

## Full Length Research Paper

# Characterization of *Aspergillus* section *Nigri* isolates from leaf litter and soil in the Atlantic Forest of Brazil

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**Section *Nigri* is among the most important from the genus *Aspergillus*, since it possesses economical and biotechnological properties. Its identification is considered quite hard and complex. Due to this fact, *Aspergillus* taxonomy has been carried out by morphological, biochemical and molecular data. Morphological characterization besides molecular analyses was performed to properly identify *Aspergillus* section *Nigri*. A set of 42 *Aspergillus* section *Nigri* isolates were obtained from Brazilian Atlantic Rainforest. From these isolates, 16.7% originated from leaf litter layer and 83.3%, from soil. The isolates were divided into three different groups according to analyzed characteristics. 26 were identified as *Aspergillus aculeatus*, 12 as *Aspergillus aculeatinus* and 4 belonged to a group called “*Aspergillus niger* Aggregate”, considered as biseriata.**

**Key words:** Fungi, taxonomy, morphological identification, polymerase chain reaction (PCR), ITS.

## INTRODUCTION

Fungi from the genus *Aspergillus* have a significant impact on modern society (Pitt and Hocking, 2009) because they cause food bio-deterioration and are potential pathogens to men and animals in general (Geiser et al., 2007). However, such microorganisms are of big concern because they have bio-technologic potential to produce chemical compound, mycotoxins, enzymes, organic acids, phenolic compounds, amongst other substances (Varga et al., 2003).

Of all the genera, *Aspergillus* species from section *Nigri*

are considered the most important (Gams et al., 1985), as they are used in industrial fermentation to hydrolyze enzymes and are source of extracellular enzymes (Abarca et al., 2004). The use of these fungi as enzymes producers is recognized by Food and Drug Administration (FDA); some species of the *Aspergillus* genus possess Generally Regarded as Safe (GRAS) status, due to their low toxicity and historical use in industrial food production and drink. A good example is the *Aspergillus niger* lineage (Abarca et al., 2004). The *Nigri* section is

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considered by many taxonomists as one of the most difficult to identify, due to high genetic diversity, which makes identification based only on phenotypic characteristics a hard work (Samson et al., 2007). Therefore, many authors have been highlighting the importance of polyphasic taxonomy, by combining different identification tools, using morphological, physiological and biochemical characteristics, as well as metabolites production and molecular data. Their main goal is to clarify the complex taxonomy of section *Nigri* species (Varga et al., 2011; Silva et al., 2011; Simões et al., 2013). New molecular approaches have contributed to such studies and have allowed significant break-throughs in fungi taxonomical organization, acknowledging the existence of an elevated biodiversity on section *Nigri* (Krijgsheld et al., 2013).

In this context, the present work aims to use morphological, biochemical and molecular data to identify *Aspergillus* species from section *Nigri* isolates from leaf litter and soil in the Atlantic Rainforest of Brazil.

## MATERIALS AND METHODS

### Leaf litter and soil samples and isolation

The collection areas of leaf litter and soil samples are located in Mid-West Minas Gerais, at Sete Lagoas city, belonging to the Atlantic Rainforest's biome of Brazil. Both samples, leaf litter and soil, were collected on two different periods, the first being humid, at the end of January and the second dry, at the beginning of September in 2012. Nine forest fragments were sampled on three different depths (leaf litter, 0 to 5 and 5 to 10 cm) with three repetitions in each depth, totaling 162 samples. After collection, the packaging containing the samples was stored at 4°C until analysis.

The isolations were performed on five grams of soil and leaf litter re-suspended in an Erlenmeyer flask with 45 mL of 0.1% peptone and Tween 80; and later agitated for 30 min on a vertical homogenizer. The cultural media used was Dichloran Rose Bengal Chloramphenicol Agar (DRBC), accomplished with aliquots of 0.1 mL from respective dilutions ( $10^{-2}$  to  $10^{-6}$ ). The Petri dishes were incubated on greenhouses at 25°C for five days (Fraga and Pereira, 2012).

### Morphological analyses

For morphological identification, the inoculations were accomplished through conidial suspension of isolates from *Aspergillus* section *Nigri* on solution containing Agar and Tween 80. From such suspension 0.2 mL was used for inoculation on three equidistant points in the Petri dishes containing Czapek yeast agar (CYA) and malt extract agar (MEA). It was done in triplicate in each medium. The incubation was performed at 25 and 37°C on CYA and 25°C on MEA for seven days; afterwards, macroscopic and microscopic characteristics were registered (Samson et al., 2007).

### Molecular analyses

#### DNA extraction

The DNA extraction of 42 isolates from section *Nigri* was carried out using four different protocols. The fungi were cultivated on PDA

(Potato dextrose agar) medium at 28°C for 5 days and about 1 cm<sup>2</sup> of mycelium from cultures was collected and used for DNA extraction. The best result was observed with the protocol indicated by Vicente (2000). The DNA was quantified in Qubit 2.0 Fluorometer (Invitrogen).

### Amplification of ITS region from rDNA by PCR

The oligonucleotides primers ITS1 (5'-TTC CGT AGG TGA ACC TGC GG- 3') and ITS4 (5' TCC TCC GCT TAT TGA TAT GC 3') (White et al., 1990) were used for molecular characterization of *Aspergillus* section *Nigri* isolates. The polymerase chain reaction (PCR) was carried out with 2 µL of DNA (between 100 and 200 ng); 10 µL of Taq buffer to PCR (10X); 6 µL of MgCl<sub>2</sub> (25 mM); 1 µL of dNTP (10 mM); 5 µL GoTaq DNA polymerase; 1.2 µL of DMSO; 1.5 µL of each primer; ITS1 (10 pmol/µL) and DNase free water to a final volume of 50 µL. The PCRs were performed on a thermo cycler (Mastercycle 22331 Hamburg Eppendorf AG). The amplification conditions were: a denaturation stage at 95°C for 4 min, followed by 30 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 30 s and extension at 72°C for 10 min. The electrophoresis was performed using 1% agarose gel stained with ethidium bromide solution (0.5 µg.mL<sup>-1</sup>) and visualized in a transilluminator (KODAK Scientific Imaging Systems).

### Amplified fragments sequencing

The PCR purified product was used for sequencing reactions on an automatic DNA sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The contiguous DNA sequences were assembled with ITS1 and ITS2 independent sequence reactions using Phred/Phrap programs (Ewing et al., 1998).

### Similarity and phylogeny analyses

The similarity research was performed in the Genbank, using Basic Local Alignment Search Tool (BLAST) program (<http://www.ncbi.nlm.nih.gov>). The ITS1-ITS4 similarly sequences were imported from BioEdit Sequence Alignment Editor program (Hall, 1999) to alignment, using CLUSTALW program (Thompson et al., 1994) and manual edition. The phylogenetic tree was built through comparison of 550 pb using the Neighbor-Joining method. The tree's topology toughness was evaluated through Kimura 2-parameter method and phylogenetic analysis was conducted on Molecular Analyses Evolutive Genetics (MEGA.5) program (Tamura et al., 2011).

## RESULTS AND DISCUSSION

### Morphological characterization

The 42 isolates were divided into 3 groups according to morphological characteristics. Group I: 26 isolates (62%); group II: 12 isolates (28.5%), and Group III: 4 isolates (9.5%). Table 1 shows the macroscopic and microscopic characteristics of *Aspergillus* section *Nigri* of isolates species. Groups I and II present all uniseriate strains; in other words, present only phialide, conidia varying from dark and light brown, echinulated on both globular and sub-globular shape and hyaline hyphae. In group III all strains were biseriate, presenting

**Table 1.** Phenotypic characters of *Aspergillus* section *Nigri* isolates obtained from leaf litter and soil in the Atlantic Rainforest of Brazil.

Isolates (groups)	Colony color		Colony diameter (mm)			Sclerotia (color)
	CYA (25°C) verso / reverse	MEA (25 °C) verso / reverse	CYA (25°C)	MEA (25°C)	CYA (37°C)	
	<b>Macroscopic</b>					
I	Dark brown/ Light Brown	Black with yellowish halo / colorless with yellowish tones	>80	55-75	15-28	Yellowish
II	Dark brown/yellowed	Black/colorless	>80	75-85	20-35	Orange
III	Dark brown to black/ colorless to yellowish with gray tones	Dark Brown to Black/colorless	50-85	54-79	47-70	-
	<b>Microscopic</b>					
	<b>Conidia</b>	<b>Vesicle</b>		<b>Conidial head</b>		
	<b>Size (µm)</b>	<b>Size (µm)</b>	<b>Shape</b>	<b>Metula (µm)</b>	<b>Phialide (µm)</b>	
I	4-5	30-75	Subglobular/ ellipsoidal	-	5-10 × 3-6	
II	3-4	40-95	Globular/subglobular	-	4-8 × 3-5	
III	3-5	45-80	Globular/subglobular	6-10 × 2-5	10-13 × 3-4	

metulae and phialide covered with a mucilaginous layer, hyaline hyphae, conidia varying from dark brown to black, globular and subglobular. Hence, based on morphological characters, the fungi were classified as: Group I = *Aspergillus aculeatus* and *Aspergillus japonicus*; Group II = *Aspergillus aculeatinus* and Group III = “*A. niger Aggregate*”, according to Samson et al. (2007).

Samson et al. (2007) observed some differences among the species of *Aspergillus* section *Nigri*, where *A. aculeatus* presented bigger vesicles in comparison to *A. japonicus*, which presented smaller vesicles, varying from 14-30 (-47) µm. The species from group I presented bigger vesicles with values similar to the *A. aculeatus* species. This is also observed by Silva et al. (2011). Maciel (2013), studying this group of fungi, used phenotypic features to generate a dendrogram gathering the lineages of *A. japonicus* and *A. aculeatus* in the same group. On CYA 37°C, the growth of lineages from Group I was also observed by Sorensen et al. (2011); and species in Group II agree with Noonim et al. (2008), where the author found values of 22-33 mm in *A. aculeatinus*. Another important characteristic not overlooked by Samson et al. (2007) and Jurjevic et al. (2012) was the sclerotia production by *A. aculeatus*. Species belonging to “*A. niger Aggregate*”, according to Varga et al. (2003) are morphologically identical; however, some authors describe differences between these species as an example, Silva et al. (2011) classified *A. niger* different from *A. tubingensis* based on sclerotia color, which varies from white and pink in *A. niger* to black in *A. tubingensis*.

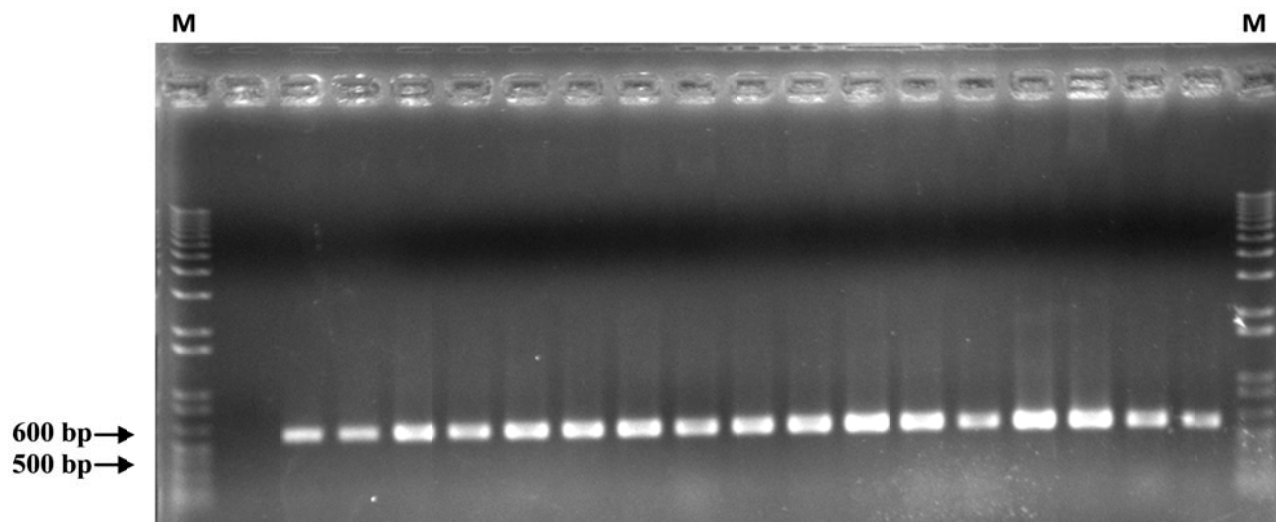
## Molecular characterization

### DNA extraction and rDNA amplification analysis

The best protocol for DNA extraction was the CIA method protocol, that successfully extracted DNA of 38 from originally 42 isolates used. This protocol distinguishes itself from the others for having low cost and for being a fast method. Despite the application of four protocols, including the use of a Commercial Kit (Quiagen, 2006) - (DNeasy Protocol), it was not possible to obtain good quality DNA from the four isolates in group III. The difficulty in the DNA extraction of these four isolates could be related to the fact that they are biserial species, according to morphological identification. These species possess a second series of cells called metulae, which may present more melanin on the hyphae and spores walls, making extraction more difficult. Bozza (2010) also observed the same extraction difficulty in this group, by using the CIA method for DNA extraction of 13 isolates from section *Nigri* on which success was obtained only in seven isolates. Regions ITS1-5, 8S- ITS2 and 18S from rDNA were amplified by PCR and all 38 isolates have shown the same band profile estimated between 500 to 600 pb (Figure 1).

### Sequence similarity analysis of ITS amplified

To identify species from section *Nigri*, the sequences ITS1 and ITS4 were compared with GenBank database via BLASTn (basic local alignment search tool). The



**Figure 1.** DNA amplification products of *Aspergillus* section *Nigri* isolates, obtained with universal primers ITS1 and ITS4. M = Molecular weight marker 1 Kb Plus DNA Ladder (Invitrogen).

phylogenetic tree of similar sequences was built using sequences ITS1 and ITS4 of *Aspergillus awamori* (*syn. A. luchuensis*) (Hong et al., 2013) to compare section *Nigri*; and the *Aspergillus flavus* species was used as an external group (Figure 2).

In a general way, similarity analysis was quite elevated (around 99%) between our isolates and the ITS sequences from database. The phylogenetic tree built from these similar sequences has shown formation of two large groups: Group I defined as *A. japonicus* and *A. aculeatus* and Group II defined as *A. aculeatinus*.

Some authors have used the primers ITS1 and ITS4 for taxonomic positioning of *Aspergillus* section *Nigri* (Nugroho et al., 2013). These ITS regions are commonly used on fungi, because they are made by variable nucleotides sequences, which allow position analysis or phylogenetic relationship among species (Fungaro, 2000). However, despite broadly used, molecular methods present some problems on what concerns the section *Nigri*, due to high level of genetic similarity between some species (Rodrigues et al., 2011).

For species description and phylogenetic inference, Samson and Varga (2009) recommended at least two sequences of genes to be used by a multilocus approach (MLST). In this way, as observed by Sanson et al. (2007) on *Aspergillus* section *Nigri*, it is necessary to utilize other genes, such as calmodulin, or part of  $\beta$ -tubulin sequences for a better classification and discrimination of both intra and inter-specific (Varga et al., 2011). Sorensen et al. (2011) described strain relations of *A. aculeatus* and *A. japonicus* with another species of *Aspergillus* section *Nigri* using polyphasic approach including ITS, calmodulin and  $\beta$ -tubulin. Such authors observed a separated grouping on the  $\beta$ -tubulin tree from these species in comparison with other species, and

have concluded that such grouping is because *A. japonicus* and *A. aculeatus* do not possess an intron on  $\beta$ -tubulin gene as compared to other species *Nigri*. This is also pointed out by Noonim et al. (2008).

## Conclusion

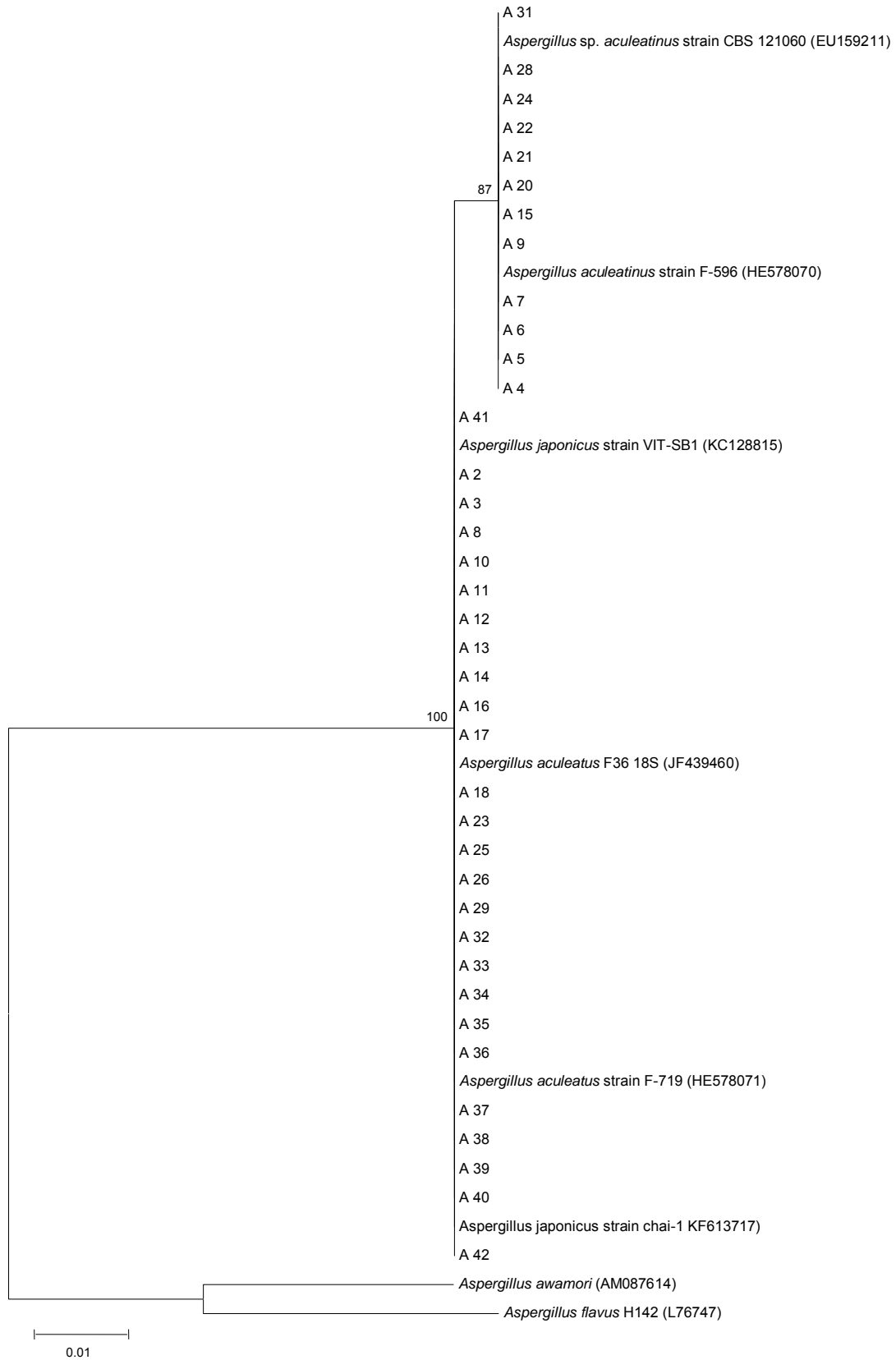
In this work, apart from using ITS1-ITS4 regions for *Aspergillus* section *Nigri* identification, it was also used for morphological characterization to aid the taxonomic positioning of the isolates. Such analyses allowed the statement that from the 42 isolates fungi from leaf litter and soil in the Atlantic Rainforest of Brazil, 26 belong to *A. aculeatus* species, 12 to *A. aculeatinus* species and 4 to "*A. niger* Aggregate". In the *A. niger* aggregate, although speciation at molecular level has been proposed, no morphological differences can be observed and species identification will therefore remain problematic.

## Conflict of interests

The authors did not declare any conflict of interest.

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**Figure 2.** Phylogenetic tree based on sequences of regions ITS from rDNA of species *Aspergillus* section *Nigri*, built by neighbor-joining method. Bootstrap values (n=1000 replicates). Scale bar = 0.01 substitutions per position.

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