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A N I N T E R N A T I O N A L J O U R N A L

Genetic Relationships among Strains of the *Aspergillus niger* Aggregate

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ABSTRACT

We analyzed the genetic relationships between 51 fungal isolates previously identified as A. niger aggregate, obtained from dried fruit samples from worldwide origin and 7 A. tubingensis obtained from Brazilian coffee beans samples. Greater fungal diversity was found in black sultanas. Aspergillus niger sensu stricto was the most prevalent species. It was found in all fruit substrates of all geographical origins. Based on Random Amplification of Polymorphic DNA (RAPD) and β-tubulin sequences data two groups of A. niger were found. In spite of the small number of isolates from Group IV an association between extrolite patterns and molecular clustering is speculated. A. tubingensis were the second most frequent species and this species were clearly subdivided into two groups. The finding of two groups for A. tubingensis strains could not yet explain the contradictions found in the literature about the capability this species for ochratoxin production, because both of them were formed by only non-ochratoxin-producing strains.

Key words: *Aspergillus niger* aggregate, ochratoxin A, dried fruits, toxigenic fungi

INTRODUCTION

Ochratoxin A (OTA) is a mycotoxin that has been detected in a variety of food products. Human exposure to this mycotoxin should be avoided or kept to minimal concentration because it has nephrotoxic effects and carcinogenic activity. Several authors reported the presence of OTA in dried fruit samples exceeding 10μg kg $^{-1}$, which is the maximum limit recommended by the European Union for this kind of substrate (Abarca et al., 2003; MacDonald et al., 1999; Iamanaka et al., 2005; Verstraete, 2008). Equally this mycotoxin has been found in roasted and instant coffee (Fujii et al., 2007).

The black *Aspergilli* species (*Aspergillus* section *Nigri*) are the main source of OTA in dried fruits (Cabañes et al., 2002; Abarca et al., 2003; Magnoli et al., 2004 Iamanaka et al., 2005).

Because there are minor differences between some species belonging to section *Nigri* the identification of some of them requires DNA-

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based analyses. The species *A. niger* sensu stricto, *A. tubingensis, A. foetidus,* and *A. brasiliensis* are morphologically identical and altogether have been called *A. niger* aggregate (Parenicova et al., 2001).

With the exception of *A. niger* sensu stricto, the potential of species within the *A. niger* aggregate to produce OTA is uncertain, probably due to the difficulty of species identification. For instance, Ueno et al. (1991) described an *A. foetidus* strain (CBS 618.78) that was able to produce OTA. Very recently, two research groups found OTA-producers isolates of *A. tubingensis* (Medina et al., 2005; Perrone et al., 2006). Nevertheless, according to Samson et al. (2004), no strains of *A. tubingensis* and *A. foetidus* sensu stricto produce OTA, and CBS 618.78 was in fact *A. niger* but not *A. foetidus*.

Recently, our research group performed an extensive work searching for the presence of toxigenic fungi in dried fruits from worldwide origin, and *A. niger* aggregate was found as the most common (Iamanaka et al., 2005). In the present study, we analyzed by Random Amplification of Polymorphic DNA (RAPD) and β-tubulin sequences, the genetic relationships among fungal isolates collected from black sultanas, dates, dried figs and prunes with the aim to enlighten the taxonomical position of the isolates previously identified as belonging to the *A. niger* aggregate.

MATERIALS AND METHODS

Strains

A total of 51 isolates, previously identified by morphological data as *A. niger* aggregate, obtained from dried fruit was analysed. Another seven *A. tubingensis* strains, isolated from Brazilian coffee beans were used to confirm the subdivision of this species in two groups (Table 1). The dried fruit samples from worldwide origin (Argentina, Chile, Iran, Turkey, Spain, Tunisia, USA, and Mexico) were purchased from different markets in Campinas and São Paulo, Brazil, as described by Iamanaka et al. (2005).

Genomic DNA extraction

Mycelium recovered from the culture fluid by filtration was pulverized to a fine powder under liquid nitrogen in a mortar. Approximately 400 mg of the ground mycelium was suspended in 800 μL of lysis buffer (200 mM Tris-HCl; 250 mM NaCl; 25 mM EDTA; 1% wv^{-1} SDS) and maintained at 65°C for 20 min. The DNA was purified in phenol: chloroform (25:24) and chloroform: isoamyl alcohol (24:1), precipitated in a 3M NaCl solution in the presence of 95% ethanol, then washed with 70% ethanol, and resuspended in ultrapure water.

PCR and sequencing

Amplification of the ITS1-5.8S-ITS2 region was performed using the ITS1 and ITS4 as forward and reverse primers, respectively (White et al., 1990). Two portions of the β-tubulin gene were amplified using the primer-pairs Bt1 and Bt2, as described by Glass and Donaldson (1995). Amplifications were performed in a Thermocycler (PTC 100, MJ Research). PCR products were directly sequenced in both forward and reverse directions in a MegaBACE TM 1000 sequencer (Amersham Biosciences). The quality of the sequences was examined using Phred/Phrap/Consed package. The sequences were aligned using the software BioEdit version 1.6.6 (Hall, 1999). Phylogenetic analyses were performed by ClustalW multiple-sequence alignment program, version 1.6 (Thompson et al., 1994).

RAPD analysis

DNA amplifications were carried out by using arbitrary primers (QIAGEN-Operon) in a PTC-100 thermocycler (MJ Research, Inc.) according to Fungaro et al. (1996). Each isolate was scored for the presence or absence of DNA bands. An UPGMA (Unweighted Pair Group Method with Arithmetic Mean) cluster based on the similarity Dice index (Sneath and Sokal, 1973) was generated using the NTSYS (Numerical Taxonomy System, Applied Biostatistics) computer application software (Rohlf, 1987).

Characterization of OTA-producing isolates

The isolates were three point inoculated into Yeast Extract Sucrose Agar (YES agar) and incubated at 25ºC for 7 days. Three agar plugs were removed from the central area of the colony, weighted and introduced into a small vial. A volume of 0.5 mL of methanol was added to the vial. After 60 min, the extracts were filtered (Millex-Millipore) and injected into the HPLC (Shimadzu 10VP system). The system has a fluorescence detector, set at 333

nm excitation and 477 nm emission. The HPLC was fitted with a Shimadzu CLC G-ODS (4 x 10 mm) guard column and Shimadzu Shimpack CLC-ODS (4.6 x 250 mm) column. The mobile phase was methanol: acetonitrile: water: acetic acid (35:35:29:10) and the flow rate was 0.8mL/min. An ochratoxin A standard (Sigma) was used for the construction of the 5-point calibration curve linear, peak areas versus mass (ng). The OTA concentration in the sample extract was determined by interpolation of resulting peak areas from the calibration graph.

Extrolite analyses

The fungi were grown for 7 days on CYA and YES agar at 25°C in the dark and five agar plugs were cut out of the colonies. These plugs were extracted for the extrolite analysis using ethyl acetate/ dichloromethane/ methanol 3:2:1 (v:v:v) added 1% formic acid (volume basis).

Extrolites were analysed by HPLC using alkylphenone retention indices and diode array UV-VIS detection as previously described by Frisvad and Thrane (1987). Authentic analytical standards were employed for the retention time index comparison with the extrolites detected. Extrolites with a characteristic UV spectrum retention index of unknown chemical structure were given a four letter code ("SPUT" and "KOTN").

Table 1 - Isolates of the *Aspergillus niger* aggregate analysed in this study. Potential to produce ochratoxin A (OTA) is indicated by + or -.

n.º	Substrate	Origin	OTA	n.º	Substrate	Origin	OTA
ITAL 166	black sultanas	Argentina	-	**ITAL 263**	dates	Spain	-
ITAL 176	black sultanas	Argentina	+	**ITAL 277**	dates	Spain	-
ITAL 177	black sultanas	Argentina	-	**ITAL 313**	dates	Spain	-
ITAL 178	black sultanas	Argentina	+	**ITAL 318**	dates	Spain	+
ITAL 180	black sultanas	Argentina	+	**ITAL 319**	dates	Spain	-
ITAL 460	black sultanas	Argentina	-	**ITAL 325**	dates	Spain	-
ITAL 461	black sultanas	Argentina	-	**ITAL 326**	dates	Spain	-
ITAL 517	black sultanas	Argentina	-	**ITAL 327**	dates	Spain	+
ITAL 353	black sultanas	Turkey	-	**ITAL 331**	dates	Spain	+
ITAL 354	black sultanas	Turkey	-	**ITAL 332**	dates	Spain	-
ITAL 356	black sultanas	Turkey	-	**ITAL 503**	dates	Spain	-
ITAL 363	black sultanas	Turkey	-	**ITAL 490**	dates	Tunisia	+
ITAL 402	black sultanas	USA	-	**ITAL 493**	dates	Tunisia	+
ITAL 403	black sultanas	USA	-	**ITAL 494**	dates	Tunisia	+
ITAL 404	black sultanas	USA	-	**ITAL 496**	dates	Tunisia	-
ITAL 405	black sultanas	USA	-	**ITAL 498**	dates	Tunisia	+
ITAL 406	black sultanas	USA	-	**ITAL 499**	dates	Mexico	+
ITAL 150	black sultanas	Iran	+	**ITAL 500**	dates	Mexico	-
ITAL 152	prunes	Chile	+	**ITAL 501**	dates	Mexico	+
ITAL 424	prunes	Argentina	-	**ITAL 528**	dates	USA	-
ITAL 425	prunes	Argentina	-	**ITAL 249**	dried figs	Turkey	+
ITAL 426	prunes	Argentina	+	**ITAL 250**	dried figs	Turkey	+
ITAL 428	prunes	Argentina	+	**ITAL 442**	coffee beans	Brazil	-
ITAL 429	prunes	Argentina	+	**ITAL 167**	coffee beans	Brazil	-
ITAL 430	prunes	Argentina	+	**ITAL 108**	coffee beans	Brazil	-
ITAL 431	prunes	Argentina	+	**ITAL 200**	coffee beans	Brazil	-
ITAL 437	prunes	Argentina	+	**ITAL 97**	coffee beans	Brazil	-
ITAL 438	prunes	Argentina	-	**ITAL 207**	coffee beans	Brazil	-
ITAL 439	prunes	Argentina	-	**ITAL 217**	coffee beans	Brazil	-

RESULTS AND DISCUSSION

Based on RAPD data the 51 isolates from the *A. niger* aggregate collected from dried fruit samples e.g. black sultanas, dates, dried figs, and prunes in different countries were divided into four major groups (I, II, III, IV). Greater fungal diversity was found in black sultanas. The 18 isolates from this substrate spanned in all four groups. The isolates collected from prunes, dates, and dried figs spanned into two, three, and one group, respectively. Group III accommodated the majority of the isolates (69%). Only group III comprised toxigenic and non-toxigenic isolates. In the other three groups, non-ochratoxigenics were found. Fig 1 exemplifies the distinct RAPD profiles of a sample of 29 random selected isolates. As shown in Fig 2, the clustering was not related to fruit type or country origin.

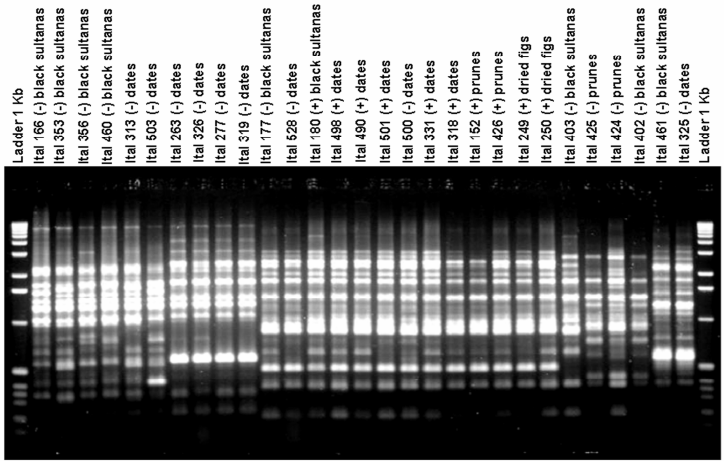

Figure 1 - Amplification of polymorphic DNA of 29 isolates de *Aspergillus niger* aggregate using the 10-mer OPX-7 primer. Isolates number are present above each lane. The isolates ITAL 166, ITAL 353, ITAL 356, ITAL 460, ITAL 313, ITAL 503, ITAL 263, ITAL 326, ITAL 277, and ITAL 319 were classified as *Aspergillus tubingensis*; the isolates ITAL 177, ITAL 528, ITAL 180, ITAL 498, ITAL 490, ITAL 501, ITAL 500, ITAL 331, ITAL 318, ITAL 152, ITAL 426, ITAL 249, ITAL 250, ITAL 403, ITAL 425, ITAL 424, and ITAL 402 were classified as *A. niger;* and the isolates ITAL 461 and ITAL 325 were classified as *A. foetidus*. Toxigenic and nontoxigenic strains are denoted (+) and (-), respectively.

Isolates from each of the RAPD-groups were submitted to sequencing analysis of a portion of the β-tubulin gene (β-tub2) for species identification. Moreover, details on the relationships among the *A. niger* aggregate isolates were of interested. Altogether, 430 nucleotides were involved in the analysis. In summary, good consensus was found between RAPD and β-tub2 sequences data (Fig 3).

The isolates from RAPD group I were recognized as *A. tubingensis*, which were clearly subdivided into two subgroups IA and IB. Three single nucleotide polymorphisms discriminated the two groups of *A. tubingensis*: one deletion (T) at position 290 and two substitutions at position 48 (A instead of G) and 63 (T instead of G). Both subgroups (IA and IB) were formed by ochratoxin not producing strains According to the literature, the potential of *A. tubingensis* to produce OTA is uncertain, probably due to the difficulty of species identification. Accensi et al. (2001) evaluated 48 *A. tubingensis* strains (termed as *A. niger* type T) for OTA production and described this species as non-producer. Analyzing grape samples, Medina et al. (2005) reported that 55% of the isolates recognized as *A. niger* aggregate were in fact *A. tubingensis;* conversely to Accensi et al. (2001) results, 14% of them were positive for OTA production. Perrone et al. (2006) published similar results, as five out of 20 *A. tubingensis* isolates collected from grape berries were OTA-producers. It is interesting to state that in our study the potential of *A. tubingensis* as OTA-producers was repeatedly investigated.

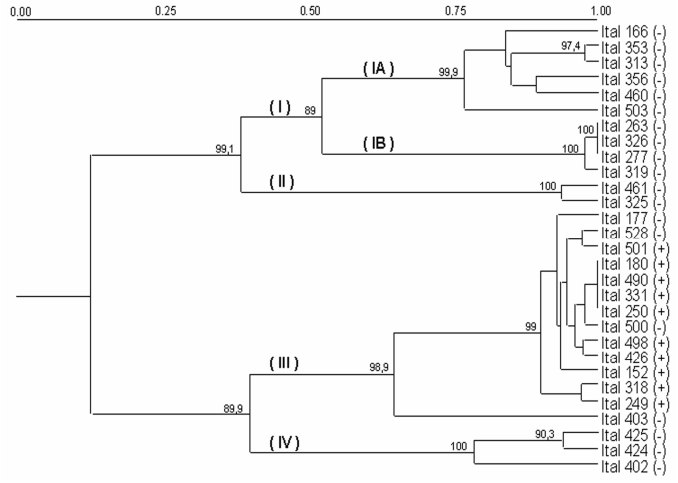

Figure 2 - Dendrogram of 29 isolates of *Aspergillus niger* aggregate based on cluster analysis with UPGMA method using Dice genetic similarity index on RAPD data obtained with three primers. The number of each node indicates the percentage bootstrap support (out of 1,000). Toxigenic and nontoxigenic strains are denoted (+) and (-), respectively.

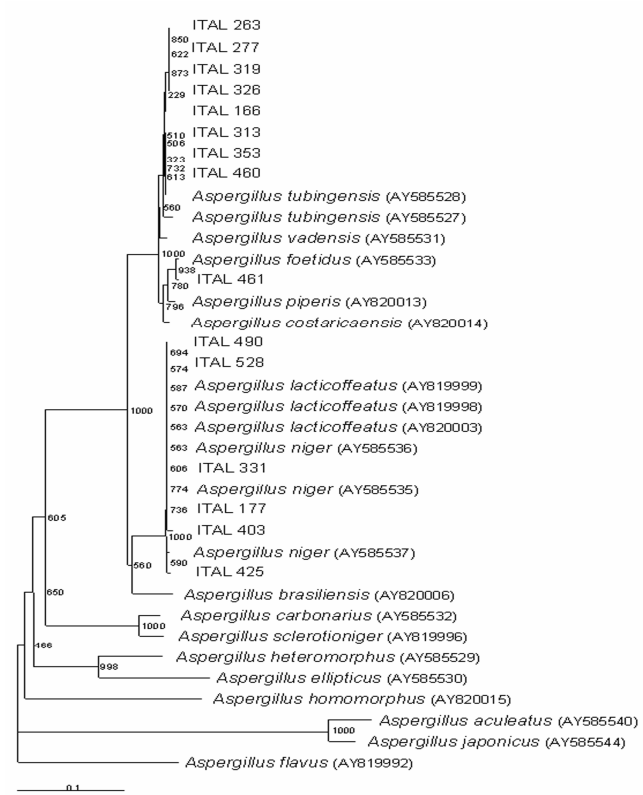

Figure 3 - Phylogenetic tree based analysis of β-tubulin gene sequences, using *Aspergillus flavus* as the outgroup. Bootstrap values (based on 1,000 bootstrap samples) are placed on the tree nodes. Species with numbers represent sequences obtained from GenBank.

Due to the small number of isolates (n=5) that were clustered into subgroup IB, and their homogeneity concerning substrate and country origin (dates from Spain), we extended our analysis to include several other *A. tubingensis* isolates. The RAPD profiles of 20 *A. tubingensis*

isolates collected from coffee beans (n=7), dates (n=6), and black sultanas (n=7) are shown in Fig. 4. Together with five isolates from dates, all from coffee beans were included in subgroup IB. Subgroup IA integrated exclusively dried fruit derived isolates i.e. from dates and black sultanas, from different countries (Argentina, Turkey, and USA).

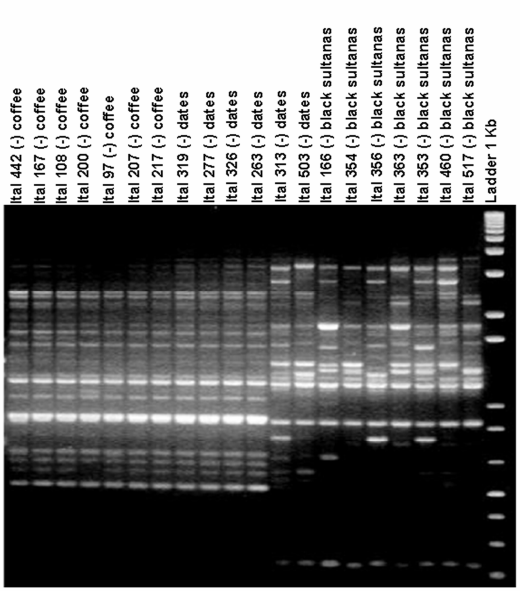

Figure 4 - RAPD profiles from 20 isolates of *Aspergillus tubingensis* using the 10-mer primer OPX-7. Isolates number are present above each lane. The isolates ITAL 442, ITAL 167, ITAL 108, ITAL 200, ITAL 97, ITAL 207, and ITAL 217 were obtained from coffee beans; and the isolates ITAL 319, ITAL 277, ITAL 326, ITAL 263, ITAL 313, ITAL 503, ITAL 166, ITAL 354, ITAL 356, ITAL 363, ITAL 353, ITAL 460, and ITAL 517 were obtained from dried fruits.

Even if β-tub2 data clearly indicated that *A. tubingensis* isolates were accommodated into two groups, we tried to get more evidence to support their separation. Another region of the β-tubulin gene (β-tub1) was sequenced and analyzed. Three nucleotide polymorphisms were found between the two groups: Group IA differ from group IB at the following positions: 148 (C→T); 300 (G→C); 402 (C→T).

Recently a PCR-RFLP analysis for the ITS region using *Nla*III restriction enzyme was described in order to distinguish *A. niger*, *A. tubingensis*, *A. carbonarius*, and *A. aculeatus* isolates from grape (Martinez-Culebras and Ramón, 2007). Interestingly, one profile (denoted as type T2) that included 30 isolates did not correspond to any referenced species, and the ITS sequence of one type T2 isolate was nearly identical to the sequences of *A. tubingensis* CBS 643.92 and CBS 127.42, except for a single nucleotide (G→T). Because of this type T2 isolates were denoted as *A. tubingensis*-like species (Martinez-Culebras and Ramón, 2007). Nevertheless, in the present study no group-specific nucleotide polymorphisms were found in the ITS sequences.

The production of secondary metabolites is usually consistent in a species, and they are commonly useful for species identification (Samson et al., 2007). Asperazine, funalenone, malformins, naphtho-γ-pyrones, pyranonigrin A, and tensidol A and B are extrolites expected to be produced by *A. tubingensis*. The extrolite profiles of the isolates belonging to Group IA and IB were investigated to find possible association between secondary metabolite and molecular patterns. All isolates from both groups produced asperazine, pyranonigrin A, funalenone, and naphtho-γ-pyrones. Tensidol B was not found in all isolates, but no association between the presence of this extrolite and molecular clustering were found. In addition, the extrolite KOTN was only found in two isolates from Group IA.

Although good consensus was found between RAPD profiles and data sequences from β-tub1 and β-tub2 for discriminating *A. tubingensis* into

two distinctly groups, it is still premature to assign a new taxonomic rank for one of them as no association between extrolite and molecular profiles was found. Additional molecular studies are necessary for it may be useful for explaining the incongruence reported about the capability of *A. tubingensis* for OTA production.

Only two isolates were gathered into RAPD-group II, both being unable to produce OTA. After β-tub2 sequence analysis, they were recognized as *A. foetidus*, which agrees with a previous publication that described *A. foetidus* as OTA non-producer (Samson et al., 2004). The extrolite patterns found in both isolates were really compatible with *A. foetidus* species, e.g. asperazine, pyranonigrin A, naphtho-γ-pyrones, and "SPUT" were found.

Based on β-tubulin sequences all isolates clustered into RAPD-group III were equally similar to *A.niger* and *A. lacticoffeatus*. The description of *A. lacticoffeatus* as a new species is recent (Samson et al., 2004). Although *A. niger* sensu stricto and *A. lacticoffeatus* could not be separated by their β-tubulin sequences, there are evident differences in their colony colour and extrolite patterns. Differently to *A. niger* sensu stricto, *A. lacticoffeatus* has sulpher yellow mycelium on YES agar and do not produce naphtho-γ-pyrones (Samson et al., 2004). The morphological and extrolite pattern observed on YES agar showed that all RAPD-group III isolates are *A. niger* sensu stricto.

Isolates clustered into RAPD-group IV were identified as *A. niger* based on β-tub2 sequences and their colony color. The β-tub2 sequences from group IV-isolates characterized in our laboratory were aligned with other *A. niger* sequences available in GenBank (http://ncbi.nlm.nig.gov). In spite of the small number of isolates from group IV (also identified as *A. niger* sensu stricto), it is important to note that the three isolates were not able to produce OTA. Additionally, all Group IV isolates do not produce orlandin and kotanin and the production of pyranonigrin A is very weak contrasting with the isolates of Group III that produce pyranonigrin A, tensidol B, funalenone, orlandin, kotanin, and naphtho-γ-pyrones. Therefore, an association between extrolite patterns and molecular clustering is here speculated.

One single nucleotide polymorphism (A→ T) into the intron 5 of β-tub2 sequence was found to differentiate Groups III and IV, but no differences were found in β-tub1 sequences. Gene faster-evolving analysis need to be performed to confirm the genetic divergence between these two groups.

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RESUMO

Neste trabalho foi analisada a relação genética entre 51 isolados obtidos de amostras de frutas secas provenientes de diferentes regiões do previamente identificados como pertencentes ao agregado A. niger e 7 isolados de Aspergillus tubingensis obtidos de amostras de café do Brasil. Maior diversidade fúngica foi encontrada em uvas passas escuras. Aspergillus niger sensu stricto foi a espécie mais frequente. Esta espécie foi encontrada em todos os substratos e origens geográficas analisadas. Baseando-se nos dados de Polimorfismo de DNA Amplificado ao Acaso (RAPD) e sequências de nucleotídeos do gene da β-tubulina, dois grupos de A. niger foram observados. Apesar do pequeno número de isolados do grupo IV uma associação entre padrão de extrólitos e agrupamento molecular foi encontrada. A. tubingensis foi a segunda espécie mais frequente e foi claramente subdivida em dois grupos. Como os grupos de A. tubingensis são formados somente por linhagens não produtoras de ocratoxina A, a identificação destes grupos não explica a controvérsia encontrada na literatura sobre a capacidade desta espécie em produzir a referida toxina.

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