ramón 2007).
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18 99 Reference strains were provided by Centraalbureau voor Schimmelcultures (CBS, Utrecht,
19
20 The Netherlands) and the Spanish Type Culture Collection (CECT, Valencia, Spain) (see
21
22 Table 1). The reference strains used in the present study were previously tested for OTA
23
24 100 production (Samson et al. 2004; Martínez-Culebras et al., 2008). They were all grown in
25
26 101 Malt Extract Agar (MEA) plates at 25 ± 1 °C for 5-7 days (Pitt & Hocking 1997).
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34 106 *Fungal and grape DNA isolation*

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36 107 Mycelia were collected from cultures growing on MEA plates frozen in liquid nitrogen and
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38 108 ground to a fine powder. Fungal DNA extractions were performed using 100 mg of powder
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40 and the commercial EZNA Fungal DNA kit (Omega bio-teck, Doraville, USA) according
41
42 109 to the manufacturer's instructions. Accurate DNA quantification for real-time PCR assays
43
44 110 was approached using the 'Fluorescent DNA Quantitation Kit' (BioRad, California, USA)
45
46 111 and a VersaFluor® spectrofluorimeter (BioRad, California, USA)
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50
51 113 For grape DNA extraction, 25 g of grapes were homogenized in 100 mL of sterile 0.1 %
52
53 114 buffered peptone water (BPW, AES Laboratoire, Combourg, France) using a pulsifier
54
55 115 equipment (Microgen bioproducts, Surrey, UK). One milliliter of pulsified grape samples
56
57 116 were centrifuged for 3 min at 13,000 g. Pellets were washed in 0.5 mL of TE (10 mmol L⁻¹
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3 117 Tris-HCl; 1mmol L⁻¹ EDTA, pH 8), centrifuged at 13,000 *g* for 3 min and resuspended in
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5 118 50 µL of sterile nanopure water. They were boiled (95 °C for 10 min) to release the DNA.
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8 119 Samples were cooled on ice for 10 min. Finally DNA isolation was performed by EZNA
9
10 120 Fungal DNA kit (Omega bio-teck, Doraville, USA), following the manufacturer's
11
12 121 recommendations and starting from step 1 of protocol B where 600 µL of buffer FG1 were
13
14 122 added to 50 µL of pellets resuspended in water. In the final step, DNA was eluted in 100
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16 123 µL of sterile nanopure water and kept at – 20 °C until used as template for PCR
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18 124 amplification.
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24 126 *Primer and TaqMan probe design*

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27 127 *A. carbonarius* specific primers and probe reported in Table 2 were designed in a previous
28
29 128 study (Selma et al. 2008). They are targeted to a conserved region in the β-ketosynthase
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31 129 (KS) domain of a PKS gene (AcKS10) from *A. carbonarius* (Genbank accession number:
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33 130 AY540952). Primers delimit a 161 bp fragment. The *A. carbonarius* TaqMan probe (AC
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35 131 probe) was labeled at the 5' and 3' ends with a 6-carboxy-fluorescein group (FAM) and 6-
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37 132 carboxy-tetramethyl-rhodamine (TAMRA), respectively.
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41 133 Specific primers and probe for *Aspergillus* strains belonging to *A. niger* aggregate
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43 134 (Table 2) were designed in the present study and they were derived from a conserved region
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45 135 in the acyl transferase (AT) domain sequences of PKS genes after comparison with
46
47 136 different *Aspergillus* and *Penicillium* species (Genbank accession numbers: EF527410 *A.*
48
49 137 *awamori*; EF527411 *A. foetidus*; EF527408 *A. niger*; EF527818 *A. ochraceus*; EF527414
50
51 138 *A. tuningensis*; EF527415 *A. tubingensis*; EF527412 *A. tubingensis*-like; EF527413 *A.*
52
53 139 *tuningensis*-like; EF527409 *A. usamii*; F527416 *A. wenti*; EF527417 *A. wenti*; EF527419
54
55 140 *P. citrinum*; EF527421 *P. griseoroseum*; EF527420 *P. verrucosum*; EF527423 *P.*
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3 141 *viridicatum*). The design of the primers and Taqman probe for real-time PCR assays was
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5 142 carried out using the Primer Express, version 2.0 software (Applied Biosystems Division,
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7 143 Perkin-Elmer Co., Foster city, CA, USA). Primers delimit a 120 bp fragment. *A. niger*
8
9 144 aggregate specific TaqMan probe (AN probe) designed in the present study was labeled at
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11 145 the 5' and 3' ends with a VIC[®] group and a non-fluorescent quencher, respectively. Primer
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13 146 and probe synthesis were ordered from ABI PRISM Primers and TaqMan Probe Synthesis
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15 147 Service (Applied Biosystems, Madrid, Spain).
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21 22 149 *Specificity of the conventional PCR assays*

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24 150 A total of 47 strains were used for specificity assays of the newly designed primers AtNig1/
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26 151 AtNig2 by conventional PCR (Table 1). They included 42 strains belonging to *Aspergillus*
27
28 152 species and 5 to other fungal species commonly isolated from grapes. PCR reactions using
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30 153 the *A. niger* aggregate specific primers AtNig1 / AtNig2 and the universal primers its5/its4
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32 154 (White et al. 1990) were performed in 50 µL as final volume, containing 200 ng of DNA,
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34 155 50 mM KCl, 10 mM Tris-HCl, 100 µM (each) dNTP, 1 µM of each primer, 2 mM MgCl₂
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36 156 and 1 U of DNA polymerase (Netzyme, Molecular Netline Bioproducts, N.E.E.D, SL,
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38 157 Valencia, Spain).
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43 158 Amplification conditions were: 35 cycles consisting of 1 min at 95 °C, 1 min at 52 °C
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45 159 and 1 min at 72 °C. Reactions were carried out in a GeneAmp PCR System 9700 (PE
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47 160 Applied Biosystems, Norwalk, CT, USA) thermal cycler. Fifteen microlitres of PCR
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49 161 products were electrophoresed through 2 % agarose (Pronadisa, Hispanlab, Madrid, Spain)
50
51 162 gel in TAE buffer [40 mM Tris-acetate, pH 7.6 and 1 mM Na acetic acid (EDTA)]. After
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53 163 electrophoresis, gels were stained with ethidium bromide (0.5 mg mL⁻¹), and the DNA
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3 164 bands visualized under UV light. Sizes were estimated by comparison with a DNA standard
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5 165 length (GeneRuler™ 100 bp DNA ladder, MBI Fermentans, Vilnius, Lithuania).
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10 167 *Specificity of the real-time PCR assays*
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12 168 A total of 39 strains were used for specificity assays of the newly designed primers AtNig1/
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14 169 AtNig2 by real-time PCR (Table 1). Real-time PCR reactions, with SYBR-Green I and
15
16 170 TaqMan probe, were performed using the SYBR-Green I Core Reagents and the TaqMan
17
18 171 Core Reagents, respectively (both from Applied Biosystems, Madrid, Spain). Amplification
19
20 172 mixtures for real-time PCR reactions with SYBR-Green I, contained in a final volume of 20
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22 173 μL , 1 x PCR Buffer, 200 μM each dATP, dCTP, dGTP and 400 μM dUTP; 1 U of
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24 174 Amperase uracil N-glycosidase; 3 mM MgCl_2 ; 0.9 U of AmpliTaq Gold DNA polymerase,
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26 175 200 nM each primer (AtNig1/ AtNig2) and 5 μL of template DNA (10 ng for specificity
27
28 176 assay). Amplification mixtures for real-time PCR reactions with TaqMan probe, contained
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30 177 in a final volume of 20 μL , 1x TaqMan Buffer A, 200 μM each dATP, dCTP, dGTP, and
31
32 178 400 μM dUTP; 1 U of Amperase uracil N-glycosidase; 3.5 mM MgCl_2 ; 1 U of AmpliTaq
33
34 179 Gold DNA polymerase, 200 nM each primer (AtNig1/ AtNig2), 5 μL of template DNA.
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36 180 The following concentrations of the AN probe were assayed: 50, 100, 150 and 200 nM.
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38 181 Real-time PCR assays, using either SYBR-Green I or TaqMan probe for fluorescence
39
40 182 emission, were carried out in a GeneAmp® 5700 Sequence Detection System (PE
41
42 183 Biosystems, Foster city, CA, USA) programmed to hold at 50 °C for 2 min and at 95 °C for
43
44 184 10 min, and to complete 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Reactions were
45
46 185 done in triplicate. PCR results were given as the increase in the fluorescence signal of the
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48 186 reporter dye detected and visualized by the GeneAmp 5700 SDS software provided with the
49
50 187 GeneAmp® 5700 SDS (Applied Biosystems). Threshold cycle (C_T) values represent the
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3 188 PCR cycle in which an increase in fluorescence, over a defined threshold, first occurred for
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5 189 each amplification plot.
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10 191 *Multiplex real-time PCR*

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12 192 Real-time PCR reactions were performed using both the SYBR-Green I Core Reagents and

13 193 TaqMan Core Reagents (Applied Biosystems Madrid Spain). Amplification mixtures for

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15 194 Real-time PCR, contained in a final volume of 20 μ L, 1x buffer (SYBR-Green I PCR

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17 195 Buffer or TaqMan PCR buffer), 200 μ M each dATP, dCTP, dGTP, and 400 μ M dUTP; 1 U

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19 196 of Amperase uracil N-glycosidase; 1 U of AmpliTaq Gold DNA polymerase; and 5 μ L of

20
21 197 template DNA. Concentration of primers, probes and $MgCl_2$ were optimized for the

22
23 198 simultaneous amplification of the two amplicons. The following concentrations were

24
25 199 assayed: 3, 3.5, 4 and 5 mM of $MgCl_2$; 100, 200, 300 and 400 nM of primers; 50, 100, 150,

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27 200 and 200 nM of TaqMan probes. Real-time PCR assays were carried out in a GeneAmp

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29 201 5700 Sequence Detection System (PE Biosystems Foster City Calif.) programmed to hold

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31 202 at 50 $^{\circ}C$ for 5 min, to hold at 95 $^{\circ}C$ for 10 min, and to complete 40 cycles of 95 $^{\circ}C$ for 15 s

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33 203 and (60-63 $^{\circ}C$) for 1 min. Several annealing temperatures were assayed to optimize the

34
35 204 multiplex amplification. Reactions were done in triplicate. SYBR Green I reactions were

36
37 205 carried out in a GeneAmp[®] 5700 Sequence Detection System (Applied Biosystems). For

38
39 206 multiplex real-time PCR reactions using TaqMan probes, an ABI Prism 7000 Sequence

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41 207 Detection System (Applied Biosystems) was used. In both cases, PCR results were given as

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43 208 the increase in the fluorescence signal of the reporter dye detected and visualized by the

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45 209 corresponding software, as indicated above.

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8 214 *Melting-curve analysis*

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10 215 Melting-curves were programmed in order to check the expected amplification product
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12 216 when using SYBR Green I approach. The thermal protocol for dissociation is defined as 15
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14 217 s at 95 °C, 20 s at 60 °C and 20 min slow ramp between 60 and 95 °C, after the real-time
15
16 218 PCR program. The data for the dissociation curve is captured during this slow ramp. The
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18 219 melting curve was visualized with the software in the 'Dissociation Window' using the
19
20 220 corresponding GeneAmp 5700 SDS software procedure (Applied Biosystems).

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27 222 *Sensitivity of the multiplex real-time PCR reaction*

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29 223 In order to establish detection limits of the multiplex TaqMan real-time PCR,
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31 224 amplifications were carried out for each targeted ochratoxigenic organism using both the
32
33 225 uniplex and the multiplex reaction. To that purpose, tenfold serial dilutions of DNA
34
35 226 extracted from the reference strains *A. carbonarius* CBS 11380 and *A. tubingensis* CBS
36
37 227 134.48 were done covering a range from 3 to 3×10^5 genome equivalents, that were
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39 228 calculated assuming it to be similar to that of *A. niger* (4.13×10^{-5} ng, for a haploid
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41 229 genome).

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45 230 Sensitivity of the multiplex real-time PCR reaction was also analyzed in the presence of
46
47 231 grape DNA and different concentrations of fungi DNAs. Grape DNA was obtained from
48
49 232 pulsed grape samples as described above (section 2.2.) and tested in triplicate by real-
50
51 233 time PCR using the *A. carbonarius* specific primers AcKS10L/ AcKS10R and the *A. niger*
52
53 234 aggregate specific primers AtNig1/ AtNig2, under the amplification conditions established
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55 235 in the study. Grape DNA that tested negative for both *A. carbonarius* and *A. tubingensis*
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3 236 was added with DNA from *A. carbonarius* CBS 11380 and *A. tubingensis* CBS 134.48 in
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5 237 different concentrations. Samples were tested in triplicate by real-time PCR and a non-
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8 238 template negative control was included in each experiment. Analysis of variance with a
9
10 239 significance level of $P \leq 0.05$ was carried out on these data using SPSS (Windows 2000,
11
12 240 Statistical Analysis).

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15 24116
17 242 **Results**18
19 243 *Specificity of the real-time PCR assays*

20 244 A new set of specific primers and a probe were designed to target a conserved region in the
21
22 245 acyl transferase (AT) domain sequences of PKS genes of all strains belonging to the *A.*
23
24 246 *niger* aggregate. Specificity was tested by conventional PCR using purified DNA from 47
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26
27 247 strains (Table 1). The expected 120 bp amplicon was obtained from strains belonging to the
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29
30 248 species included in the *A. niger* aggregate (*A. awamori*, *A. foetidus*, *A. niger*, *A. tubingensis*,
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32 249 *A. tubingensis*-like, and *A. usamii*). The 120 bp amplicon was also obtained using DNA
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34
35 250 from *A. lacticofeatus* and *A. pulverulentus*, two OTA-producing species closely related to
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37
38 251 the *A. niger* aggregate (Sanson et al. 2004; Perrone et al. 2006) (Table 1). Amplification of
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40
41 252 the 5.8S-ITS region using universal primers its5/its4 was obtained in all strains, thus
42
43 253 confirming the DNA suitability when negative amplification results were obtained using the
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45
46 254 specific primers AtNig1/AtNig2.

47
48 255 Specificity of the newly designed primers AtNig1/AtNig2 was also tested by SYBR
49
50 256 Green I and TaqMan real-time PCR using 10 ng of template DNA/reaction of 39 strains
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52
53 257 representing the species included in the study (Table 1). The melting curve analysis showed
54
55 258 a T_m value 80.5 ± 0.5 in the *A. niger* aggregate species. Strains belonging to other genera,
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58 259 showed no melting curve, revealing that no amplification products were obtained.
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3 260 Specificity of primers AcKS10L/AckS10R used for the real-time PCR detection of *A.*
4
5 261 *carbonarius* had previously been demonstrated by Selma et al. (2008).
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10 263 *Set up of the multiplex real-time PCR reaction*
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12 264 Optimized reaction conditions for the duplex real-time PCR were obtained by using 3.5
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14 265 mM MgCl₂, 200 nM of *A. niger* aggregate specific primers and 100 nM of *A. carbonarius*
15
16 266 specific primers. Melting curve analysis of amplicons corresponding to strains belonging to
17
18 267 *A. niger* aggregate and *A. carbonarius* showed T_m values of 80.5±0.5 and 83.4±0.4,
19
20 268 respectively, thus allowing the differentiation between the two targeted organisms (Figure
21
22 269 1). For the TaqMan assay, reaction annealing temperature allowing both primers and probes
23
24 270 to hybridize was established at 60 °C. Primer and MgCl₂ concentration was maintained
25
26 271 identical to the SYBR Green I reaction. Optimized probe concentration was 200 nM for AN
27
28 272 probe targeting *A. niger* aggregate and 150 nM for AC probe targeting *A. carbonarius*.
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36 274 *Sensitivity of the multiplex Taqman real-time PCR compared to uniplex real-time PCR*
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38 275 *assay*
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40
41 276 Tenfold serial dilutions of DNA extracted from the reference strains *A. carbonarius* CBS
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43 277 11380 and *A. tubingensis* CBS 134.48 covering 6 log orders, were used for multiplex and
44
45 278 uniplex TaqMan real-time PCR assays in order to establish detection limits of the multiplex
46
47 279 real-time PCR reaction compared to the uniplex real-time PCR (Table 3). For *A.*
48
49 280 *carbonarius*, C_T values obtained by multiplex real-time PCR were slightly higher than those
50
51 281 obtained with the uniplex (Table 3). Linear regression analysis of C_T values corresponding
52
53 282 to *A. carbonarius* DNA suspensions yielded R² values above 0.99 and the slopes were
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55 283 -2.57 with uniplex and -2.56 with multiplex. For *A. tubingensis*, no differences were
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3 284 observed between C_T values obtained by multiplex real-time PCR and uniplex real-time
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5 285 PCR. At the lowest concentration tested (3 genome equivalents/reaction), a slight
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7 286 amplification signal was obtained in 1 out of 3 replicates with the uniplex reaction and no
8
9
10 287 amplification was observed with the multiplex real-time PCR. Linear regression analysis of
11
12 288 C_T values corresponding to *A. tubingensis* DNA suspensions yielded R^2 values above 0.99
13
14
15 289 and the slopes were -2.73 with uniplex and -2.67 with multiplex. These results indicate no
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17 290 differences in sensitivity when using either the two sets of primers and probes, in separate,
18
19
20 291 or in the same reaction.
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23 24 293 *Co-amplification using different amounts of target DNA*

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26
27 294 To evaluate the sensitivity of the multiplex real-time PCR, a combination of 10, 10^2 , 10^3
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29 295 and 10^4 genome equivalents of *A. carbonarius* and *A. tubingensis* was analyzed (Table 4).
30
31 296 Moreover, these combinations were assayed with and without the presence of grape
32
33 297 purified DNA. Amplification was observed in all combinations with both SYBR Green and
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35
36 298 TaqMan. However, when both targets were in very different amounts (10 and 10^4 or 10^2
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38 299 and 10^4 genome equivalents) the one that was in less concentration obtained significantly
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41 300 higher C_T value ($P < 0.01$) compared to the corresponding C_T when both were at the same
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43 301 concentration (Table 4). On the other hand, C_T values obtained in the presence of grape
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46 302 DNA were very similar to those observed when only conidial purified DNA was present,
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48 303 indicating that the grape DNA, extracted as previously described, does not interfere in the
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51 304 real-time PCR reaction.
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54 55 306 **Discussion**

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3 307 The PCR reaction is a well recognized rapid technique widely used for detection of
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5 308 foodborne pathogens. Recently, it is also being applied for detection of toxinogenic
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8 309 microorganisms such as mycotoxin producing fungi. PCR detection of ochratoxigenic fungi
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10 310 has been approached using primers targeted to single species, i.e. *A. carbonarius* (Mulè et
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12 311 al. 2006; Atoui et al. 2007; Selma et al. 2008), *A. ochraceus* (Dao et al. 2005), *P. nordicum*
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14 312 (Geisen et al. 2004) or to several species including citrinin producers (Dao et al. 2005).
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16 313 Sartori et al. (2006) developed a multiplex conventional PCR for the simultaneous detection
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18 314 of *A. carbonarius*, *A. niger* and *A. ochraceus* in green coffee. In the present study we have
19
20 315 developed a rapid, specific and sensitive duplex real-time PCR assay that allows the
21
22 316 simultaneous detection of members of the *A. niger* aggregate and *A. carbonarius*, which are
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24 317 the main species responsible for OTA contamination in grapes and wine (Abarca et al.
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26 318 2003; Samson et al. 2004; Battilani et al. 2006; Martínez-Culebras & Ramon 2007).

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29 319 With this aim, we combined the previously published *A. carbonarius*-specific real-time
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31 320 PCR (Selma et al. 2008) with a newly designed real-time PCR targeted to the acyl
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33 321 transferase (AT) domain of a PKS gene of the species belonging to the *A. niger* aggregate.
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35 322 Grapes isolates belonging to the *A. niger* aggregate (*A. niger*, *A. tubingensis* and *A.*
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37 323 *tubingensis*-like) were successfully amplified using the primer pair AtNig1/AtNig2.
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39 324 Additionally, strains belonging to the species *A. awamori*, *A. foetidus* and *A. usarii*, which
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41 325 are also included into the *A. niger* aggregate, were also amplified by these primers. OTA-
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43 326 producing strains of the aforementioned species have commonly been found in different
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45 327 foods and feeds, including grapes (Chulze et al. 2006; Ponsone et al. 2006). The newly
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47 328 developed primers also amplified DNA from *A. lacticofeatus* and *A. pulverulentus*, two
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49 329 OTA-producing species of the *Aspergillus* section *Nigri* closely related to the *A. niger*
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51 330 aggregate (Sanson et al. 2004; Perrone et al. 2006). Nevertheless, these two *Aspergillus*
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3 331 species have never been isolated from grapes. Negative results with DNA from other
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5 332 *Aspergillus* section *Nigri* and additional fungal species commonly found in grapes
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8 333 confirmed the specificity of the newly designed primers.
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10 334 For the development of a suitable duplex real-time PCR, relative concentration of
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12 335 primers and probes, cycling temperatures and amounts of template DNA and Taq DNA
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14 336 polymerase have to be optimized (Markoulatos et al. 2002). In this study, SYBR Green I
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16 337 system, was used for the optimization of the reaction as it is the simplest and least
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18 338 expensive of the real-time PCR methods. By using SYBR Green, amplification products
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20 339 can be identified by their different T_m , by analysing the melting curve of the amplicon
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22 340 (Wittwer et al. 2001). The presence of a single peak at the corresponding T_m , indicates the
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24 341 specificity of the reaction. In a duplex real-time PCR, both amplicons can be distinguished
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26 342 by differences of at least 1 °C in T_m (Lipsky et al. 2001; Ririe et al. 1997). The duplex real-
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28 343 time PCR developed in this study yielded two peaks with clearly different T_m values: 80.5
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30 344 ± 0.5 for *A. niger* aggregate and 83.4 ± 0.4 for *A. carbonarius*, which confirmed the
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32 345 specificity of the two sets of primers. As a later approach, the duplex assay was adapted to
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34 346 be used in the TaqMan system by adding hybridization probes labeled with two different
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36 347 fluorescent dyes (FAM and VIC).
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43 348 Following optimization of the reaction conditions, the TaqMan duplex real-time PCR
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45 349 developed, was compared with both single real-time PCR reactions using purified DNA.
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47 350 This assay revealed very similar C_T values at any *A. tubingensis* DNA concentration with
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49 351 similar efficiency for uniplex and multiplex assays using as template only 1 target. For *A.*
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51 352 *carbonarius*, C_T values obtained by multiplex real-time PCR were slightly higher than those
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53 353 obtained with the uniplex but not significant differences in the reaction efficiency were
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55 354 found. Both approaches uniplex and duplex allowed the detection of 30 genome
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3 355 equivalents/reaction. Although, the uniplex assay was able to detect as far as 3 genome
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5 356 equivalents/reaction in some replicates, C_T values obtained for this concentration were 40
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8 357 or very close to 40 which is at the border line to be considered positive. Thus, the
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10 358 amplification of the two targets is efficient in the presence of the other competing primers
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12 359 and probe keeping the detection limit in 30 genome equivalents/reaction for both of them.
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15 360 These results are in the same range as the detection limit reported for the single detection of
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17 361 *A. carbonarius* with the TaqMan real-time PCR system developed by Mulè et al. (2006),
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19 362 and about 1 log order lower (more sensitive) than the detection limits reported for the single
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21 363 detection of *A. carbonarius* with the SYBR-Green I system used by Atoui et al. (2007).

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24 364 The suitability of this duplex real-time PCR system to detect *A. carbonarius* and *A.*
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26 365 *niger* aggregate strains, was evaluated by co-amplifying both targets present in different
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28 366 proportions using template DNA from both species with and without grape DNA. Both
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30 367 targets were detected in all combinations when using either SYBR Green or TaqMan.
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32 368 However, when both targets are in very different amounts (10 and 10^4 genome
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34 369 equivalents/reaction) the one that is in less concentration renders a C_T value very close to
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36 370 40 which indicates the preferential amplification of one of the targets in a multiplex PCR.
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38 371 These results were also obtained when both DNA were present in 10^2 and 10^4 genome
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40 372 equivalents/reaction. This fact can be induced in the presence of very low template
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42 373 concentration, as in this case, due to fluctuation in the interactions of PCR reagents,
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44 374 particularly in the early cycles (Mutter & Boyton 1995). As concentration and C_T values do
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46 375 not show a linear correlation when applied to very different amounts of target DNA,
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48 376 quantification would not be possible in these cases.

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50 377 The advantages of the multiplex real-time PCR developed in this study, besides the
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52 378 advantages of the real-time PCR, i.e. highly sensitive and reliable detection technique
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379 (Schaad & Frederick 2002), are the simultaneous detection of the main OTA producing
380 species in grapes, *A. carbonarius* and *A. niger* aggregate. To our knowledge this is the first
381 report on a multiplex real-time PCR reaction for the simultaneous detection of *A.*
382 *carbonarius* and *A. niger* aggregate in grape.

383 This procedure provides a fast and accurate tool to monitor in a single reaction the
384 presence of OTA producing species in grapes which, to some extent, will facilitate OTA
385 contamination surveys to guarantee food safety in the wine industry.

386

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3 496 **Figure legends**
4

5 497 Figure 1. Melting-curve analysis corresponding to the two amplicons generated by SYBR
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8 498 Green I multiplex real-time PCR showing the peaks and T_m values of *A. carbonarius* and
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10 499 *A. niger* aggregate.
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517 **Table I.** Fungal strains used for specificity assay of primers AtNig1/ AtNig2 and results
 518 of specificity by conventional PCR and real-time PCR.

Species	Strain	Source of isolation	OTA ^b	PCR	
<i>Aspergillus awamori</i>	CECT 2907 ^a	Bran, USA	+	+	
	CBS 101701 ^a	Unknown substratum, Japan	+	+	
<i>Aspergillus carbonarius</i>	CBS 113.80 ^a	Cocoa, Nigeria	ND	-	
	CBS111.26 ^a	Unknown substratum	ND	-	
	W04-20 ^a	Wine grape, Spain	+	-	
	W04-30 ^a	Wine grape, Spain	+	-	
	W04-32 ^a	Wine grape, Spain	+	-	
	W04-35 ^a	Wine grape, Spain	+	-	
	W04-40 ^a	Wine grape, Spain	+	-	
<i>Aspergillus costaricensis</i>	CBS 115574 ^a	Soil, Costa Rica	-	-	
<i>Aspergillus foetidus</i>	CBS 101708	Unknown substratum	+	+	
	CBS565.65 ^a	Unknown substratum, Japan	+	+	
<i>Aspergillus heteromorphus</i>	CBS117.55 ^a	<i>Trichophyton</i> contaminated culture, Brazil	-	-	
<i>Aspergillus homomorphus</i>	CBS101889 ^a	Soil, Israel	-	-	
<i>Aspergillus japonicus</i>	CBS114.51 ^a	Unknown substratum	-	-	
<i>Aspergillus lacticofeatus</i> ^T	CBS101883 ^{Ta}	Coffee, Venezuela	+	+	
<i>Aspergillus níger</i>	CECT 2090 ^a	Unknown substratum	ND	+	
	CECT 2091	Soil, Canada	ND	+	
	W04-19	Wine grape, Spain	+	+	
	W04-136 ^a	Wine grape, Spain	+	+	
	W04-140 ^a	Wine grape, Spain	+	+	
	W04-150 ^a	Wine grape, Spain	+	+	
<i>Aspergillus ochraceus</i>	CECT 2948 ^a	Unknown substratum	+	-	
	CBS 236.67 ^a	Unknown substratum	+	-	
<i>Aspergillus piperis</i>	CBS 112811 ^T	Black pepper, Denmark	-	-	
<i>Aspergillus pulverulentus</i>	CBS 558.65 ^a	Unknown substratum	+	+	
<i>Aspergillus tubingensis</i>	CBS 136.52	Kuro-koji, Japan	-	+	
	CBS134.48 ^a	Unknown substratum	ND	+	
	W04-12 ^a	Wine grape, Spain	+	+	
	W04-28	Wine grape, Spain	+	+	
	W04-53	Wine grape, Spain	ND	+	
	W04-57 ^a	Wine grape, Spain	ND	+	
	W04-109 ^a	Wine grape, Spain	ND	+	
	W04-118 ^a	Wine grape, Spain	ND	+	
	W04-146 ^a	Wine grape, Spain	ND	+	
	<i>Aspergillus tubingensis-like</i>	W04-17 ^a	Wine grape, Spain	+	+
		W04-22 ^a	Wine grape, Spain	+	+
W04-67 ^a		Wine grape, Spain	ND	+	
W04-77 ^a		Wine grape, Spain	ND	+	
W04-79 ^a		Wine grape, Spain	ND	+	
W04-132 ^a	Wine grape, Spain	ND	+		
<i>Aspergillus usamii</i>	CBS 101700 ^a	Unknown substratum	+	+	
<i>Botrytis cinerea</i>	W04-186 ^a	Wine grape, Spain	-	-	
<i>Cladosporium</i> spp.	W04-122 ^a	Wine grape, Spain	-	-	
<i>Fusarium</i> spp.	W04-101 ^a	Wine grape, Spain	-	-	
<i>Neopetromyces muricatus</i>	CBS 113177	Unknown substratum, USA	+	-	
<i>Penicillium</i> spp.	W04-28 ^a	Wine grape, Spain	-	-	

519 ND: OTA production was not determined. W04: Strains isolated from Spanish vineyards. CBS: Centralbureau voor
 520 Schimmelcultures. T: Type strain. CECT: Spanish Type Culture Collection. ^a Strains tested by SYBR Green I and
 521 TaqMan RTi-PCR using AtNig1/ AtNig2 primers. ^bOTA production was tested in previous studies (Martínez-
 522 Culebras and Ramón 2007, Martínez-Culebras et al. 2008)

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Table II. Primers and probes used in this study.

	Size	Oligonucleotide sequence	Tm	Location
AtNig1	22	5'-GAC TGA GCC CAG ATG ACC TAC A-3'	63.9	124-145 ^a
AtNig2	18	5'-CGC TGT CGC CGG ATA CTG-3'	66.4	244 to 227 ^a
AN probe ^b	31	5'- VIC-TTG ACT ATT GCA TGT TTT AAT AGC CCR AAG AAC C-MGB-3'	67.0	177 to 147 ^a
AcKS10R	22	5'-CCC TGA TCC TCG TAT GAT AGC G-3'	65.1	362 to 340 ^c
AcKS10L	22	5'-CCG GCC TTA GAT TTC TCT CAC C-3'	65.4	201-223 ^c
AC probe ^d	23	5'-FAM-AGA ACG CTG ATG GGT ATG CGC GG-TAMRA-3'	70.0	275 to 252 ^c

^aPrimers and probes designed in this study, to target a conserved region in the acyl transferase domain of polyketido synthase gene.

^bTaqman (MGB) probe labeled at the 5' end with VIC[®] and at the 3' end with a dark quencher.

^cPrimers and probes designed in a previous study (Selma et al. 2008), to target a conserved region in the β -ketosynthase domain of a polyketido synthase gene (Genbank accession number: AY540952).

^dTaqman probe labeled at the 5' end with 6-carboxy-fluorescein (FAM) and at the 3' end with 6-carboxy-tetramethyl-rhodamine (TAMRA).

531 **Table III.** Comparison of C_T values obtained by [TaqMan](#) real-time PCR uniplex and multiplex assays using as template [only 1 target](#), purified
 532 DNA of *A. carbonarius* CBS 113.80 [or](#) *A. tubingensis* CBS 134.48 from 3×10^5 to 3 equivalents/reaction.

Genome equivalents/reaction ^a	<i>A. carbonarius</i>		<i>A. tubingensis</i>	
	Uniplex ^d	Multiplex ^e	Uniplex ^f	Multiplex ^g
3.0×10^5	26.32 ± 0.39^b	27.49 ± 0.60	27.13 ± 0.07^b	27.67 ± 0.09
1.5×10^5	27.00 ± 0.30	27.81 ± 0.49	27.24 ± 0.08	27.66 ± 0.01
3.0×10^4	27.80 ± 0.19	28.41 ± 0.48	29.73 ± 0.26	29.73 ± 0.73
3.0×10^3	30.82 ± 0.48	30.29 ± 0.21	33.79 ± 0.64	33.13 ± 0.23
3.0×10^2	34.09 ± 0.49	34.12 ± 0.30	36.20 ± 0.24	35.95 ± 0.82
30	36.56 ± 0.50	37.81 ± 1.20	39.09 ± 0.07	38.59 ± 0.52
3	39.25 ± 0.69	(-)^h	39.37^h	(-)^c

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533 ^aCalculated assuming that genomic DNA weight of *A. carbonarius* CBS 113.80 and *A. tubingensis* CBS 134.48 is similar to that of *A. niger* (4.13×10^{-5} ng for
 534 a haploid genome). ^b C_T values (mean \pm SD) corresponding to three replicates. ^c~~Negative, no amplification. Standard curves obtained from genomic DNA serial~~
 535 ~~dilutions: ^d $y = -2.57x + 40.05$; ^e $y = -2.56x + 40.69$; ^f $y = -2.73x + 42.27$; ^g $y = -2.67x + 42.04$. ^h C_T value corresponding to the replicate out of 3 that showed~~
 536 ~~amplification.~~

537 **Table IV.** Co-amplification of different amounts of purified DNA from *A. carbonarius*
 538 CBS 113.80 and *A. tubingensis* CBS 134.48 in the presence of grape DNA.

	DNA ^a		SYBRGreen		TaqMan ^b	
	<i>A. carbonarius</i>	<i>A. tubingensis</i>	<i>A. carbonarius</i>	<i>A. tubingensis</i>	<i>A. carbonarius</i>	<i>A. tubingensis</i>
Without grape DNA	10 ⁴	10	+	+	24.30 ± 0.71	36.34 ± 0.63
	10 ⁴	10 ²	+	+	23.20 ± 0.17	33.36 ± 1.70
	10 ⁴	10 ³	+	+	22.90 ± 0.17	24.90 ± 0.21
	10 ⁴	10 ⁴	+	+	22.92 ± 0.26	21.71 ± 0.87
	10	10 ⁴	+	+	38.93 ± 0.12	21.86 ± 0.14
	10 ²	10 ⁴	+	+	35.71 ± 1.30	22.04 ± 0.47
	10 ³	10 ⁴	+	+	27.20 ± 0.26	21.94 ± 0.19
	10 ²	10 ²	+	+	29.97 ± 0.12	28.51 ± 0.07
With grape DNA	10 ⁴	10	+	+	23.12 ± 0.18	30.13 ± 0.20
	10 ⁴	10 ²	+	+	22.98 ± 0.12	28.92 ± 0.64
	10 ⁴	10 ³	+	+	22.79 ± 0.16	24.91 ± 0.72
	10 ⁴	10 ⁴	+	+	23.40 ± 0.39	22.39 ± 0.25
	10	10 ⁴	+	+	38.07 ± 0.56	22.13 ± 0.03
	10 ²	10 ⁴	+	+	33.09 ± 1.40	22.37 ± 0.06
	10 ³	10 ⁴	+	+	27.26 ± 0.38	22.44 ± 0.17
	10 ²	10 ²	+	+	30.87 ± 0.21	30.52 ± 1.30

539 ^aGenome equivalents/reaction. Genomic DNA weight of *A. carbonarius* CBS 113.80
 540 and *A. tubingensis* CBS 134.48 was calculated assuming them to be similar to that of *A.*
 541 *niger* (4.13×10⁻⁵ ng for a haploid genome).

542 ^bC_T values (mean ± SD) corresponding to three replicates.

543

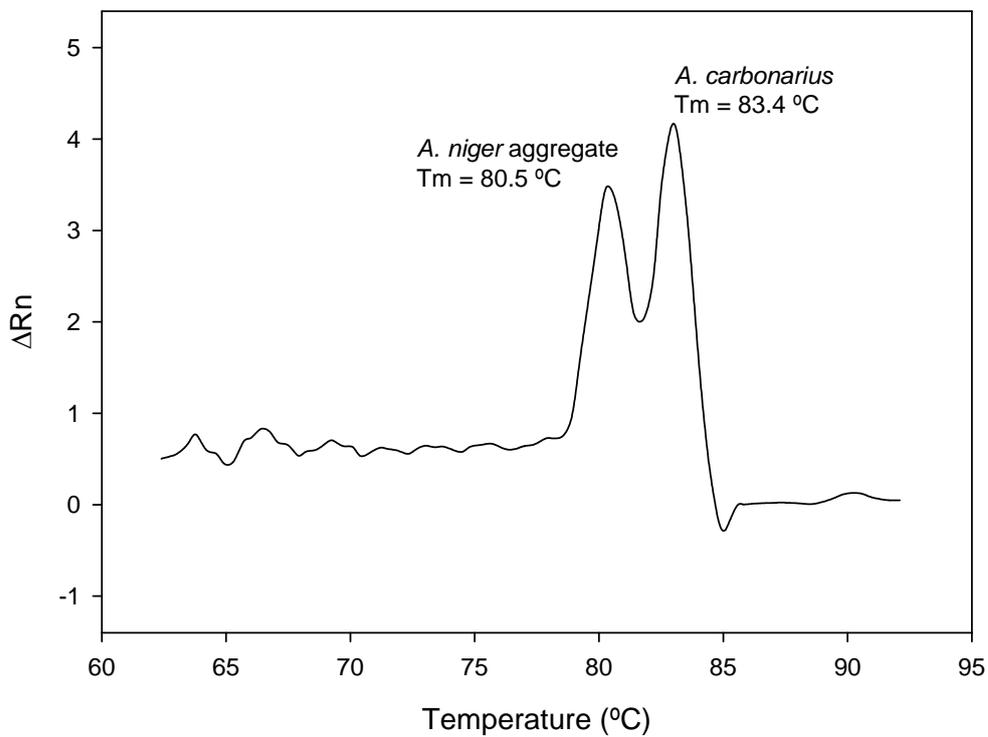
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551 Fig 1.



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Review Only