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TAXONOMY AND PHYLOGENETIC ANALYSIS OF *ASPERGILLUS* SECTION *NIGRI* ISOLATED FROM YERBA MATE IN MISIONES (ARGENTINA)

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ABSTRACT

Members of *Aspergillus* section *Nigri* are distributed worldwide and are potentially ochratoxin A producers; some of them are used at industrial level as source of extracellular enzymes and organic acids. The taxonomy of this section comprises one of the most complex and confusing of genus *Aspergillus*. The objectives of present work were to study the phylogeny of *Aspergillus* section *Nigri* and their taxonomy by amplifying ITS1, 5.8S and ITS2 regions. We analyzed 14 strains of *Aspergillus* section *Nigri* isolated from different commercial forms of yerba mate, classified according to morphology. Genomic DNA was extracted, the ITS regions were amplified with universal primers ITS1 and ITS4, and the obtained fragments were sequenced. The obtained sequences were aligned and edited to a size of 532bp, to carry out phylogenetic analyses using TNT program. We used 100 RAS+TBR cycles to search for the most parsimonious tree, which was supported by booststrap and Jackknife with 1000 resampled matrices. From the analysis of parsimony, one hundred trees were obtained with 568 steps. Bootstrap and Jackknife analyses exhibited similar topology and minor differences in their supported values, showing that section *Nigri* is distributed in two general clusters: one composed of *A. niger*, *A. carbonarius* and *A. heteromorphus* clades, and the other composed of *A. aculeatus* and *A. homomorphus* clades, separated from different sections of genus *Aspergillus*.

Key words: *Aspergillus* section *Nigri*; taxonomy; ribosomal DNA

RESUMEN

Las especies de *Aspergillus* sección *Nigri* están distribuidas mundialmente y son consideradas potenciales productores de ocratoxina A; algunas de ellas se usan a nivel industrial como fuentes de enzimas extracelulares y ácidos orgánicos. La taxonomía de esta sección comprende una de las más complejas y confusas del género *Aspergillus*. El objetivo del presente trabajo fue estudiar la filogenia de *Aspergillus* sección *Nigri* y su taxonomía mediante la amplificación de la región ITS1, 5,8S e ITS2. Analizamos 14 cepas de *Aspergillus* sección *Nigri* asiladas de diferentes formas comerciales de yerba mate, clasificadas de acuerdo a su morfología. Se extrajo ADN genómico, las regiones ITS fueron amplificadas con los cebadores universales ITS1 e ITS4, y los fragmentos obtenidos fueron secuenciados. Las secuencias obtenidas fueron alineadas y editadas a un tamaño de 532pb, para realizar un análisis filogenético con el programa TNT. Para averiguar el árbol más parsimonioso, usamos 100 ciclos RAS+TBR, respaldados con 1000 repeticiones por análisis Bootstrap y Jackknife. Del análisis de parsimonia, se obtuvieron 100 árboles con 568 pasos. Ambos análisis, Bootstrap y Jackknife, mostraron topología similar y diferencias mínimas en sus valores respaldados, mostrando que la sección *Nigri* está distribuida en dos grupos generales: uno compuesto por los clados *A. niger*, *A. carbonarius* y *A. heteromorphus*; y el otro compuesto por los clados *A. aculeatus* y *A. homomorphus*, separados de las demás secciones del género *Aspergillus*.

Palabras clave: *Aspergillus* sección *Nigri*; Taxonomía; ADN ribosomal

INTRODUCTION

The three major genera of mycotoxin-producing fungi are *Aspergillus*, *Fusarium* and *Penicillium*. These fungi frequently contaminate foods, usually causing severe biological alterations to human and animal health (Franco, 2005; Castrillo, 2010).

Members of *Aspergillus* section *Nigri* are the most important in the genus. They are ubiquitously distributed worldwide, growing on a wide variety of substrates and being considered as responsible fungi for food spoilage (Domsch *et al.*, 1980; Pitt and Hocking, 1997; Abarca *et al*., 2004; Geiser *et al.*, 2007). Some of them are a common source of extracellular enzymes and organic acids that are used in food processing (Yokotsuka and Sasaki, 1998; Abarca *et al.*, 2004). Moreover, *Aspergillus niger* products hold the GRAS (Generally Recognized As Safe) status from the FDA (Federal Drugs and Foods Administration, USA) (Abarca *et al.*, 2004). However, the potential capacity to produce mycotoxins by these species suppose a risk for human and animal health. Due to their biotechnological importance and because they are responsible of food spoilage, the identification of such strains needs to be unambiguous (Abarca *et al.*, 2004).

The taxonomy of black aspergilli constitutes one of the most complex and confusing in genus *Aspergillus*; therefore, several taxonomic schemes have been proposed. Classical taxonomy for classification of species is primarily based on morphological criteria, such as forms and conidial ornamentations, size of conidia, conidiophore structures, and coloration of colonies, among others (Klich, 2002). Molecular techniques have contributed to these studies and allowed significant advances in fungal taxonomic organization. Ribosomal DNA (rDNA) amplification was one of the first applications of the Polymerase Chain Reaction (PCR) in mycology (White *et al.*, 1990). The rDNA unit is constituted of regions that possess highly preserved genes, as the 18S and 28S regions, and universal primers were designed to allow internal transcribed spacers amplification of any fungus, independently of genus. The amplified region comprises ITS1, 5.8S and ITS2 regions, which can have variable nucleotide sequences (Fungaro, 2000), and are commonly used to examine phylogenetic positions or relationships at species or intraspecies level (Lee *et al.*, 2000).

For many years, several authors have discussed the composition of section *Nigri*, taking into account morphological data and using rDNA sequences for phylogenetic analyses. Raper and Fennell (1965) produced the most comprehensive monograph of the genus and all *Aspergillus* species with conidial heads in some shades of black were included in the same group. They accepted twelve species and two varieties for *Aspergillus* section *Nigri*. Al-Musallam (1980) revised the taxonomy of this group of fungi using cluster analysis involving all available morphological and cultural parameters, and established five easily distinguishable species and the *A. niger* clade. The apparently insignificant differences between the members of *A. niger* clade was the decisive reason why Al-Musallam suggested that some of the species recognized by Raper and Fennell (1965) should be reclassified as varieties. Later, Kozakiewicz (1989) made a new proposal based upon conidial ornamentations, suggested alterations in the *A. niger* clade, and distinguished 16 taxa.

The previously mentioned classifications of *Aspergillus* section *Nigri* were solely based on morphological criteria and the delimitation of some taxa is problematic because they are distinguished by relatively small differences in variable characters (Parenicova *et al.,* 2000; Varga *et al.*, 2003; Abarca *et al.*, 2004; Samson *et al.*, 2004).

New molecular approaches have shown that there is a high biodiversity in *Aspergillus* section *Nigri*, but that species are occasionally difficult to recognize based solely on their phenotypic characters (Samson *et al.*, 2007). Using this type of approaches, 19 species of *Aspergillus* section *Nigri* were accepted (Samson *et al.*, 2007; Noonim *et al.*, 2008; Perrone *et al.*, 2008). More recently, Varga *et al.* (2011) reported that *Aspergillus* section *Nigri* includes 26 taxa.

Changes in the species concept of black aspergilli according to different authors are shown in Table 1.

As a further contribution, the aims of this paper were to study the phylogeny of *Aspergillus* section *Nigri* and their taxonomy by amplifying ITS1, 5.8S and ITS2 regions.

Raper and Fennel (1965)	Al-Musallam (1980)	Kozakiewicz (1989)	Samson *et al.* (2007); Perrone *et al.* (2008)	Varga *et al.* (2011)
A. japonicus Saito	*A. japonicus* var. *japonicus* Saito	*A. japonicus* Saito	*A. aculeatinus* – CBS 121060 – ITS EU159211	*A. acidus* - CBS 564.65 – ITS AJ280009 = *A. coreanus* - CBS 119384 – ITS FJ491684
A. aculeatus Iizuka	*A. japonicus* var. *aculeatus* _Iizuka_ Al-Musallam	*A. atroviolaceus* Moss.	*A. aculeatus* - CBS 172.66 – AJ279988	*A. aculeatinus* - CBS 121060 – ITS EU159211
A. carbonarius _Bainier_ Thom	*A. carbonarius* _Bainier_ Thom	*A. carbonarius* _Bainier_ Thom	*A. brasiliensis* - CBS 101740 – AJ280010	*A. aculeatus* - CBS 172.66 – ITS AJ279988
A. heteromorphus Batista and Maia	*A. heteromorphus* Batista and Maia	*A. fonsecaeus* Thom and Raper	*A. carbonarius* – CBS 111.26 – ITS DQ900605	*A. awamori* - CBS 557.65 – ITS AM087614
A. ellipticus Raper and Fennell	*A. ellipticus* _Raper and Fennell_ Al-Musallam	*A. heteromorphus* Batista and Maia	*A. costaricaensis* – CBS 115574 – ITS DQ900602	*A. brasiliensis* - CBS 101740 – ITS AJ280010
A. niger van Tieghem	*A. helicothrix* Al-Musallam	*A. ellipticus* Raper and Fennell	*A. ellipticus* – CBS 707.79 – ITS AJ 280014	*A. carbonarius* - CBS 111.26 – ITS DQ900605
A. ficuum _Reichard_ Hennings	*A. niger* aggregate:	*A. helicothrix* Al-Musallam	*A. foetidus* – CBS 564.65 – ITS AJ280009	*A. costaricaensis* - CBS 115574 - DQ900602
A. tubingensis _Schöber_ Mosseray	*A. niger* var. *niger* van Tiegh.	*A. niger* var. *niger* van Tiegh.	*A. heteromorphus* – CBS 117.55 – ITS AJ280013	*A. ellipticus* - CBS 707.79 – ITS AJ280014
A. phoenicis _Corda_ Thom	*A. niger* var. *niger* f. *hennebergii* _Blochwitz Al-Musallam	*A. niger* var. tubingensis _Moss._ Kozakiewicz	*A. homomorphus* - CBS 101889 – ITS EF166063	*A. eucalypticola* - CBS 122712 – ITS EU482439
A. pulverulentus _McAlp_ Thom	*A. niger* var. *phoenicis* _Corda_ Al-Musallam	*A. niger* var. *phoenicis* _Corda_ Al-Musallam	*A. ibericus* - CBS 121593 – ITS AY656625	*A. fijiensis* - CBS 119.49 – ITS FJ491679
A. awamori Nakazawa	*A. niger* var. *phoenicis f. pulverulentus* McAlp Al-Musallam	*A. niger* var. *pulverulentus* _McAlp_ Kozakiewicz	*A. japonicus* - CBS 114.51 – ITS AJ279985	*A. foetidus* - CBS 121.28 – ITS FJ491683 = *A. lacticoffeatus* - CBS 101883 – ITS DQ900604
A. foetidus _Naka._ Thom and Raper	*A. niger* var. *awamori* _Nakazawa_ Al-Musallam	*A. niger* var. *awamori* _Nakazawa_ Al-Musallam	*A. lacticoffeatus* - CBS 101883 – ITS DQ900604	*A. heteromorphus* - CBS 117.55 – ITS AJ280013
A. foetidus var. *pallidus* Naka., Simo and Watanabe	*A. niger* var. *nanus* _Mont._ Al-Musallam	*A. niger* var. *ficuum* _Reich._ Kozakiewicz	*A. niger* - CBS 554.65 – ITS AJ223852	*A. homomorphus* - CBS 101889 – ITS EF166063
A. foetidus var. *acidus* Naka., Simo and Watanabe	*A. niger* var. *usamii* _Sakaguchi et al._ Al-Musallam	*A. citricus* var. *citricus* _Wehmer_ Moss.	*A. piperis* - CBS 112811 – ITS DQ900603	*A. ibericus* - CBS 121593 – ITS AY656625
	A. niger var. *intermedius* _Speg._ Al-Musallam	*A. acidus* Kozakiewicz	*A. sclerotiicarbonarius* - CBS 121057 – ITS EU159216	*A. indologenus* - CBS 114.80 – ITS AL 280005
	A. foetidus Thom and Raper	*A. citricus* var. *pallidus* _Naka., Simo and Watanabe Kozakiewicz	*A. sclerotioniger* - CBS 115572 – ITS DQ900603	*A. japonicus* - CBS 114.51 – ITS AJ279985
			A. tubingensis - CBS 134.48 – ITS AJ223853	*A. neoniger* - CBS 115656 – ITS FJ491682
			A. uvarum - CBS 121591 – ITS AM745751	*A. niger* - CBS 554.65 – ITS AJ223852
			A. vadensis - CBS 113365 – ITS AY585549	*A. piperis* - CBS 112811 – ITS DQ900603
				A. saccharolyticus - CBS 127449 – ITS HM853552
				A. sclerotiicarbonarius - CBS 121057 – ITS EU159216
				A. sclerotioniger - CBS 115572 – ITS DQ900606
				A. tubingensis - CBS 134.48 – ITS AJ223853
				A. uvarum – CBS 121.595 – ITS JQ 316520
				A. vadensis - CBS 113365 – ITS AY585549
				A. violaceofuscus - CBS 123.27 – ITS FJ491678

Table 1. Species concepts of black aspergilli according to various authors.

MATERIALS AND METHODS

Biological material

The strains used in this study were 14 isolates belonging to *Aspergillus* section *Nigri* of three commercial yerba mate forms: five strains from milled yerba mate, without ageing (referred as YMCHA); four strains from elaborated yerba mate (referred as YMA); and 5 strains from composed yerba mate (referred as YMCA). In addition, two *Aspergillus carbonarius* OTA+ standard strains with known capacity to produce ochratoxin were included, which were provided by the University of Buenos Aires (UBA).

The section *Nigri* strains initially were classified by microbiological classic methods, using Klich (2002) codes, by taking into account main macro- and micro-morphology criteria.

Extraction of genomic DNA

The fungal strains isolated from different commercial yerba mate forms were sown in YES (Yeast Extract Sucrose) liquid medium in order to obtain large amounts of mycelium. Mycelia was filtered and washed with Tris-HCl 0.1 M (pH 8) EDTA 0.02 M (pH 8). DNA extraction was carried out with buffer solution (Tris-HCl 0.1 M pH8, NaCl 1.5 M, EDTA 0.05 M pH8) at 60º C, containing Proteinase K 0.1mg/mL, β-mercaptoethanol 10mM and SDS 2 % (p/v). DNA was purified with chloroform: isoamilyc alcohol (24:1 v/v) and potassium acetate 3M, and then was precipitated with isopropyl alcohol (Fonseca, 2012).

Amplification of ribosomal DNA

Ribosomal DNA fragments (ITS1 – 5.8S – ITS2) of 14 isolates of section *Nigri* and two *A. carbonarius* OTA+ standard strains, were amplified and sequenced. For the amplification reaction primers ITS1, 5' TTCGTAGGTGAACCTGCGG, and ITS4, 5' TCCTCCGCTTATTGATATGC (White *et al.*, 1990) were used. The amplification reactions were prepared in a final volume of 20 µL, containing buffer 1X; MgCl2 2.5 mM; dNTP 200 µM; 10 pMol of each primer; *Taq* DNA polymerase 0.5 U and template DNA 5 ng/µL. PCR cycling was programmed to 35 cycles after an initial denaturation of 4 min at 94º C. Each amplification cycle consisted of the following steps: denaturation (94º C, 40 s), hybridization (50º C, 40 s), and extension (72º C, 40 s). Finally, a final extension of 10 min at 72º C was realized.

Agarose gels at 1% (p/v) and 2% (p/v) were performed, containing 10 mg/mL of ethidium bromide, visualized with UV transiluminator, and finally photographed with digital camera. Gels containing 1% (p/v) agarose were used to visualize genomic DNA and those with 2 % (p/v) agarose were used to visualize PCR products.

The concentration of DNA samples was semi-quantified by comparison to the intensity of the marker.

Sequencing of the regions ITS1 – 5.8S – ITS2

Sequencing reactions of PCR products were performed by Macrogen sequencing services (Kumchun-ku, Seoul, Korea).

Bioinformatic study of the obtained sequences

The obtained sequences were compared with those deposited in Database of the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov), with BLAST Search tool. Sequenced ITS1-5.8S-ITS2 regions and the sequences of interest retrieved from NCBI database were aligned by using the CLUSTAL W sequence editor on-line version (Thompson *et al.*, 1994). The alignments were trimmed, overhangs were removed and gaps were corrected, prior to phylogenetic calculations with BIOEDIT software (Sequence Alignment Editor), for obtaining sequences of the same size and also for comparing the same regions. The sequences were submitted to Database of the National Center for Biotechnology Information (accession numbers: JF318957, JF436881, JF436882, JF436883, JF436884, JF436885, JF436886, JF436887, JF436888, JF436889, JF436890, JF436891, JF436892, JF436893, JF436894, JF436895).

To allow an appropriate phylogenetic analyses, sequences of *Aspergillus* section *Nigri* that were

cited in Abarca *et al.* (2004) and Varga *et al.* (2011) were included in the present study (accession numbers: AJ280009, EU159211, AM087614, AJ280010, DQ900605, FJ491684, DQ900602, AJ280014, EU482439, FJ491679, FJ491683, AJ280013, EF166063, AY656625, JX291167.1, JQ316520.1, AJ279985, DQ900604, FJ491682, AJ223852, DQ900603, EU159216, HM853552, DQ900606, AJ223853, AY585549, FJ491678, L76747.1, AB000535, AF128852, AF203800, AJ000933, AY373938).

The parsimony analyses were performed with TNT version 1.1 (Willi Hennig Society Edition). Two methods were used: Bootstrap (Felsenstien, 1985) and Parsimony Jackknifing (Farris *et al.*, 1996). Both analyses included 1000 resampled matrices. The existence of local optima for data sets beyond 40–50 taxa has long been well-informed (Maddison, 1991). In all analyses, *Emericella nidulans* CBS 12135 (AJ000933) was used as an out-group.

RESULTS

Analysis of genomic DNA amplification

Nucleic acid extraction of 14 fungal isolates from different commercial yerba mate forms (previously identified as species of *Nigri* section) and two *A. carbonarius* OTA+ standard strains were analyzed.

The PCR reaction with primers ITS1 and ITS4 amplified a fragment of approximately 600 bp for each isolate. The size of the amplified product was in close agreement with the expected fragment size in *Aspergillus* species (563 to 613 bp, according to White *et al.*, 1990).

Bioinformatics study of the obtained sequences

The obtained sequences of each isolated were compared with deposited sequences in NCBI database, using BLAST as a search tool. In most cases, the high identity values obtained allowed us to assure that these species belong to *Aspergillus* section *Nigri*, always with identity values above 98 %. Seven strains of yerba mate (referred as YMCHA 73, YMCHA 71, YMCHA 55, YMA 120, YMA 119, YMA 10 and YMCA 29) were morphologically and molecularly defined as *A. niger*; three strains (referred as YMCHA 69, YMCA 18 and YMCA 2) were defined as *A. tubingensis*; and one strain (referred as YMCHA 63) was defined as *A. brasiliensis*; all strains belong to the *A. niger* clade. In addition, two standard strains (referred as PATRON I and PATRON II) were defined as *A. carbonarius*, that belongs to the *A. carbonarius* clade (Figure 1). However, three isolates of yerba mate (referred as YMCA 11, YMA 2 and YMCA 9) were morphologically defined only as *Aspergillus* section *Nigri* species, and molecularly defined as *Aspergillus* spp. (Figure 1).

Phylogenetic Analyses

Good quality sequences of approximately 600 bp were first aligned and then trimmed, and sequences 532 bp in size were obtained, prior to carrying out phylogenetic calculations.

Study of parsimony was done using Bootstrap and Jackknife analyses. Parsimony analysis resulted in 100 equally parsimonious trees 568 steps long, performing 100 RAS + TBR cycles. Our data set consisted of 134 parsimony informative characters. Both, analyses, Bootstrap and Jackknife, yielded nearly the same topology, and presented minimal differences in support values. Our trees showed that all isolated strains of yerba mate are included in the *A. niger* and *A. carbonarius* clades, except for the isolated strain referred as YMCA 11 (Figure 1).

DISCUSSION

Until a few years ago, systematic studies of microorganisms were based almost exclusively on morphological criteria (classical microbiological classification). However, the number of tools actually used in systematic studies has increased. Nowadays, both morphological and molecular criteria are taken into account (Perez Valencia *et al.*, 2005).

In this study, and for taxonomic organization of fungi, were considered the classical microbiological classification by morphological criteria and molecular techniques by amplification of ITS1,

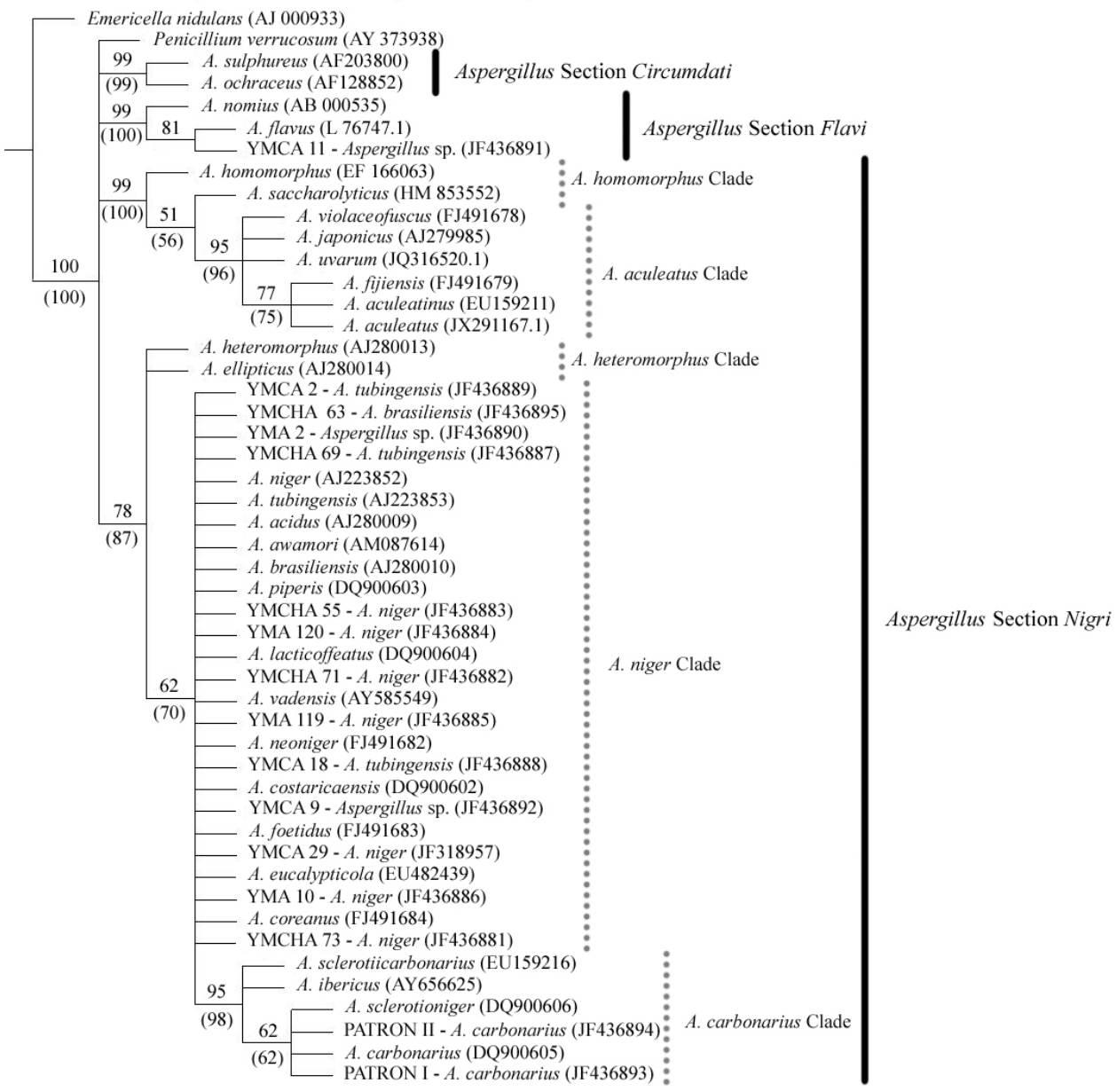

Figure 1. Phylogenetic analysis. Group support, assessed with 1000 Bootstrapping and Parsimony Jackknifing replicates. Numbers above branches correspond to Bootstrap support. Jackknife supports are given in parentheses.

5.8S and ITS2 regions. These regions are commonly used in fungi because they are composed of variable nucleotides sequences that allow the examination of phylogenetic positions or relationships at species or intraspecies level (Fungaro, 2000; Lee *et al.*, 2000).

In *Aspergillus* section *Nigri* there are very subtle differences between species, being for this reason one of the most difficult groups for classification and identification. Consequently, classical microbiological criteria are insufficient for classification (Abarca *et al.*, 2004; Varga *et al.*, 2011). In this study, and by applying morphological criteria, we could accurately classify the isolated strains at the species level. However, it was necessary to confirm the morphological classification with molecular information obtained by amplification of ITS1, 5.8S and ITS2 regions and by contrasting them with those in NCBI database. Classical microbiological classification and molecular data ensured the correct strain classification at the genus level.

Most of the isolated strains belonged to *Aspergillus* section *Nigri*, invariably with high identity values, except in three cases (YMCA 11, YMA 2 y YMCA 9) which could only be morphologically defined as *Aspergillus* section *Nigri* and molecularly defined as *Aspergillus* spp., reflecting the confusing and complex taxonomy of this section. These exceptional cases either may belong to the *A. niger* aggregate or the bioinformatics analysis using solely the ITS conserved regions was insufficient for the correct molecular identification of *Aspergillus* section *Nigri*. Some authors have cited other conserved regions as useful for molecular classification, such as the calmodulin or parts of β -tubulin sequences (Peterson, 2000; Abarca *et al.*, 2004; Varga *et al.*, 2011).

With respect to the phylogenetic analysis of *Aspergillus* section *Nigri*, in previous studies with amplification of ITS regions, Parenicova *et al.* (2000) reported that *A. carbonarius* could be easily distinguished from other black aspergilli. They cited that *A. carbonarius* forms a separate clade within the biseriate black aspergilli. Then, Parenicova *et al.* (2001) reported that ITS sequences could be used to distinguish uniseriate aspergilli in a separate clade from a general clade containing biseriate species and species belonging to section *Flavi* and *Circumdati*; but could not be used for differentiation within uniseriate species.

In this study, were observed four different general clades of *Aspergillus* strains, section *Flavi*, section *Circumdati*, and two general clusters of section *Nigri*: one general cluster containing *A. aculeatus* and *A. homomorphus* clades; and the other general cluster with *A. heteromorphus* clade and *A. niger* clade which includes *A. carbonarius* clade. *A. carbonarius* formed a separate branch into *A. niger* clade (biseriate species), as reported by Parenicova *et al*. (2000). But then, it was observed that in *Aspergillus* section *Nigri*, both biseriate (*A. niger*, *A. carbonarius*, *A. heteromorphus* and *A. homomorphus* clades) and uniseriate species (*A. aculeatus* clade) formed two general clades separated from section *Flavi* clade and section *Circumdati* clade (Figure 1). It was not observed that the uniseriate species formed a separate clade from all others, as previously cited by Parenicova *et al.* (2000).

Based on phylogenetic analysis of ITS sequence data, Varga *et al.* (2011) reported that *Aspergillus* section *Nigri* formed two general clusters: one cluster containing *A. aculeatus* and *A. homomorphus* clades and the other cluster containing *A. heteromorphus* clade and *A. niger* clade with *A. carbonarius* clade, as observed in this study.

In this phylogenetic analysis it is possible to visualize that some of the isolated strains of yerba mate belong to *A. niger* clade so it can be argued that YMA 2 and YMCA 9 strains, which were morphologically defined as *Aspergillus* section *Nigri* and molecularly defined as *Aspergillus* spp., also belong to section *Nigri* clade. Likewise, it was observed that YMCA 11 strain belongs to section *Flavi*; it was molecularly referred as *A. flavus*, being the only strain that was poorly characterized by classical microbiological methods (Figure 1).

In addition, parsimony analyses performed with Bootstrap and Jackknife methods, revealing minimal differences between the values obtained with both methods; this allows us to assure that the phylogenetic analyses performed were consistent and also they were in accordance with other current reports (Varga *et al.*, 2011).

Moreover, in this study, and in order to observe whether toxin production had implications for phylogeny, were considered sequences of known species with OTA capability, as *Penicillium*

verrucosum, Aspergillus ochraceus and patterns I and II strains. The results did not show that these species were part of the same clade, the ochratoxin A production does not seem to have implications on the phylogeny of ochratoxin A-producing species when ITS sequences were considered (Figure 1). Some authors reported that a chloroperoxidase gene presumably takes part in ochratoxin biosynthesis in *P. verrucosum*, so the use of this sequence on phylogenetic analysis can be useful for discriminate the ochratoxin A-producing species from others (Geiser *et al*., 2007; Varga *et al.*, 2011).

Our results show that *Aspergillus* section *Nigri* has a polyphyletic origin and that it is composed of two general clusters, one containing *A. aculeatus* and *A. homomorphus* clades and the other containing *A. heteromorphus* clade and *A. niger* clade which includes *A. carbonarius* clade. Also, the uniseriate species (*A. aculeatus* clade) did not appear as a different group in relation to the biseriate species in *Aspergillus* genus phylogeny. We can also conclude that the taxonomic classification of *Aspergillus* section *Nigri* still remains as confusing and complex topic, and to date, it is necessary to take several precautions for studying the phylogeny of *Aspergillus* section *Nigri.*

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