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Strain-specific polyketide synthase genes of *Aspergillus niger*

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ABSTRACT

In silico comparison of 34 putative *pks* genes in *Aspergillus niger* strain CBS 513.88 versus *A. niger* strain ATCC 1015 genome revealed significant nucleotide identity (>95% covering a minimum of 99% of the gene sequence) for 31 of these genes (approximately 91%). *A. niger* CBS 513.88 harbors three putative *pks* genes (An01g01130, An11g05940, and An15g07920), for which nucleotide identity was not found in *A. niger* ATCC 1015. To compare the results of the in silico analysis with the in vivo situation, experimental data were obtained for a large number of *A. niger* strains obtained from different substrates and geographical regions. Three putative *pks* genes that were found to be variable between the two *A. niger* strains using bioinformatics tools were in fact strain-specific genes based on experimental data. The PCR amplification signals for the An01g01130, An11g05940, and An15g07920 *pks* genes were detected in only 97%, 71%, and 26% of the strains, respectively. Southern blot analyses confirmed the PCR data. Because one of the strain-specific *pks* genes (An15g07920) is located in a putative ochratoxin cluster, we focused our investigation on that region. We assessed the ochratoxin production capability of the 119 *A. niger* strains and found a positive association between the presence of this *pks* gene and the capability of the respective strain to produce ochratoxin.

1. Introduction

Filamentous fungi are a well-known source of several small molecules derived from secondary metabolism. These small molecules range from beneficial antibiotics to harmful toxins (Palmer and Keller, 2010). Genes associated with secondary metabolite biosynthesis are often found physically linked or clustered within a given region of a genome, and most of the time they are near telomeric regions. These clusters usually harbor genes encoding polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS), which are adjacent to genes encoding hydrolases, oxidases, methylases, transporters, and regulatory proteins. Because PKS and NRPS are typically associated with secondary metabolism, a convenient approach to find a new biosynthetic pathway is to search genome sequences for those genes first, and subsequently examine adjacent regions (Turner, 2010). The recent availability of the complete sequences of several genomes from filamentous fungi has revealed a surprisingly large number of PKS and NRPS genes, indicating that there are a number of secondary metabolites that have yet to be discovered and structurally elucidated (von Döhren, 2009).

Aspergillus niger is one of the most important microorganisms used for biotechnological purposes. For many decades, it has been used to produce citric acid as well as for a source of extracellular enzymes and in the traditional process of oriental food manufacturing. This species has been labeled with generally recognized as safe (GRAS) status from the US Food and Drug Administration; however, it may also produce metabolites that are harmful to humans (as reviewed by Gautam et al., 2011). Some isolates of *A. niger* are able to produce ochratoxin A and/or fumonisin, and the current general consensus within the research community is that new and unknown isolates of fungi should be carefully checked for mycotoxin production before their use in industrial applications (Abarca et al., 1994; Frisvad et al., 2007).

Analysis of multiple genome sequences from a single species is an exciting field of biological research, which provides new ways to understand evolution, adaptation, and population structure. In addition,

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these studies can be used for more applied research, such as the discovery of new gene products. This type of analysis is currently available for several bacteria, but is very limited for fungi, since few strains within the same species have had their genomes completely sequenced.

To date, only three strains of *A. niger* (CBS 513.88, ATCC 1015, and ATCC 9029) have had their genome sequences determined; however, public genome websites are only available for CBS 513.88 (http://www.ncbi.nlm.nih.gov/genomeprj/19263) and ATCC 1015 (http://genome.jgi-psf.org/Aspni5/Aspni5.home.html) strains. The ATCC 1015 strain is used for industrial production of citric acid, and CBS 513.88 is an early ancestor of ATCC 1015. A total of 33 PKS, 15 NRPS, and 9 hybrid PKS/NRPS genes have been identified throughout the genome of *A. niger* ATCC 1015 (Fisch et al., 2009). In the CBS 513.88 strain, 34 PKS, 14 NRPS, and 7 hybrid PKS/NRPS genes have been found, and most of them are located in clusters (Pel et al., 2007). Although comparative analyses among genomes of *Aspergillus* species have suggested that some secondary metabolism gene clusters are species-specific (i.e. no orthologs in other species, as reviewed by Fedorova and Nierman (2010), studies that aim to identify intraspecific variation in the context of genes involved in secondary metabolism have only just begun (Sun et al., 2007; Fedorova et al., 2008; Fisch et al., 2009; Andersen et al., 2011).

In this study, bioinformatic approaches were applied to the *pks* gene inventory of two *A. niger* genomes (CBS 513.88 and ATCC 1015) in order to identify strain-specific gene clusters for secondary metabolism pathways. To compare results of the in silico analysis with the in vivo situation, experimental data were collected from a large number of *A. niger* strains obtained from different substrates and geographical regions.

2. Material and methods

2.1. Strains

We analyzed a total of 119 *A. niger* isolates. The isolates were provided by two Brazilian institutions: 87 from Instituto de Tecnologia de Alimentos (ITAL, Campinas) and 32 from Universidade Estadual de Londrina (UEL, Londrina). Of them, 35 were collected from dry fruits (black sultanas, dates, prunes, and dried figs), 22 were collected from Brazil nuts, and 62 were isolated from coffee beans (Table 1).

2.2. Ochratoxin A production

The ochratoxin A (OTA) production of each isolate was assessed using the method described by Filtenborg et al. (1983). Briefly, isolates were three-point inoculated into Yeast Extract Sucrose Agar (YES Agar) and incubated at 25 °C for 7 d. Three agar plugs were removed from the central area of the colony, weighed, and introduced into a small vial. Methanol (0.5 mL) was then added to the vial. After 60 min, the extracts were filtered (Millex-Millipore) and injected into a high performance liquid chromatography (HPLC) system (Shimadzu 10VP). This system has a fluorescence detector that was operated at an excitation wavelength of 333 nm and an emission wavelength of 477 nm. The HPLC was fitted with a Shimadzu CLC G-ODS (4×10 mm) guard column and a Shimadzu Shimpack CLC-ODS (4.6×250 mm) column. The mobile phase was methanol:acetonitrile:water:acetic acid (35:35:29:1), and the flow rate was 0.8 mL/min, according to Vargas et al. (2005). An OTA standard (Sigma) was used for the construction of a five-point calibration curve, peak area versus mass (ng). The OTA concentration in the sample extract was determined by interpolation of the rcesulting peak areas from the calibration graph.

2.3. DNA extraction

Conidia of each isolate were inoculated into liquid minimal medium (Pontecorvo et al., 1953) and incubated at 28 °C for 24 h at 180 rpm. Genomic DNA was extracted according to Azevedo et al. (2000) and quantified using a fluorimetric method (Dyna Quant, Phamarcia).

2.4. Partial β-tubulin gene sequence analyses

Amplification of a β-tubulin gene region was performed using the following primer pair: Bt2a (5′ GGT AAC CAA ATC GGT GCT GCT TTC 3′) and Bt2b (5′ ACC CTC AGT GTA GTG ACC CTT GGC 3′), as described by Glass and Donaldson (1995). Standard amplification reactions and cycling protocols were adopted, and amplicons submitted to direct sequencing in both directions (forward and reverse) in a MegaBaceTM 1000 Molecular Dynamics system (Amersham, Pharmacia Biotech). The quality of the sequences was examined using the software package Phred/Phrap/Consed. Phylogenetic analysis was performed by using CLUSTAL W multiple-sequence alignment program version 1.6 (Thompson et al., 1994).

2.5. Survey of polyketide synthase genes by PCR

Based on the nucleotide sequence from *A. niger* strain CBS 513.88 deposited in the NCBI database (www.ncbi.nlm.nih.gov/), six primer pairs (Table 2) were designed and used in an attempt to amplify regions of the polyketide synthase genes (*pks*) from 119 wild-type strains. Amplifications were performed in a PTC 100 thermal cycler (MJ Research Inc.) using a 25 μL reaction volume containing 5 ng of DNA template, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each PCR primer, and 1 U of *Taq* DNA polymerase (Invitrogen Life Technologies). To confirm the presence of PCR-compatible DNA, amplification of the βt2 region of the β-tubulin gene was carried out using the primer pair described by Glass and Donaldson (1995).

2.6. Cloning and sequencing

PCR products were cloned using the TOPO TA Cloning Kit for sequencing (Invitrogen Life Technologies). The recombinant plasmids were purified using the PureLink Quick Plasmid Miniprep Kit (Invitrogen Life Technologies). The inserts and PCR products were sequenced as described by Sanger et al. (1977) using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) on a MegaBACE 1000 (Amersham Biosciences). The quality of the sequences was examined using the Phred/Phrap/Consed package.

2.7. Southern blot analysis

For each strain, 4 μg of genomic DNA was digested with *Hind*III, electrophoresed, and transferred to a nylon membrane, as described by Sambrook and Russell (2001). A 556 bp fragment of the C-methyltransferase (C-MeT) domain from the *pks* gene was obtained by PCR from genomic DNA using the sequences 5′TCCTACGACTTCACCGACAT and 5′CATTTCGTTGATCCCATCG as forward and reverse primers, respectively. The fragment was radioactively labeled by random priming with the Random-Priming DNA Labelling System Kit (Invitrogen Life Technologies) and used as a probe. Blots were hybridized at 65 °C. Two high stringency washes were done at 65 °C with 0.5× SSC and 0.1% (w/v) SDS each for 30 min, followed by two washes with 0.1× SSC and 0.1% (w/v) SDS each for 30 min. The results were visualized by autoradiography.

Table 1
Isolates code, capability to produce ochratoxin A (OTA), and substrate from which 119 *Aspergillus niger* isolates were collected.

Code	OTA	Substrate	Code	OTA	Substrate	Code	OTA	Substrate
ITAL150	+	Black sultanas	ITAL424	−	Prunes	UEL128	−	Coffee beans
ITAL176	+	Black sultanas	ITAL425	−	Prunes	UEL132	−	Coffee beans
ITAL178	+	Black sultanas	ITAL438	−	Prunes	UEL136	−	Coffee beans
ITAL180	+	Black sultanas	ITAL439	−	Prunes	UEL139	−	Coffee beans
ITAL249	+	Dried figs	ITAL7	−	Coffee beans	UEL140	−	Coffee beans
ITAL250	+	Dried figs	ITAL104	−	Coffee beans	UEL11.03	−	Coffee beans
ITAL318	+	Dates	ITAL105	−	Coffee beans	UEL11.07	−	Coffee beans
ITAL327	+	Dates	ITAL106	−	Coffee beans	UEL11.18	−	Coffee beans
ITAL331	+	Dates	ITAL109	−	Coffee beans	UEL11.25	−	Coffee beans
ITAL490	+	Dates	ITAL113	−	Coffee beans	UEL12.07	−	Coffee beans
ITAL493	+	Dates	ITAL114	−	Coffee beans	UEL12.17	−	Coffee beans
ITAL494	+	Dates	ITAL121	−	Coffee beans	UEL12.33	−	Coffee beans
ITAL498	+	Dates	ITAL123	−	Coffee beans	UEL12.41	−	Coffee beans
ITAL499	+	Dates	ITAL126	−	Coffee beans	UEL13.02	−	Coffee beans
ITAL501	+	Dates	ITAL185	−	Coffee beans	UEL13.09	−	Coffee beans
ITAL152	+	Prunes	ITAL186	−	Coffee beans	UEL14.09	−	Coffee beans
ITAL426	+	Prunes	ITAL215	−	Coffee beans	UEL15.03	−	Coffee beans
ITAL428	+	Prunes	ITAL219	−	Coffee beans	UEL15.06	−	Coffee beans
ITAL429	+	Prunes	ITAL413	−	Coffee beans	ITAL103a	−	Brazil nuts
ITAL430	+	Prunes	ITAL418	−	Coffee beans	ITAL153	−	Brazil nuts
ITAL431	+	Prunes	ITAL444	−	Coffee beans	ITAL156	−	Brazil nuts
ITAL437	+	Prunes	ITAL446	−	Coffee beans	ITAL157	−	Brazil nuts
ITAL103	+	Coffee beans	ITAL448	−	Coffee beans	ITAL158	−	Brazil nuts
ITAL119	+	Coffee beans	ITAL642	−	Coffee beans	ITAL160	−	Brazil nuts
ITAL148	+	Coffee beans	ITAL777	−	Coffee beans	ITAL161	−	Brazil nuts
ITAL213	+	Coffee beans	ITAL1248	−	Coffee beans	ITAL162	−	Brazil nuts
ITAL477	+	Coffee beans	UEL3	−	Coffee beans	ITAL228	−	Brazil nuts
ITAL643	+	Coffee beans	UEL6	−	Coffee beans	ITAL229	−	Brazil nuts
ITAL1240	+	Coffee beans	UEL10	−	Coffee beans	ITAL231	−	Brazil nuts
ITAL 1242	+	Coffee beans	UEL29	−	Coffee beans	ITAL232	−	Brazil nuts
ITAL159	+	Brazil nuts	UEL31	−	Coffee beans	ITAL233	−	Brazil nuts
ITAL177	−	Black sultanas	UEL32	−	Coffee beans	ITAL234	−	Brazil nuts
ITAL402	−	Black sultanas	UEL45	−	Coffee beans	ITAL236	−	Brazil nuts
ITAL403	−	Black sultanas	UEL50	−	Coffee beans	ITAL240a	−	Brazil nuts
ITAL404	−	Black sultanas	UEL67	−	Coffee beans	ITAL269	−	Brazil nuts
ITAL405	−	Black sultanas	UEL74	−	Coffee beans	ITAL276	−	Brazil nuts
ITAL406	−	Black sultanas	UEL102	−	Coffee beans	ITAL277	−	Brazil nuts
ITAL496	−	Dates	UEL107	−	Coffee beans	ITAL278	−	Brazil nuts
ITAL500	−	Dates	UEL118	−	Coffee beans	ITAL279	−	Brazil nuts
ITAL528	−	Dates	UEL120	−	Coffee beans			

2.8. Bioinformatic analyses

The secondary metabolite gene clusters, in which most of the *pks* genes are contained (Pel et al., 2007), were annotated using the Secondary Metabolite Unique Regions Finder (SMURF) tool. This web-based software systematically predicts clustered secondary metabolite genes based on their genomic context and domain content (www.jcvi.org/smurf/). The sequences of 34 *pks* genes identified in the genome of the CBS 513.88 strain of *A. niger* (Pel et al., 2007) were confronted with the genome of the ATCC 1015 strain of *A. niger* and *Aspergillus carbonarius* ITEM 5010 v3 by searches (BlastN) within the JGI database (http://genome.jgi-psf.org/Aspni5/Aspni5.home.html).

The *pks* gene distribution along *A. niger* chromosomes and distances to either centromere or telomere were accessed using Ensembl Genome Browser (http://fungi.ensembl.org/index.html). Conserved domains were identified using the InterProScan sequence search tool (http://www.ebi.ac.uk/Tools/pfa/iprscan/). In addition, proteins within clusters were functionally annotated by searches in public databases, including UniProt and GenBank.

Table 2
Target gene, designation, sequence, annealing temperature, and expected amplicon size of the primer pairs used in the study.

Gene	Domain	Primer pair designation	Primer sequence (5′-3′)[a]	Ta (°C)	Amplicon size (bp)
An01g01130	KS	PKS01KS	F-CACTCCACCTTGCTTGTAA R-GCCCGTTGCTCTTTGCGAA	58	536
An04g04340	KS	PKS04KS	F-GTGATGTAGCGTCCAAGCCT R-GAGAGCCGATTAGCAAGAA	60	382
An11g05940	KS	PKS11KS	F-TACGAGGCTATGGAAAATG R-AGAGTGGCTGACCTGGCGG	58	863
An15g07920	KS	PKS15KS	F-CAATGCCGTCCAACCGTATG R-CCTTCGCCTCGCCCGTAG	60	776
An15g07920	C-MeT	PKS15C-MeT	F-GCTTTCATGGACTGGATG R-CATTTCGTTGATCCCATCG	62	998
An18g00520	AT	PKS18AT	F-AATCCCCTATCTTCCTCCAG R-GTTTACAGCAGCAATGACAGC	62	440

[a] F, forward primer and R, reverse primer

3. Results

3.1. Screening of pks genes

Using the SMURF software, 67 putative clusters involved in secondary metabolite biosynthesis were predicted in *A. niger* strain CBS 513.88. At least 34 of these clusters harbor one or two putative *pks* genes. Among these, one gene (3%) is located at the end of the chromosome (<3 kb), two (6%) are subtelomeric (<90 kb), and thirty-one (91%) are located away from subtelomeric regions.

In silico comparison of the 34 DNA sequences of *A. niger* strain CBS 513.88 to the *A. niger* strain ATCC 1015 genome revealed significant nucleotide identity (>95% covering a minimum of 99% of the gene sequences) for 31 of the sequences (approximately 91%). Three putative *pks* genes (An01g01130, An11g05940, and An15g07920) were unique to *A. niger* CBS 513.88 (Table 3). The upstream and downstream regions of the three unique *pks* genes in the ATCC 1015 and CBS 513.88 strains were compared. An01g01130 was found to be located in a 22 kb cluster positioned in a region on chromosome II that was far from the telomeric region (~1.2 Mb from the chromosome end). This CBS 513.88 cluster contains five putative ORFs. Of these, only An01g01130 is missing in ATCC 1015, which is a gene annotated similar to a *pks* gene from *Gibberella moniliformis*. This missing region is 9 kb in size.

The second missing *pks* gene (An11g05940) is located in a cluster positioned on the middle of the left arm of chromosome VII. The predicted cluster has a size of 48 kb and harbors 14 putative ORFs, of which three are missing in ATCC 1015: An11g05940, An11g05950, and An11g05960. This missing region is 20 kb long. The third unique *pks* gene (An15g07920) is located on the extremity of chromosome III of *A. niger* in a 31 kb cluster that harbors 10 putative genes, which has been annotated as a putative ochratoxin cluster by Pel et al. (2007). Five ORFs were missing in the *A. niger* ATCC 1015: An15g07880, a putative FAD-binding oxidoreductase; An15g07890, a putative transcription factor; An15g07900, a putative cytochrome P450; An15g07910, a putative NRPS, and An15g07920, which is evidently a PKS. This missing region is approximately 22 kb in size. Very recently, the genome sequences of the CBS 513.88 strain were improved and a re-annotation is in progress. Therefore, we re-analyzed this region containing the ORF An15g07920 based on the NCBI annotation updated on 31-May-2011 (Fig. 1). The most significant difference between the first and the last versions was the abolishment of ORF An15g07890. Because *A. carbonarius* is an important OTA-producer and belongs to section *Nigri*, we also included in our analysis an equivalent genomic region. This *A. carbonarius* genomic region (~24 kb in size) harbors homolog genes to *A. niger* CBS 511.88 encoding putative FAD-binding oxidoreductase, cytochrome-P450, non-ribosomal peptide synthetase and polyketide synthase that are organized in the same order and direction of transcription (Fig. 1).

We also attempted to compare the strain-specific *pks* gene with others that have been previously described and are possibly involved in known pathways. As shown in Fig. 2, we found that the putative An15g07920 product grouped together with an *Aspergillus ochraceus* PKS previously described as involved in the biosynthesis of OTA (O'Callaghan et al., 2003; Dao et al., 2005) and with an *A. carbonarius* putative product (scaffold 12) (http://genome.jgi-psf.org, as of 31-May-2011). As can be seen in Fig. 2, other PKS sequence (JGI 505925) described as involved in OTA biosynthesis in *A. carbonarius* (Gallo et al., 2009) clustered very close to the *A. niger* An18g00520 putative product, and somewhat more distant from a second *A. ochraceus* PKS sequence (A3KLM2) also described as involved in OTA biosynthesis (Bacha et al., 2009). In silico comparison of the An18g00520 nucleotide sequences of *A. niger* CBS 513.88 and ATCC 1015 strains revealed this gene is present in both genomes.

Table 3
A comparison of 34 predicted *pks* sequences present in *Aspergillus niger* strain CBS 513.88 against *A. niger* strain ATCC 1015 genome, *performed using JGI BLAST search*.

PKS in the genome of *A. niger* CBS 513.88	Linear mRNA size (bp)	Chromosome	Nomenclature updated by NCBI	Coverage over the genome of *A. niger* ATCC 1015 (%)	Identity over the genome of *A. niger* ATCC 1015 (%)
An01g01130	7191	II	ANI_1_2198014	No homolog	
An01g06930	7443	II	ANI_1_2672014	100	99.35
An01g06950	6849	II	ANI_1_2676014	99.97	97.63
An02g00450	7491	IV	Discontinued record	100	99.80
An02g09430	7390	IV	ANI_1_2894024	100	99.98
An03g01820	6405	VI	ANI_1_1088034	100	99.81
An03g05140	7536	VI	ANI_1_1400034	100	99.89
An03g05440	6429	VI	ANI_1_1430034	99.98	97.53
An03g06380	7563	VI	ANI_1_1546034	100	99.87
An04g04340	7392	VI	ANI_1_1824184	99.92	99.73
An04g09530	4992	VI	ANI_1_2226184	100	99.94
An04g10030	7845	VI	ANI_1_2294184	99.69	99.35
An07g01030	3810	IV	ANI_1_1462064	100	99.75
An09g01290	7365	I	ANI_1_1056084	100	98.67
An09g01860	7854	I	ANI_1_1132084	100	98.11
An09g01930	7677	I	ANI_1_1146084	100	97.99
An09g05340	7314	I	ANI_1_1476084	100	99.41
An09g05730	6462	I	ANI_1_726084	100	99.98
An10g00140	5340	V	ANI_1_136174	99.93	98.14
An11g03920	8247	VII	ANI_1_1892094	99.44	98.38
An11g04280	7026	VII	ANI_1_1948094	100	95.96
An11g05570	7509	VII	ANI_1_2050094	99.97	98.21
An11g05940	7248	VII	ANI_1_2098094	No homolog	
An11g07310	5382	VII	ANI_1_2242094	100	98.01
An11g09720	7437	VII	Discontinued record	100	98.71
An12g02050	7456	III	ANI_1_1474104	99.77	96.57
An12g02670	8496	III	ANI_1_1558104	100	99.91
An12g02730	7059	III	ANI_1_1566104	100	99.99
An12g07070	6714	III	ANI_1_2048104	100	99.98
An13g02430	6810	II	ANI_1_724114	99.94	99.95
An13g02960	2712	II	ANI_1_756114	100	99.98
An15g04140	7698	III	ANI_1_1430134	100	99.77
An15g05090	7425	III	ANI_1_1564134	99.96	99.91
An15g07920	7557	III	ANI_1_1836134	10.24	89.73

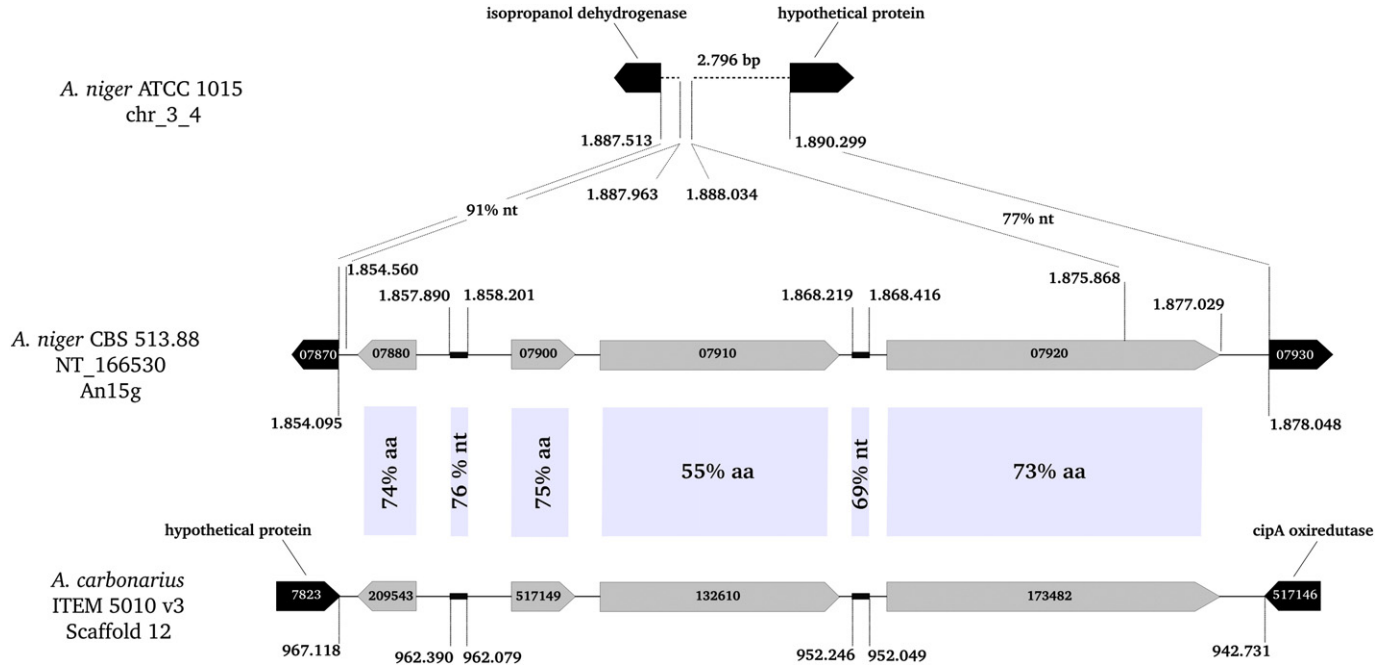

Fig. 1. Comparative sequence analysis of the putative ochratoxin gene cluster harboring PKS An15g07920 of *A. niger* strain CBS 513.88, and equivalent genomic regions of *A. niger* strain ATCC 1015 and *A. carbonarius* ITEM 5010 v3. Each line represents the genomic context of equivalent regions. Gray arrows represent genes within the cluster; and black arrows, bordering genes common to all genomes. Annotations ID either from JGI or NCBI are shown inside arrows. Genomic coordinates are depicted along the dotted lines. Amino acid sequence similarities between orthologs are shown within shaded areas as well as similarities in nucleotide sequences of intergenic regions. As indicated, *A. niger* strain ATCC 1015, carries a truncated version of An15g07920.
Based on the NCBI annotation updated on 31-May-2011.

3.2. Survey of pks genes in vivo

To compare the results of the in silico analyses with the in vivo situation, PCR was conducted on a large number of *A. niger* strains obtained from different substrates and geographical regions. Firstly, because the taxonomy of *A. niger* aggregate is not very clear and species identification remains problematic, we confirmed the taxonomic status of each strain by morphological and molecular data (Fig. 3).

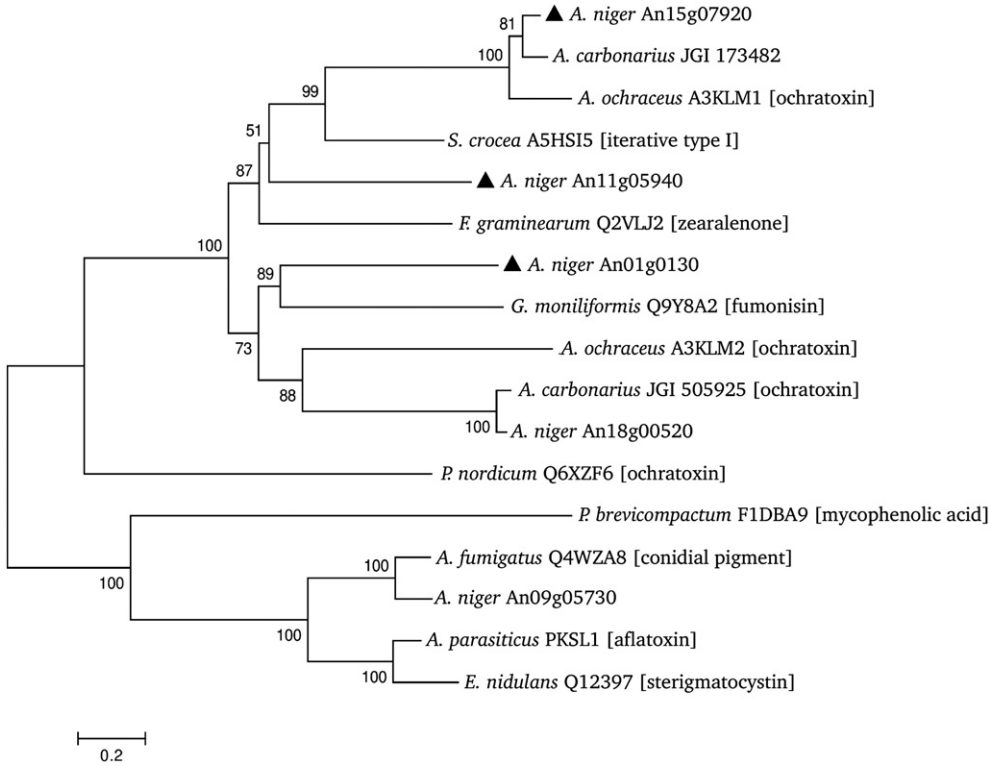

Fig. 2. Sequence similarity relationships among selected PKSs. Type I PKSs were selected from different organisms and various predicted functions in addition to five PKSs of *A. niger* and two of *A. carbonarius*. Black triangles represent *pks* genes missing in *A. niger* strain ATCC 1015. The tree was inferred by Neighbor-Joining analysis, with distances computed using the JTT matrix-based method and bootstrap consensus tree from 1000 replicates. Analyses were conducted in MEGA5 (Tamura et al., 2011).

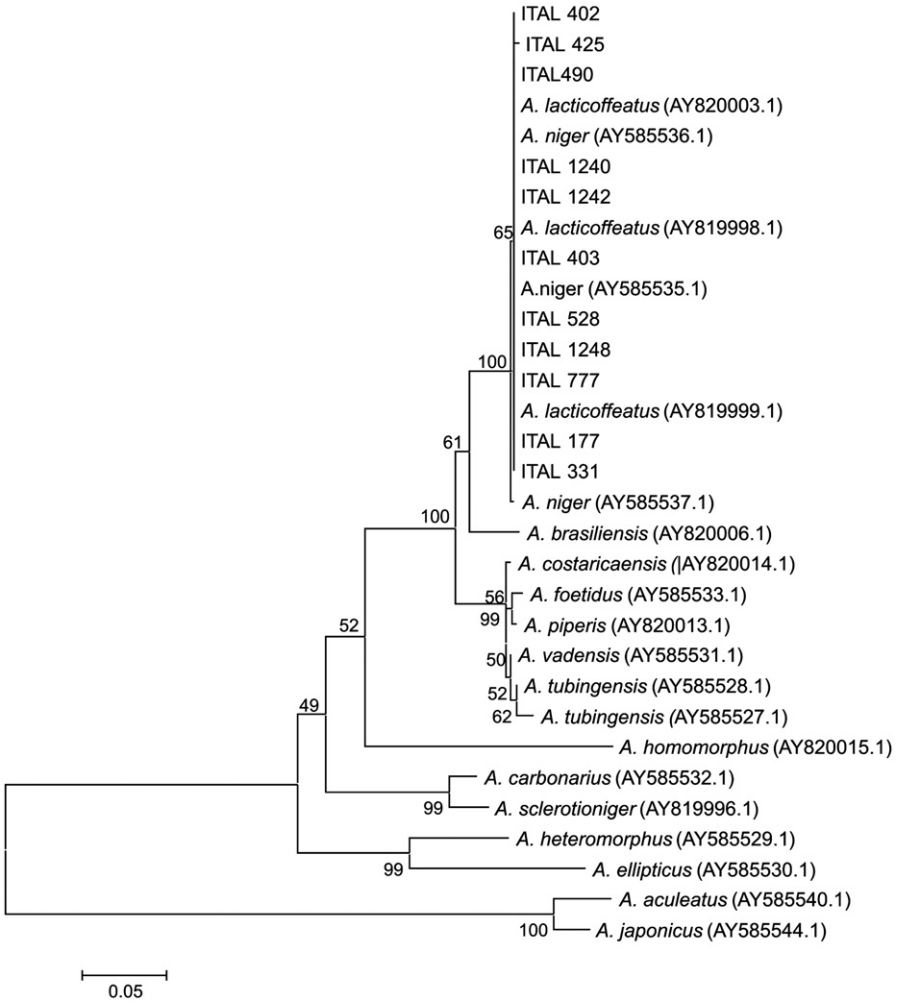

Fig. 3. A phylogenetic tree of *Aspergillus* from section *Nigri* based on β-tubulin gene sequences. Numbers above the branches correspond to bootstrap values generated by CLUSTAL X 1.6.

Evidence of morphology and *β-tub2* sequences were complementary and indicated that all strains were in fact *A. niger* sensu stricto. For instance, *Aspergillus lacticoffeatus* and *A. niger* sensu stricto could not be separated based on the β-tub2 sequences; however, there are clear differences in their colony color (Samson et al., 2004).

PCR was carried out on 119 wild-type strains of *A. niger* to test for the presence of five *pks* genes. Concerning the three *pks* genes that were found to be present in CBS 513.88 but absent in ATCC 1015, we detected amplification signals for An01g01130, An11g05940, and An15g07920 *pks* genes in only 97%, 71%, and 26% of the wild-type strains, respectively (data not show). These results show that differences in *pks* gene content of the two fully sequenced genomes of *A. niger* cannot be merely attributed to the classical strain improvement programs they were submitted in the past. The other two *A. niger pks* genes that were in vivo analyzed in our study were An04g04340 and An18g00520. These genes were in silico found in both genomes CBS 513.88 and ATCC 1015. Their respective putative protein sequences showed high identity with *A. ochraceus* (A3KLM2) and *A. carbonarius* (JGI 505925), previously described as involved in OTA biosynthesis (Bacha et al., 2009; Gallo et al., 2009). Our results showed that the genes An04g04340 and An18g00520 are present in 96% and 100% of the wild-type strains, respectively.

3.3. Association between An15g07920 and strain capability of OTA production

Because of the cluster in which An15g07920 *pks* is located was annotated as a putative ochratoxin cluster, we focused our investigation on assessing the OTA production capability of each of the strains included in this study. In our sample, 26% (31/119) of the strains were able to produce OTA (Table 1). Our goal was to examine the possible association between the presence of the *pks* locus and the capability of the respective strain to produce OTA. The predicted domains of β-ketoacyl synthase (KS), acyl transferase (AT), dehydratase (DH), C-methyltransferase (C-MeT), enoylreductase (ER), ketoreductase (KR), and acyl carrier protein (ACP) were found in the hypothetical protein encoded by An15g07920. When a primer pair (PKS15C-MeT) was used to amplify a DNA fragment corresponding to the C-MeT PKS domain, a single 998 bp band was detected in all strains that produced OTA (31); this band was not detected in any of the non-producing strains (88). Equally, when a primer pair (PKS15KS) was used to amplify a DNA fragment corresponding to the β-ketoacyl synthase domain, which is another PKS domain, a single 766 bp band was only detected in the 31 OTA producing strains (Fig. 4). Sequencing analysis of both amplicons confirmed their *pks* identity.

Finally, to prove that the An15g07920 is a strain-specific locus, a DNA segment of the corresponding C-MeT domain (556 bp) was used as a probe in a Southern blot analysis. In agreement with the abovementioned PCR data, hybridization signals were only detected in the 31 OTA producing strains (Fig. 5).

4. Discussion

The in silico analysis in this study showed that the CBS 513.88 genome harbors three putative *pks* genes (An01g01130, An11g05940,

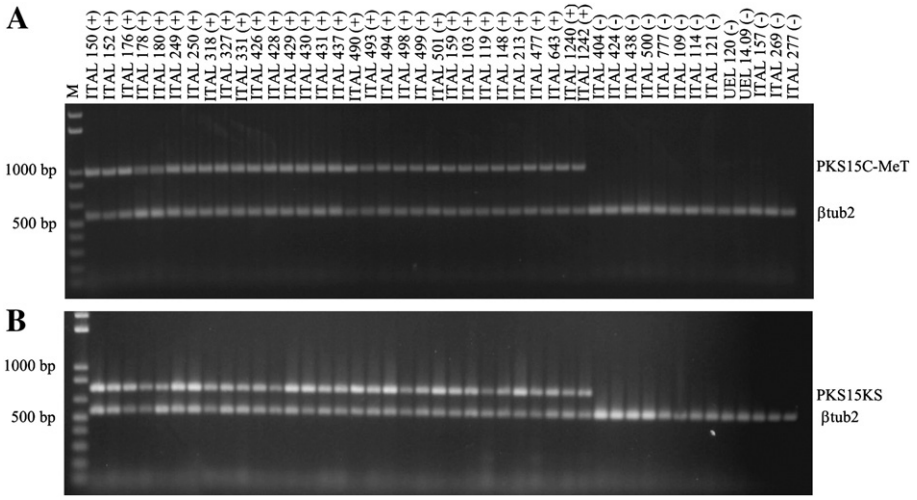

Fig. 4. Association between An15g07920 and OTA production capability of each strain, as demonstrated by PCR performed with two primer pairs, PKS15C-MeT (A) and PKS15KS (B). PCR detected a 998 bp band and a 776 bp band, respectively, in the ochratoxin producing strains only. The 554 bp-amplicon corresponding to the βt2 region of the β-tubulin gene confirm the presence of PCR-compatible DNA in all strains.

and An15g07920), which are not present in ATCC 1015. These differences cannot be associated with low-coverage genome sequencing, since the ATCC 1015 genome was sequenced with a higher coverage than that of CBS 513.88. Although the studies that have sought to identify variation in gene content within fungal species are very restricted, some interesting information is already available in the literature. In *A. niger*, it has been previously demonstrated that the nucleotide sequence identity between strains CBS 513.88 and ATCC 1015 is 99.3% (Rokas et al., 2007). Similarly, the comparative genomic analysis of *A. niger* strains ATCC 9029 and CBS 513.88 showed that approximately 600 CDSs of CBS 513.88 have no orthologs in ATCC 9029 (Sun et al., 2007). Among these CBS 513.88 unique genes, some encode enzymes of secondary metabolism.

According to Turner (2010), some secondary metabolite gene clusters are located near chromosome ends, which are sites of the most frequent rearrangements in many eukaryotic genomes. This is the case for the *pks* gene An15g07920, which has no orthologs in ATCC 1015 and was found positioned in a subtelomeric region (≤3 kb). However, the two other *pks* missing genes in ATCC 1015 (An01g01130 and An11g05940) are distant from the telomere (>1 Mb), meaning that the deletion cannot only be explained by subtelomeric rearrangements. Rokas et al. (2007) stated that variable loci are randomly distributed along the chromosomes of *Aspergillus fumigatus*, although they display a clear subtelomeric bias.

When annotating the *A. niger* CBS 513.88 genome, Pel et al. (2007) did not suggest any function for the clusters that harbor the genes An01g01130 and An11g05940. However, for the cluster

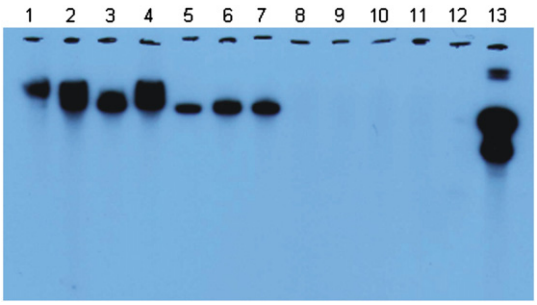

Fig. 5. Association between An15g07920 and OTA production capability of each strain, as demonstrated by Southern blot analysis of randomly selected *A. niger* OTA producing (lanes 1–7) and OTA non-producing strains (lanes 8–12). For hybridization, a fragment of the *pks* gene was labeled with ^{32}P and used for the assay. Lane 13 shows the plasmid with the probe, which served as the positive control.

where An15g07920 is located, an ochratoxin biosynthesis putative function was predicted. When comparing the three CBS 513.88 *pks* products, which do not have orthologs in ATCC 1015, to other products previously reported to be involved in known pathways, we observed that the products of the An01g01130 and An11g05940 genes were not sufficiently related in phylogenetic terms to those with functions already specified. In contrast, the An15g07920 product is closely related to the PKS involved in OTA biosynthesis (A3KLM1) described by O'Callaghan et al. (2003) and with a PKS from *A. carbonarius* (JGI 173482).

Chemically, OTA is a 2-(5-chloro-8-hydroxy-3-methyl-1-oxo-isochroman-7-yl) carbonylamino-3-phenyl-propanoic acid that was classified by the International Agency for Research on Cancer (IARC, 1993) as a possible human carcinogen (group 2B). It was isolated in 1965 from a culture of *A. ochraceus* (van der Merwe et al., 1965), but subsequent studies have revealed that some other fungal species, including *A. niger* sensu stricto, are able to produce this toxin as well (reviewed by El Khoury and Atoui, 2010).

Recently, Huffman et al. (2010) proposed a biosynthetic pathway for OTA. Their hypothesis includes a PKS involved in the synthesis of the PK dihydroisocoumarin, a methyltransferase (or a C-MeT domain within the PKS), and a P450-type oxidation enzyme for the formation of the carboxyl group at C7. In addition, they hypothesize that an NRPS catalyzes the ligation of phenylalanine with the PK, and a halogenase (chloroperoxidase) incorporates the chlorine atom. However, none of the biosynthetic steps has been genetically or biochemically established. In our study, we found that all of the genes included in the 22 kb region, which are present in CBS 513.88 but absent in ATCC 1015, have a predicted function that is expected in the OTA synthesis mechanism: PKS (An15g07920), NRPS (An15g07910), cytochrome P450 (An15g07900), and FAD-binding oxidoreductase (An15g07880). However, a gene encoding the enzyme responsible for the chlorination of OTA was not identified in this cluster.

The predicted PKS enzymatic domains: β-ketoacyl synthase (KS), acyl transferase (AT), dehydratase (DH), C-methyltransferase (C-MeT), enoylreductase (ER), ketoreductase (KR), and acyl carrier protein (ACP) were found in the hypothetical protein encoded by An15g07920 suggesting that it yields products that are highly reduced. Interestingly, a C-MeT domain is within the putative PKS encoded by An15g07920, which is important for OTA biosynthesis. *A. carbonarius* is an important ochratoxigenic species that is closely related to *A. niger*. In general, this species produces larger amounts of OTA than *A. niger*, and most *A. carbonarius* strains have the ability to produce the toxin. A comparison of the gene cluster that contains

the PKS An15g07920 in *A. niger* strain CBS 513.88 against the strain ITEM 5010 of *A. carbonarius* revealed the same organization of ORFs, high sequence similarity, but in a different genomic context.

It is important to note that when the annotation of the CBS 513.88 genome was completed, descriptions of gene involvement in OTA biosynthesis were only available for *A. ochraceus* and *Penicillium nordicum* (Karolewiez and Geisen, 2005; O'Callaghan et al., 2003). Based on the sequence identity of An15g07920 with the *A. ochraceus pks* gene product reported by O'Callaghan et al. (2003), a putative ochratoxin biosynthesis function was predicted for this ORF. Additional evidence of the involvement of certain genes involved in OTA biosynthesis was later discovered. Bacha et al. (2009) reported that a disruption of the gene *awks1* (GenBank Accession Number: AY583209) inhibited the biosynthesis of OTA in *Aspergillus westerdijkiae* (a recently dismembered species of *A. ochraceus*). Given that *awks*1 product (A3KLM2) is different from the PKS (A3KLM1) described by O'Callaghan et al. (2003), the authors concluded that two PKS proteins may be involved in OTA biosynthesis in *A. westerdijkiae*. In fact, two groups of PKS enzymes that are hypothesized to be involved in OTA biosynthesis can be observed in our phylogenetic analyses (Fig. 2).

The Blast comparisons showed that among all *pks* predicted for the *A. niger* strain CBS 513.88 genome, the best match (54% identity at the deduced amino acid level) to that described by Bacha et al. (2009) was the putative product of *pks* gene An04g04340. This gene was detected in silico in both *A. niger* genomes (CBS 513.88 and ATCC 1015), and was detected in 96% of the strains analyzed in the present study. Moreover, an association between the PCR profile and OTA production was not detected (data not shown). In *A. carbonarius*, a correlation between the expression of the *ACpks* gene and OTA production was reported by Gallo et al. (2009), suggesting that this gene product is involved in OTA biosynthesis. The Blast comparisons showed that among all the predicted *pks* genes in the *A. niger* CBS 513.88 genome, the best match (86% identity at the deduced amino acid level) to that described by Gallo et al. (2009) was the putative *pks* gene An18g00520. Similarly, this gene was detected in silico in both *A. niger* genomes (CBS 513.88 and ATCC 1015), and was detected in 100% of the strains analyzed in the present study (data not shown).

Additional data showed that one *A. ochraceus* putative P450-type monooxygenase gene (P450-B03) has an expression profile that is very similar to that of the *pks* involved in OTA biosynthesis (O'Callaghan et al., 2006), suggesting that these genes may be clustered in the *A. ochraceus* genome. Based on the Blast analysis, we observed that among all P450 genes predicted in the *A. niger* CBS 513.88 genome, the most similar (with 61% identity) to *A. ochraceus* P450-B03 was the putative P450 gene An15g07900, which was located adjacent to An15g07920 but also absent in the ATCC 1015 genome.

OTA production in *A. niger* sensu stricto was previously found to be restricted to only 3–10% of the strains (Taniwaki et al., 2003), and the cause of this variation was unknown until now. Our PCR-based experimental data showed that the putative *pks* gene annotated as An15g07920 in *A. niger* CBS 513.88 exists in only 26% of the wild-type *A. niger* strains analyzed in this study. Interestingly, this gene was exclusively detected in the 31 OTA producing strains, which suggests a clear association between the molecular profiles and OTA phenotype. Andersen et al. (2011) examined the exometabolomic profiles of 11 *A. niger* strains, including CBS 513.88 and ATCC 1015. Among several secondary metabolites analyzed, only ochratoxin and nigragillin were produced by the CBS 513.88 strain but not the ATCC 1015 strain. These findings provide additional evidence of the association between the presence of An15g07920 and the capability of OTA production. Very recently, Castellá and Cabanes (2011) also showed the importance of the An15g07920 *pks* gene for specific detection of OTA-producing strains of *A. niger* aggregate.

Based on the structural complexity of PKS family proteins, functional studies such as analyses facilitated by disrupted mutants remain a necessity to elucidate OTA biosynthetic steps in *A. niger* in both genetic and biochemical terms. However, we expect that our results showing a clear association between the gene An15g07920 and an OTA positive phenotype can leverage the characterization of the OTA biosynthetic process. We also suggest that two additional *pks* genes (An01g01130 and An11g05940), which until now have not had an identified function, are strain-specific genes. This information possibly will provide clues for designing and building future studies.

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