International Journal of Food Microbiology 165 (2013) 163–168

Contents lists available at SciVerse ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Multilocus sequence analysis of *Aspergillus* Sect. *Nigri* in dried vine fruits of worldwide origin

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ARTICLE INFO

Article history:
Received 22 February 2013
Received in revised form 24 April 2013
Accepted 29 April 2013
Available online 14 May 2013

Keywords:
Food microbiology
Biodiversity
Housekeeping genes
Phylogeny

ABSTRACT

Dried vine fruits may be heavily colonized by *Aspergillus* species. The molecular biodiversity of an *Aspergillus* population (234 strains) isolated from dried vine fruit samples of worldwide origin were analyzed by investigating four housekeeping gene loci (calmodulin, β-tubulin, elongation factor 1-α, RPB2). *Aspergillus* Sect. *Nigri* was dominant and the strains were identified as *A. tubingensis* (138), *A. awamori* (38), *A. carbonarius* (27), *A. uvarum* (16) and *A. niger* (11). Four *Aspergillus flavus* strains were also identified from Chilean raisins. Two clusters closely related to the *A. tubingensis* species with a significant bootstrap (60% and 99%) were identified as distinct populations. Among the four loci, RPB2 showed the highest genetic variability. This is the first complete study on the worldwide distribution of black Aspergilli occurring on dried vine fruits identified by a molecular approach.

1. Introduction

Species of *Aspergillus* Sect. *Nigri* are considered opportunistic fungi (saprophytes) of agronomically important crops, including grapes and dried vine fruit (Somma et al., 2012), and are the main fungi responsible for contamination of the grape-wine chain with mycotoxins, particularly ochratoxin A and fumonisins, that can affect human and animal health (Nielsen et al., 2009). The correct identification of *Aspergillus* species based on molecular methodologies is of primary concern in predicting potential mycotoxin contamination of grapes and derived products. Black Aspergilli isolated from grapes have been extensively studied using molecular methods (Perrone et al., 2006b, 2007; Dachoupakan et al., 2009; Chiotta et al., 2011), including the description of new species peculiar to grapes such as *A. ibericus* and *A. uvarum* (Serra et al., 2006; Perrone et al., 2008), however no comprehensive study is available on the molecular biodiversity of black Aspergilli occurring on dried vine fruit.

Individual reports on the occurrence and identification of *Aspergillus* Sect. *Nigri* on dried vine fruit are mostly focused on a small number of strains (Varga et al., 2010) or limited geographical area such as Spain (Abarca et al., 2003), Brazil (Iamanaka et al., 2005), Argentina (Da Rocha et al., 2002) and California (Palumbo et al., 2011). There is a lack of data from important dried vine fruit producing countries such as Turkey, Chile and Iran. Dried vine fruits include raisins, currants and sultanas, according to berry colour (white for sultanas and white or red for raisins) and origin (e.g. currants from Greece, sultanas from Turkey, raisins from USA, Turkey, Greece and Australia).

Housekeeping genes, particularly the calmodulin gene, have proved to be highly useful in discriminating species belonging to Section *Nigri* since they contain some species-specific traits, suitable for diagnostic purposes (Samson et al., 2007).

The present work aims to investigate molecular biodiversity in 4 loci (calmodulin, β-tubulin, elongation factor 1-α, RPB2) of a large population of Aspergilli isolated from dried vine fruits of worldwide origin.

2. Materials and methods

2.1. Fungal culture

A total of 234 *Aspergillus* strains randomly isolated from dried vine fruit samples of worldwide origin [Turkey (21) Chile (7); Iran (2); USA (2); China (2) and South Africa (1)] collected during 2009–2011 (Fig. 1) and twenty-four reference type strains from *Aspergillus* Section *Nigri* and Section *Flavi* (*A. tubingensis* ITEM7040, *A. acidus* ITEM4507, *A. costaricaensis* ITEM7555, *A. vadensis* ITEM7561, *A. niger* ITEM4501, *A. lacticoffeatus* ITEM7559, *A. awamori* ITEM4509, *A. brasiliensis* ITEM7048, *A. ibericus* ITEM4776, *A sclerotioniger* ITEM7560, *A. carbonarius* ITEM4503, *A. ellipticus* ITEM4505, *A. heteromorphus* ITEM7045, *A. homomorphus* ITEM7556, *A. aculeatus* ITEM7046, *A. japonicus* ITEM7034, *A. uvarum* ITEM4834, *A. nominus* ITEM7533, *A. parasiticus* ITEM7531, *A. sojae* ITEM7532, *A. minisclerotioniger* ITEM9596, *A. parvisclerotioniger* ITEM9594, *A. oryzae* ITEM7529, *A. flavus* ITEM7526) were obtained from the ITEM Collection (CNR-ISPA, Bari, Italy). Supplemental information about isolates can be recovered from the ITEM electronic catalogue (http: www.ispa.cnr.it/Collection). The

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http://dx.doi.org/10.1016/j.ijfoodmicro.2013.04.027

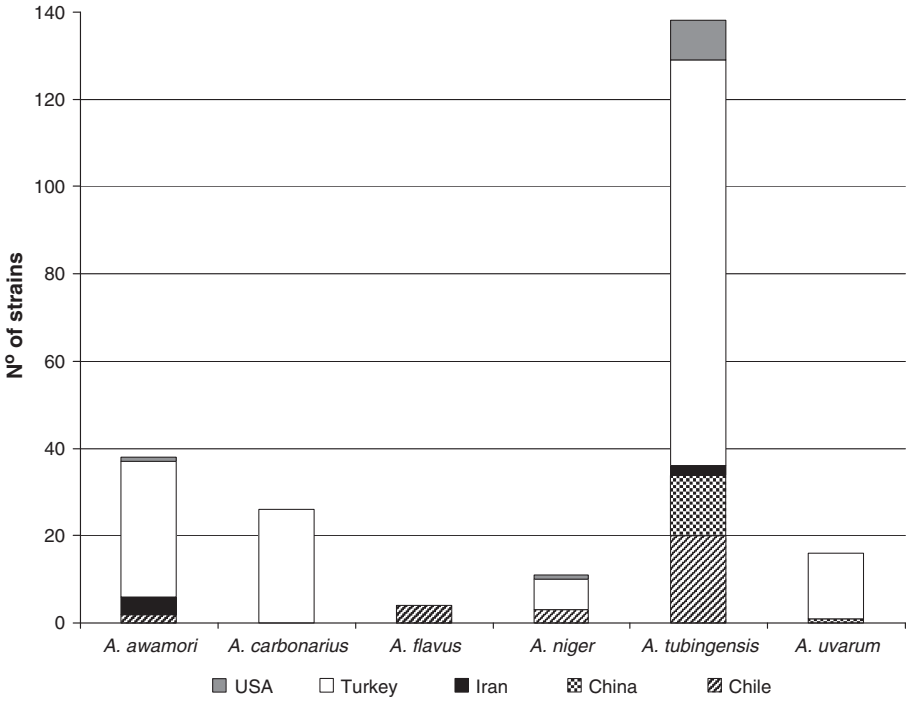


Fig. 1. Geographical origin of *Aspergillus* strains isolated world-wide from dried vine fruits.

mycological analysis of each sample in relation to mycotoxin contamination will be reported in another paper.

2.2. DNA extraction and sequencing

For mycelium production, a suspension of spores from each fungal strain was grown in Wickerham medium, containing 40 g glucose, 5 g peptone, 3 g yeast extract, 3 g malt extract and water up to 1 L. Mycelia were filtered and lyophilized for total DNA isolation. The fungal DNA was extracted by mechanical grinding using 5 mm iron beads in a Mixer Mill MM 301 (Retsch), and a "Wizard® Magnetic DNA Purification System for Food" kit (Promega), with some modifications, starting from 10 mg of lyophilized mycelium. The quality of genomic DNA was determined by electrophoresis and quantification using a Spectophotometer ND-1000 (Nano Drop).

Beta-tubulin (benA, ca. 450 nt), calmodulin (calM, ca. 650 nt), translation elongation factor-1 alpha (EF-1α, ca. 700 nt) and RNA polymerase II (RPB2, ca. 1100 nt) were amplified respectively using PCR conditions and primers described in literature: primers BT2a and BT2b (Glass and Donaldson, 1995), CL1 and CL2A (O'Donnell et al., 2000), A-EF_F/A-EF_R (Perrone et al., 2011), 5 F and 7CR (Liu et al., 1999). After amplification, the products were purified with the enzymatic mixture EXO/SAP (Exonuclease I, *E. coli*/Shrimp Alkaline Phosphatase). Bidirectional sequencing was performed for all loci and isolates. Sequence reactions were performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit for both strands, purified by gel filtration through Sephadex G-50 (Amersham Pharmacia Biotech) and analyzed on the "ABI PRISM 3730 Genetic Analyzer" (Applied Biosystems).

Alignment of the four loci was performed using the software package BioNumerics 5.1 from Applied Maths, with manual adjustments where necessary evaluated by an experienced human eye.

2.3. Sequence data analysis

The DNA sequences were aligned by the Clustal W algorithm (Thompson et al., 1994) using MEGA version 5 (Tamura et al., 2011): each locus was firstly aligned separately and concatenated into a super-gene alignment, which is then analyzed to generate the composite phylogenetic tree. The analysis involved 259 super-gene sequences. Phylogenetic analysis was performed in MEGA version 5 both by the Neighbor-Joining (NJ) method (Saitou and Nei, 1987) and the Maximum Likelihood (ML) method based on the Tamura-Nei model (Tamura and Nei, 1993). For NJ, evolutionary distances were computed using the Tamura–Nei method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset "complete deletion option". Bootstrap values were calculated from 1000 replications of the bootstrap procedure using programs within the MEGA 5 package which refers to tests of the reliability of an inferred tree (Felsenstein, 1985).

Maximum Likelihood analysis was performed with an initial tree(s) for the heuristic search obtained automatically as follows: when the number of common sites was <100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise, the BIONJ method with MCL distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories ($+G$, parameter = 0.5610)). The rate variation model allowed for some sites to be evolutionarily invariable ($[+I]$, 48.3317% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All ambiguous positions were removed for each sequence pair. There were a total of 2556 positions in the final dataset.

Sequence diversity and polymorphism were analyzed using the DnaSP v5 software (Librado and Rozas, 2009).

3. Results

3.1. Sequencing analysis

A multilocus analysis for 4 housekeeping genes covering 2556 nucleotides was performed on 234 strains isolated from raisins worldwide (Supplementary data, Table S1) in comparison with 24 reference type strains from *Aspergillus* Sections *Nigri* and *Flavi*. The evolutionary history was inferred using the Neighbor-Joining method.

Sequence diversity analysis performed by DnaSP software v5 showed the presence of 86 different haplotypes in the datasets analyzed when the loci were combined in a multilocus sequence alignment. In Table 1, the results of this analysis were summarized and included the sequence characters for each locus analyzed, the number of haplotypes and haplotype diversity, the variable sites and the parsimony-informative sites. The results of this analysis were then used to select the strains to be analyzed for phylogenetic studies among the 234 strains isolated. Both the percentage of variable sites and parsimony-informative sites varied for each data set: benA nucleotide sequence showed the highest percentage of variable sites, calM nucleotide sequences the highest percentage of informative sites, while EF-1α had the lowest of both (Table 1). Consequently, after a preliminary analysis using the MEGA5 software package with the Neighbor-Joining method, a deeper phylogenetic analysis was performed both with Neighbor-Joining and Maximum Likelihood. The best substitution model among six evolutionary models and four rates among sites were calculated using the 86 haplotypes characterizing the black Aspergilli population and the type strains studied. The optimal tree with the sum of branch length = 1.29532670 and the highest log likelihood (−14280.8455) is shown in Fig. 2, the phylogenetic tree which combines data from RPB2, EF-1α, calM and benA. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. As first result we assessed a higher variability within the *A. niger* and *A. tubingensis* groups compared with *A. carbonarius* and *A. uvarum*. Furthermore, regarding the genetic variability, we found the highest number of haplotypes (Table 1) in RPB2, followed by calM; EF-1α and benA, however, showed the highest percentage of informative variable sites. The analysis of polymorphic sites revealed a region (about 400 bp length) including the most parsimony informative sites, located between nucleotide 200 and 600 of the 878 bp analyzed in RPB2. An additional analysis (Supplementary data, Fig. S1) was performed on the variation in nucleotide composition among the haplotypes in each locus: benA, calM and RPB2 loci showing a quite well-distributed variability among the haplotypes compared with the EF-1α locus.

Moreover, data obtained allowed assessment of the mycological population belonging to *Aspergillus* genus most frequently occurring on dried vine fruits of worldwide origin (from Turkey, China, Chile and USA).

The predominant group of black Aspergilli occurring on dried vine fruits was *A. tubingensis* with 116 strains clustering with the *A. tubingensis* type strains ITEM 7040, with two additional clusters closely related to the *A. tubingensis* species with a bootstrap value of 60% for group 1 including 16 strains, and a higher bootstrap of 99% for group 2 including 6 strains, respectively named *A. tubingensis* group 1 and 2 on the phylogram. The phylogenetic species *A. awamori* (Perrone et al., 2011) was the second most represented group among this population, with 38 strains compared to *A. niger* (8 strains), and three strains (ITEM 10953, 11930 and 12900) among the *A. niger* group with atypical results. Twenty-seven strains clustered with a high bootstrap with the *A. carbonarius* type strain, while none belonged to the closely related species of the clade *A. ibericus* and *A. sclerotioniger*. Among the uniseriate group, no *A. japonicus* or *A. aculeatus* were identified, all the uniseriate strains isolated (16) belonging to the *A. uvarum* cluster, confirming a recent result reported by Varga et al. (2010). The four strains morphologically identified as *A. flavus* belonged, with a high bootstrap value, to the *A. flavus* type strain ITEM 7526. In particular, on analyzing sequence variability and polymorphism among the black Aspergilli population on dried vine fruits, using the DnaSP software v5, we found 62 different haplotypes (H) with a diversity Hd = 0.968. In particular, 26 H representing 116 strains belonging to *A. tubingensis*, 10 H of 16 strains belonging to *A. tubingensis* group 1, 2 H of 6 strains belonging to *A. tubingensis* group 2, 5 H of 11 strains belonging to *A. niger* of which 3 H represent 3 atypical *A. niger* strains. *A. awamori* that was the second group of represented species had 7 H representing 38 strains. There were nine *A. carbonarius* haplotypes, representing 27 strains and 3 H of *A. uvarum* belonging to 16 strains, evidencing a higher homogeneity of these two species compared with the *A. niger*/*A. tubingensis* clade. In particular, *A. tubingensis* is the most represented species, being the only one distributed in all of the countries in the survey and the population with the highest number of haplotypes. Among these, most were related to variability in a single or in two strains, while seven haplotypes represented different groups within the *A. tubingensis* population (Fig. 2); by contrast, the haplotype of ITEM 7040 contained strains from four countries (8 from Turkey, 6 from China, 5 from Chile and 2 from USA) and the haplotype of ITEM 10634 contained 26 strains, all of which came from Turkey. The 2 haplotypes representing the four *A. flavus* strains isolated were not included in this analysis but are present in the phylogenetic tree (Fig. 2).

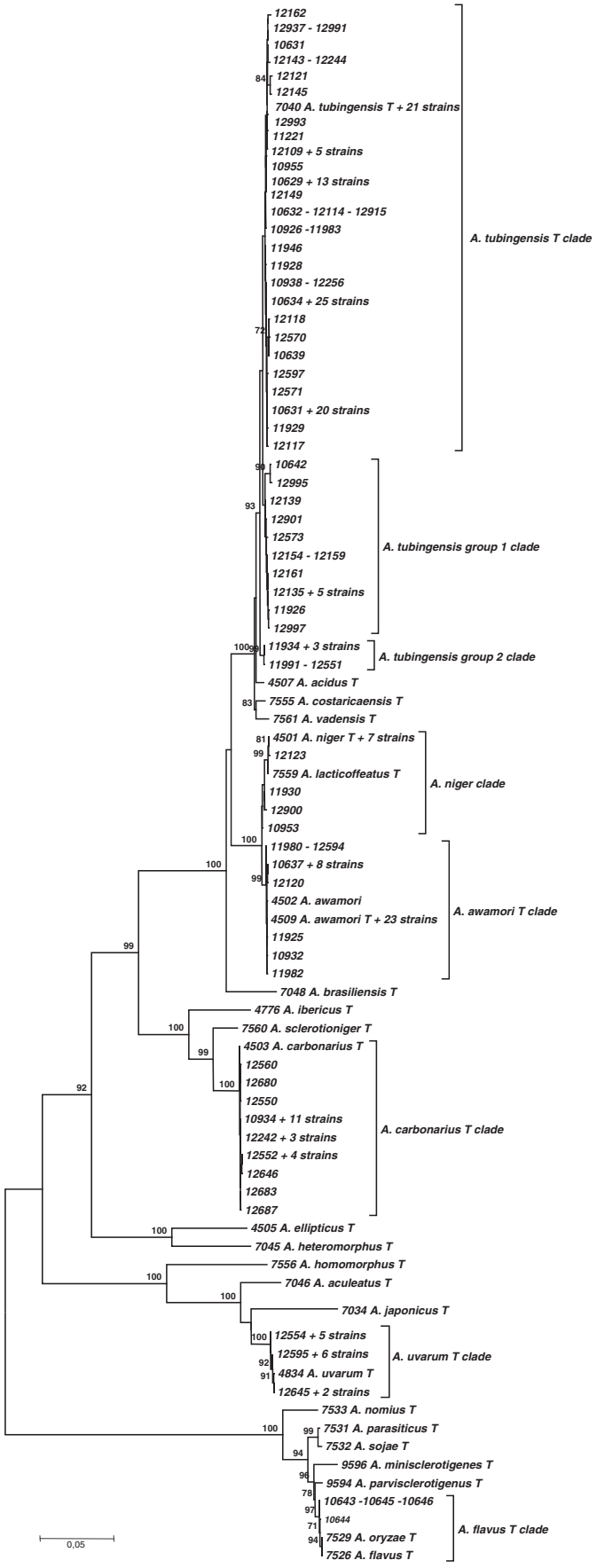
Geographic differentiations in the haplotypes within the species, in particular in *A. tubingensis*, the larger group, was not detected when isolates from all five countries were included in the analysis.

4. Discussion

Black Aspergilli are one of the more difficult groups in terms of classification and identification, and several taxonomic schemes have been proposed. New molecular approaches have shown that there is a high biodiversity, but that species are occasionally difficult to recognize based solely on their phenotypic characters (Samson et al., 2007). In this regard, the taxonomy of black Aspergilli is not completely resolved; however, accurate identification at species level is of great importance due to the different toxin profiles of each individual taxon. In recent years, to test taxonomic hypotheses based on phenotypic analysis of *Aspergillus*, several investigations have been conducted using DNA or RNA sequence information, mostly taking an established group (Raper and Fennell, 1965, 1977) or section (Gams et al., 1985) and to a greater or lesser extent resolving the taxonomic hypotheses within the group (Peterson, 1995; Frisvad et al., 2004; Varga et al., 2005). More recently, Varga et al. (2011) used a polyphasic approach including morphological, physiological, ecological and molecular (sequence analysis of parts of the β-tubulin and calmodulin genes and the ITS region) analyses in order to investigate the broad relationships among Aspergilli, leading to the description of four new species in the *Nigri* section. Gene sequencing is one of the most robust and informative techniques for fungal diagnosis. Multilocus sequence typing (MLST) has emerged as a powerful new DNA-typing tool for the evaluation of intraspecies genetic relatedness. This method relies on DNA sequence analysis of nucleotide polymorphisms in housekeeping genes and has shown a high degree of

Table 1
Sequence characteristics and phylogenetic information for RPB2, EF-1α, calM, benA and combined MLS.

Domain	Region	Sites	Net sites	% GC	No. of variable sites	No. of informative sites	No. of mutations (Eta)	Nucleotide diversity	No of haplotypes	Haplotype diversity
RPB2	1-985	985	878	52.8	354	293	435	0.07155	44	0.886
EF-1α	987-1636	650	649	57.8	107	79	121	0.02057	35	0.713
calM	1638-2205	568	486	53.8	271	249	316	0.08683	37	0.872
benA	2207-2702	496	323	58.0	259	202	180	0.05822	23	0.663
MLS	1-2702	2702	2336	55.1	991	823	1052	0.05872	86	0.959



intraspecies discriminatory power for bacterial and fungal pathogens. This method compares nucleotide polymorphisms within regions of four to seven genes which are under selective pressure to retain function. Polymorphisms giving rise to allelic variants are recorded as bar codes of integers which together constitute a strain sequence type (ST). The advantages of MLST as a typing approach are its portability (sequencing can be done anywhere, with identical results for identical DNA samples) and its archive ability (results can be stored in a central web database). MLST is increasingly being applied as a routine typing tool that enables international comparison of isolates. MLST data have also been exploited in evolutionary and population analyses that estimate recombination and mutation rates and investigate evolutionary relationships among bacteria that are classified as belonging to the same genus.

For our study on MLST, proposed as a practical tool for typing *Aspergillus* Sect. *Nigri* isolated from dried vine fruits of worldwide origin, we examined 4 loci of nuclear DNA markers already used for molecular identification of fungal species: "calmodulin (calM)" (O'Donnell et al., 2000), "β-tubulin (benA)" (Geiser et al., 2007), "elongation factor 1-α (EF-1α)" (O'Donnell et al., 2008) and "second largest subunit of RNA polymerase II (RPB2)" (Ertz et al., 2009).

In this work we characterized molecularly 234 *Aspergillus* strains isolated from raisins from 5 different geographical origins and we examined their DNA variability in calM, benA, EF-1α and RPB2. The most predominant species belong to *Aspergillus* Sect. *Nigri*, in particular *A. tubingensis*, followed by *A. awamori A. carbonarius*, *A. uvarum* and *A. niger*. The analysis of DNA variability showed RPB2 as locus with the highest number of haplotypes and haplotype diversity with the most variable sites located near the 5 F primer (a region about 400 bp length), suggesting it as a major candidate tract for barcoding in *Aspergillus*. Among 86 different haplotypes identified in *Aspergillus* spp. isolated from raisins, 62 represented the population of *Aspergillus* Sect. *Nigri*. The distribution and composition of the haplotypes differ among the species also when geographical origin is considered. In particular it suggests lack of isolation in relation to the distance, due to the presence of a same haplotype in different continents (i.e. haplotype ITEM 7040 containing 21 strains from Asia, North and South America, Fig. 2 and Supplementary data, Table S1); Conversely, the singular haplotype ("private") within the same geographical region and species, was indicative of genetic isolation.

Analytical techniques are available to address the broad question of how all fungi are related and also the very narrow question of where species boundaries occur (Taylor et al., 2000; Sites and Marshall, 2003). The issue of what constitutes a species continues to be debated (e.g. Rieppel, 2007), but the phylogenetic species concept with recognition by concordance of independent gene trees is a very attractive option. Because these species are defined in terms of genetics, monophyly can be assured. Moreover, an additional benefit of genetically defined species is represented by its suitability to set rapid detection techniques that identify species through polymorphisms in DNA (Susca et al., 2007a, 2007b; Mulè et al., 2006; Bufflier et al., 2007).

The DNA sequences with concordance analysis provide the necessary information to define species and provide sequence data for DNA-based detection and identification of medically, industrially and agriculturally important species (Hinrikson et al., 2005; Page and Kurtzman, 2005; Serra et al., 2006), but will also be valuable for putting other data into phylogenetic perspective. The phylogenetic results revealed 3 distinct clades within the *A. tubingensis* analyzed group, suggesting that it probably contains 3 distinct cryptic species,

Fig. 2. Neighbor Joining tree obtained with Maximum Composite Likelihood algorithm derived from combined RPB2, TEF, CaM and BenA data. 62 haplotypes representative of the molecular biodiversity of 234 strains of *Aspergillus* population isolated from dried vine fruits are compared with 24 type strains of *Aspergillus Nigri* and *Flavi* Sections. Numbers at nodes are bootstrap values, only value >70% are reported.

which should be corroborated by further analyses which are currently in progress.

Of the *Aspergillus* species identified in this study, *A. carbonarius* is the one causing greatest concern due to the high percentage of OTA producers and due to its ability to produce high levels of OTA (Abarca et al., 2001; Belli et al., 2004; Perrone et al., 2006a). Interestingly, while the other species were found distributed among the different countries and samples, *A. carbonarius* was isolated only from raisins collected from Turkey, thus confirming this species' great adaptability and presence in the Mediterranean basin, as well as the higher risk for OTA contamination generally reported for this region. Other species that may be considered of concern are *A. niger* and *A. awamori*, since recently some strains of these two species have been reported to be fumonisin B2 and B4 producers (Logrieco et al., 2009; Mogensen et al., 2010; Frisvad et al., 2011) and responsible for fumonisin contamination in dried vine fruit samples (Varga et al., 2010). Nevertheless, the percentage of fumonisin-producing strains of *A. niger* reported in the mentioned studies was variable and a discontinuous distribution of fumonisin-producing strains and the absence of at least part of the fumonisin biosynthetic gene cluster has been reported in *A. niger* (Susca et al., 2010). Furthermore, the presence of four strains of *A. flavus* from Chilean raisins has to be noted. This species is considered highly toxigenic, with some strains able to produce aflatoxins. In this respect, aflatoxins may occur as common contaminants of dried vine fruits in some countries such as India (Saxena and Mehrotra, 1990), Egypt (Youssef et al., 2000), and Greece (Apergi et al., 1998) even at very high levels.

Further studies must be focused on the ability to produce mycotoxins in these strains molecularly identified at species level, in order contribute to defining the toxicity of the species and the potential mycotoxin risk in dried vine fruits.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ijfoodmicro.2013.04.027.

Acknowledgments

We would like to thank Vincenzo Ricci, Filomena Epifani and Alessandra Villani for their technical assistance and Dr. Giuseppe Calcagni of Fundacio Privada International Tree Nut for providing most of the samples. This study was supported by EC KBBE-2007-222690-2 MYCORED.

References

Abarca, M.L., Accensi, F., Bragulat, M.R., Cabañes, F.J., 2001. Current importance of ochratoxin A-producing *Aspergillus* spp. Journal of Food Protection 64, 903–906.

Abarca, M.L., Accensi, F., Bragulat, M.R., Castella, G., Cabanes, F.J., 2003. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried wine fruits from the Spanish market. Journal of Food Protection 66, 504–506.

Apergi, E., Gardikis, J.P., Panagiotopoulou, V.Y., 1998. Occurrence of aflatoxins B1, B2, G1 and G2 in imported goods in Greece during 1995. In: Miraglia, M., Van Egmond, H., Brera, C., Gilbert, J. (Eds.), Mycotoxins and phycotoxins – developments in chemistry, toxicology and food safety. Fort Collins, CO, Alaken, pp. 105–110.

Belli, N., Pardo, E., Marin, S., Farrè, G., Ramos, A.J., Sanchis, V., 2004. Occurrence of ochratoxin A and toxigenic potential of fungal isolates from Spanish grapes. Journal of the Science of Food and Agriculture 84, 541–546.

Bufflier, E., Susca, A., Baud, M., Mulè, G., Brengel, K., Logrieco, A., 2007. Detection of *Aspergillus carbonarius* and other black Aspergilli from grapes by DNA OLISA microarray. Food Additives and Contaminants 24, 1138–1147.

Chiotta, M.L., Susca, A., Stea, G., Mulè, G., Perrone, G., Logrieco, A., Chulze, S.N., 2011. Phylogenetic characterization and ochratoxin A — fumonisin profile of black *Aspergillus* isolated from grapes in Argentina. International Journal of Food Microbiology 149, 171–176.

Da Rocha, R., Palacios, V., Combina, M., Fraga, M.E., De, O.R., Magnoli, C.E., Dalcero, A.M., 2002. Potential ochratoxin A producers from wine grapes in Argentina and Brazil. Food Additives and Contaminants 19, 408–414.

Dachoupakan, C., Ratomahenina, R., Martinez, V., Guiraud, J.P., Baccou, J.C., Schorr-Galindo, S., 2009. Study of the phenotypic and genotypic biodiversity of potentially ochratoxigenic black Aspergilli isolated from grapes. International Journal of Food Microbiology 132, 14–23.

Ertz, D., Miadlikowska, J., Lutzoni, F., Dessein, S., Raspé, O., Vigneron, N., Hofstetter, V., Diederich, P., 2009. Towards a new classification of the Arthoniales (Ascomycota) based on a three-gene phylogeny focussing on the genus *Opegrapha*. Mycological Research 113, 141–152.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Frisvad, J.C., Frank, J.M., Houbraken, J.A.M.P., Kuijpers, A.F.A., Samson, R.A., 2004. New ochratoxin A producing species of *Aspergillus* section *Circumdati*. Studies in Mycology 50, 23–43.

Frisvad, J.C., Larsen, T.O., Thrane, U., Meijer, M., Varga, J., Samson, R.A., Nielsen, K.F., 2011. Fumonisin and ochratoxin production in industrial *Aspergillus niger* strains. PLoS One 6 (8), e23496.

Gams, W., Christensen, M., Onions, A.H.S., Pitt, J.I., Samson, R.A., 1985. Infrageneric taxa of *Aspergillus*. In: Samson, R.A., Pitt, J.I. (Eds.), Advances in *Penicillium* and *Aspergillus* systematics. Plenum Press, New York, pp. 55–64.

Geiser, D.M., Klich, M.A., Frisvad, J.C., Peterson, S.W., Varga, J., Samson, R.A., 2007. The current status of species recognition and identification in *Aspergillus*. Studies in Mycology 59, 1–10.

Glass, N.L., Donaldson, G.C., 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 61, 1323–1330.

Hinrikson, H.P., Hurst, S.F., Lott, T.J., Warnock, D.W., Morrison, C.J., 2005. Assessment of ribosomal large-subunit D1–D2, internal transcribed spacer 1 and internal transcribed spacer 2 regions as targets for molecular identification of medically important *Aspergillus* species. Journal Clinical Microbioly 43, 2092–2103.

Iamanaka, B.T., Taniwaki, M.H., Menezes, H.C., Vicente, E., Fungano, M.H.P., 2005. Incidence of toxigenic fungi and ochratoxin A in dried fruits sold in Brazil. Food Additives and Contaminants, Part A 22, 1258–1263.

Librado, P., Rozas, J., 2009. DnaSP v5: A Software for Comprehensive Analysis of DNA Polymorphism Data. Bioinformatics 25, 1451–1452. http://dx.doi.org/10.1093/bioinformatics/btp187.

Liu, Y.L., Whelen, S., Hall, B.D., 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. Molecular Biology and Evolution 16, 1799–1808.

Logrieco, A., Ferracane, R., Haidukowsky, M., Cozzi, G., Visconti, A., Ritieni, A., 2009. Fumonisin B2 production by *Aspergillus niger* from grapes and natural occurrence in must. Food Additives and Contaminants 26, 1495–1500.

Mogensen, J.M., Larsen, T.O., Nielsen, K.F., 2010. Widespread occurrence of the mycotoxin fumonisin B2 in wine. Journal of Agricultural and Food Chemistry 58, 4853–4857.

Mulè, G., Susca, A., Logrieco, A., Stea, G., Visconti, A., 2006. Development of a quantitative real-time PCR assay for the detection of *Aspergillus carbonarius* in grapes. International Journal of Food Microbiology 111 (Suppl. 1), S28–S34.

Nielsen, F.K., Mogensen, J.M., Johansen, M., Thomas, O.L., Frisvad, C.J., 2009. Review of secondary metabolites and mycotoxins from the *Aspergillus niger* group. Analytical and Bioanalytical Chemistry 395, 1225–1242.

O'Donnell, K., Nirenberg, H.I., Aoki, T., Cigelnik, E., 2000. A multigene phylogeny of the *Gibberella fujikuroi* species complex: detection of additional phylogenetically distinct species. Mycoscience 41, 61–78.

O'Donnell, K., Sutton, D.A., Fothergill, A., McCarthy, D., Rinaldi, M.G., Brandt, M.E., Zhang, N., Geiser, D.M., 2008. Molecular phylogenetic diversity, multilocus haplotype nomenclature, and in vitro antifungal resistance within the *Fusarium solani* species complex. Journal of Clinical Microbiology 46, 2477–2490.

Page, B.T., Kurtzman, C.P., 2005. Rapid identification of Candida species and other clinically important yeast species by flow cytometry. Journal of Clinical Microbiology 43, 4507–4514.

Palumbo, J.D., O'Keeffe, T.L., Vasquez, S.J., Mahoney, N.E., 2011. Isolation and identification of ochratoxin A-producing *Aspergillus* section *Nigri* strains from California raisins. Letters in Applied Microbiology 52, 330–336.

Perrone, G., Mulè, G., Susca, A., Battilani, P., Pietri, A., Logrieco, A., 2006. Ochratoxin A production and AFLP analysis of *Aspergillus carbonarius*, *Aspergillus tubingensis*, and *Aspergillus niger* strains isolated from grapes in Italy. Applied and Environmental Microbiology 72, 680–685.

Perrone, G., Susca, A., Epifani, F., Mulè, G., 2006. AFLP characterization of Southern Europe population of *Aspergillus* Section *Nigri* from grapes. International Journal of Food Microbiology 111 (Suppl. 1), S22–S27.

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J.C., Meijer, M., Noonim, P., Mahakarnchanakul, W., Samson, R.A., 2007. Biodiversity of *Aspergillus* species in some important agricultural products. Studies in Mycology 59, 53–66.

Perrone, G., Varga, J., Susca, A., Frisvad, J.C., Stea, G., Kocsubé, S., Tóth, B., Kozakiewicz, Z., Samson, R.A., 2008. *Aspergillus uvarum* sp. nov., an uniseriate black *Aspergillus* species isolated from grapes in Europe. International Journal of Systematic and Evolutionary Microbiology 58, 1032–1039.

Perrone, G., Stea, G., Epifani, F., Varga, J., Frisvad, J.C., Samson, R.A., 2011. *Aspergillus niger* contains the cryptic phylogenetic species *A. awamori*. Fungal Biology 115, 1138–1150.

Peterson, S.W., 1995. Phylogenetic analysis of *Aspergillus* sections *Cremei* and *Wentii*, based on ribosomal DNA sequences. Mycological Research 99, 1349–1355.

Raper, K.B., Fennell, D.I., 1965. The genus *Aspergillus*. Williams & Wilkins Co., Baltimore.

Raper, K.B., Fennell, D.I., 1977. The genus *Aspergillus*. Krieger Publishing Company, Huntingon, NewYork, USA.

Rieppel, O., 2007. Species: kinds of individuals or individuals of a kind. Cladistics 23, 373–384.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4, 406–425.

Samson, R.A., Noonim, P., Meijer, M., Houbraken, J., Frisvad, J.C., Varga, J., 2007. Diagnostic tools to identify black Aspergilli. Studies in Mycology 59, 129–146.

Saxena, J., Mehrotra, B.S., 1990. The occurrence of mycotoxins in some dry fruits retail marketed in Nainital district of India. Acta Alimentaria 19, 221–224.

Serra, R., Cabanes, F.J., Perrone, G., Castella, G., Venancio, A., Mule, G., Kozakiewicz, Z., 2006. *Aspergillus ibericus*: a new species of section *Nigri* isolated from grapes. Mycologia 98, 295–306.

Sites, J.W., Marshall, J.C., 2003. Delimiting species: a renaissance issue in systematic biology. Trends in Ecology and Evolution 18, 462–470.

Somma, S., Perrone, G., Logrieco, A., 2012. Diversity of black Aspergilli and mycotoxin risks in grape, wine and dried vine fruits. Phytopathologia Mediterranea 51, 135–152.

Susca, A., Stea, G., Mulè, G., Perrone, G., 2007. Polymerase chain reaction (PCR) identification of *Aspergillus niger* and *Aspergillus tubingensis* based on the calmodulin gene. Food Additives and Contaminants 24, 1154–1160.

Susca, A., Stea, G., Perrone, G., 2007. Rapid polymerase chain reaction (PCR)-single-stranded conformational polymorphism (SSCP) screening method for the identification of *Aspergillus* section *Nigri* species by the detection of calmodulin nucleotide variations. Food Additives and Contaminants 24, 1148–1153.

Susca, A., Proctor, R.H., Mulè, G., Stea, G., Ritieni, A., Logrieco, A., Moretti, A., 2010. Correlation of mycotoxin fumonisin B2 production and presence of the fumonisin biosynthetic gene fum8 in *Aspergillus niger* from grape. Journal of Agricultural and Food Chemistry 58, 9266–9272.

Tamura, K., Nei, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10, 512–526.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution 28, 2731–2739.

Taylor, J.W., Jacobson, D.J., Kroken, S., Kasuga, T., Geiser, D.M., Hibbett, D.S., Fisher, M.C., 2000. Phylogenetic species recognition and species concepts in fungi. Fungal Genetics and Biology 31, 21–32.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22 (22), 4673–4680.

Varga, J., Tóth, B., Kocsubé, S., Farkas, B., Szakács, G., Téren, J., Kozakiewicz, Z., 2005. Evolutionary relationships among *Aspergillus terreus* isolates and their relatives. Antonie Van Leeuwenhoek 88, 141–150.

Varga, J., Kocsubé, S., Suri, K., Szigeti, G., Szekeres, A., Varga, M., Tóth, B., Bartók, T., 2010. Fumonisin contamination and fumonisin producing black Aspergilli in dried vine fruits of different origin. International Journal of Food Microbiology 143, 143–149.

Varga, J., Frisvad, J.C., Kocsubé, S., Brankovics, B., Tóth, B., Szigeti, G., Samson, R.A., 2011. New and revisited species in *Aspergillus* section *Nigri*. Studies in Mycology 69, 1–17.

Youssef, M.S., Abo-Dahab, N.F., Abou-Seidah, A.A., 2000. Mycobiota and mycotoxin contamination of dried raisins in Egypt. African Journal of Mycology and Biotechnology 8, 69–86.